ACSL4-mediated membrane phospholipid remodeling induces integrin β1 activation to facilitate triple-negative breast cancer metastasis

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Abstract

Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer and has a poor prognosis and a high propensity to metastasize. Lipid metabolism has emerged as a critical regulator of tumor progression and metastasis in other cancer types. Characterization of the lipid metabolic features of TNBC could provide important insights into the drivers of TNBC metastasis. Here, we showed that metastatic TNBC tumors harbor more unsaturated phospholipids, especially long-chain polyunsaturated fatty acids, at the sn-2 position of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) compared to primary tumors. Metastatic TNBC tumors upregulated ACSL4, a long-chain polyunsaturated acyl-CoA synthetase that drives the preferential incorporation of polyunsaturated fatty acids into phospholipids, resulting in the alteration of membrane phospholipid composition and properties. Moreover, ACSL4-mediated phospholipid remodeling of the cell membrane induced lipid-raft localization and activation of integrin β1 in a CD47-dependent manner, which led to downstream focal adhesion kinase (FAK) phosphorylation that promoted metastasis. Importantly, pharmacological inhibition of ACSL4 suppressed tumor growth and metastasis and increased chemosensitivity in TNBC models *in vivo*. These findings indicate that ACSL4-mediated phospholipid remodeling enables TNBC metastasis and can be inhibited as a potential strategy to improve the efficacy of chemotherapy in TNBC.

Statement of significance

ACSL4 upregulation in triple-negative breast cancer alters cell membrane phospholipid composition to increase integrin β1 activation and drive metastasis, indicating that targeting ACSL4 could potentially block metastasis and improve patient outcomes.

Introduction

As one of the most common cancers in women, breast cancer exhibits significant heterogeneity in pathological characteristics, disease progression, and therapeutic response (1). Triple-negative breast cancer (TNBC) accounts for 12-17% of all breast cancer cases (2), and is characterized by the lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Patients with TNBC have worse prognosis than those with other subtypes because of their higher rates of recurrence, metastasis, and limited therapeutic options (3). Therefore, a better understanding of the aggressive and metastatic properties of TNBC is of great clinical importance in the search for better therapeutic strategies and for identifying molecular targets.

Lipids play crucial roles in various biological processes within the body, such as providing energy storage, acting as signaling molecules, and acting as structural components of membranes (4). An increasing number of studies have shown that abnormal lipid metabolism can promote tumorigenesis, colonization, and the metastatic capacity of tumor cells. For example, brain metastatic cancer cells have been reported to take advantage of the high-fat microenvironment in the brain for metastasis (5). Recent studies have revealed that pancreatic cancer cells facilitate the accumulation of newly synthesized lipids in lipid droplets, which serve as energy depots to fuel cancer cell metastasis (6). Notably, the lipid composition characteristics of metastatic cancer cells and the mechanisms by which abnormal lipid metabolites promote metastasis have not been clearly elucidated.

Lipid rafts are membrane microdomains enriched in specific lipids (such as cholesterol and sphingolipids) and clusters of signaling molecules that function as cellular signaling platforms (7,8). There is growing evidence that specific constituents of the lipid bilayer, such as phospholipids and cholesterol, regulate the biophysical properties (including curvature, charge, and fluidity) of the plasma membrane and signaling molecule activity (9-12). However, it is not well understood whether abnormal lipid metabolites regulate the localization and activity of oncogenic receptors in lipid rafts by affecting plasma membrane composition and biophysical properties.

In this study, we revealed that metastatic TNBC cells have significant lipid metabolism features characterized by increased polyunsaturated phospholipids. We found that acyl-CoA synthetase long-chain family member 4 (ACSL4) is aberrantly upregulated in TNBC cells and is correlated with poor outcomes and metastasis in patients with TNBC. ACSL4 activates polyunsaturated fatty acids (PUFA) and catalyzes PUFA to PUFA-CoA, which is esterified into phospholipids, resulting in increased phospholipid unsaturation. ACSL4-mediated phospholipid remodeling contributes to changes in membrane biophysical properties, such as membrane fluidity, thus facilitating the enrichment and interaction of integrin β1 and CD47 in lipid rafts and activating integrin β1/focal adhesion kinase (FAK) signaling in a CD47-dependent manner to promote TNBC metastasis. In addition, a combination of the clinical therapeutic drugs paclitaxel and cisplatin with the ACSL4 inhibitor, PRGL493, significantly decreased TNBC growth and metastasis in vivo. Taken together, these results highlight the pivotal role of ACSL4-mediated membrane phospholipid remodeling in regulating TNBC metastasis and potentially provide a novel strategy for the treatment of TNBC.

Materials and methods

Cell culture and reagents

Human breast cancer cell lines (HCC1806, BT-549, Hs578T, MDA-MB-231, MDA-MB-468, HCC1937, BT-474, MCF-7, T47D, BT-483, HCC1954 and SKBR3), and other cancer cell lines (OVCAR-8 and HCT8) were purchased from American Type Culture Collection (ATCC). All cell lines were authenticated through short tandem repeat profiling and were used for less than six months within 15 to 20 passages. The cell lines were *Mycoplasma*-free and authenticated by monthly PCR analysis. All cell lines were cultured in the ATCC-recommended medium containing 10% fetal bovine serum (Gibco) and 1% streptomycin/ penicillin (#BL505A, Biosharp) at 37°C with 5% CO_2. Detailed information on the chemical reagents used in this study is provided in Supplementary Table S1.

Clinical breast cancer samples

Human breast cancer and paired adjacent normal tissues (at least 5 cm away from the tumor), primary and paired metastatic tumor tissues were collected from patients with breast cancer without previous radiotherapy or chemotherapy at the First Affiliated Hospital of Chongqing Medical University. Paclitaxel-sensitive and resistant TNBC tissues were obtained from TNBC patients who underwent resection after chemotherapy at the First Affiliated Hospital of Chongqing Medical University. Written informed consent was obtained from all of the patients. All procedures were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Chongqing Medical University (Reference Number 2021085). Human breast cancer tissue microarray (TMA) chips (TMA1, #HBre-Duc170Sur-01; TMA2, #HBreD055CD01) were purchased from the Biobank Center of the National Engineering Center for Biochip in Shanghai (also known as Shanghai Outdo Biotech Company).

Plasmids, small interfering RNA (siRNAs), and lentivirus-mediated RNA interference

shRNAs (GeneChem, China), specifically against ACSL4 (shACSL4#1 and shACSL4#2) and the control (shNC), were cloned into the GV493 vector (GeneChem, China). The cells were infected with a lentiviral vector (GeneChem, China) expressing shACSL4 or shNC for 12 h. The infected cells were cultured in a medium containing puromycin for two weeks to establish the engineered cells. The shRNA-resistant version of ACSL4 (VectorBuilder, China) was achieved by a series of point mutations in the shRNA#1-targeting sequence without changing amino acids, and then inserted into a VB220414-1121ggm vector (VectorBuilder, China), which was used as an ectopic ACSL4 in ACSL4-deficient TNBC cells. siRNA oligonucleotides against ITGB1, CAV1, and CD47 were purchased from GenePharma (Shanghai, China) and transfected into cells using Lipofectamine 3000 (#L3000015, Invitrogen) according to the manufacturer's instructions. The detailed sequences are provided in Supplementary Table S2.

RNA extraction and Reverse-transcriptional quantitative PCR (qRT-PCR)

Total RNA was isolated from tumor tissues using RNAiso Plus reagent (#9108, Takara) following the manufacturer's instructions, and reverse transcription was performed using the PrimeScript RT Reagent Kit (#RR047A, Takara). Gene expression was detected by qRT-PCR using the SYBR Green qPCR Master Mix (#HY-K0501, MCE) and a CFX96 Real-time PCR Detector (Bio-Rad). β-actin was used as a loading control to normalize target mRNA levels. The primers used in this study are provided in Supplementary Table S2.

Immunohistochemistry (IHC) staining and score

IHC was performed according to the SP kit instructions (#PV-9000, Zsgb-bio). The slides were baked overnight at 60°C. Next, the tissue slides were washed with PBS after deparaffinization and hydration and then boiled in citrate buffer at 100°C for 15 min. After blocking endogenous peroxidase, slides were incubated at 4°C overnight with Ki-67, ACSL4, p-FAK, and Activated-ITGB1 antibodies. After washing with PBS, the slides were incubated with a secondary antibody for 30 min at room temperature.

Sections were stained with DAB (#ZLI-9018, Zsgb-bio) and counterstained with Hematoxylin Staining Solution (#C0107, Beyotime) according to the manufacturer's instructions. Detailed information on the antibodies used is provided in Supplementary Table S1.

The strength of IHC staining was scored as follows: 0, no staining; 1, weak; 2, moderate; 3, strong. The percentage of stained cells was scored as follows:0, no staining; 1, <10%; 2, 10–50%; and 3, >50% tumor cells. The IHC score was calculated by multiplying the staining score with the percentage score. Samples with IHC scores greater than 4 were considered to have high ACSL4, Act-ITGB1, or p-FAK expression.

Co-immunoprecipitation (Co-IP) analysis

The primary antibody, 3 μg ITGB1 (#sc-374429, Santa Cruz), was bound to 50 μl of Protein A/G magnetic beads (#B23201, Bimake) for 15 min and washed three times. The breast cancer cell protein was extracted using cell lysis buffer for western and IP (#P0013, Beyotime), and the protein supernatant from each sample was incubated with the antibody-crosslinked beads overnight at 4°C. The next day, the beads were washed twice with the IP lysis buffer. Supernatants were collected for western blot analysis.

Immunofluorescence staining (IF) and Lipid raft labeling

TNBC cells were fixed with 4% paraformaldehyde on slides for 15 min. The slides were then blocked with BSA and incubated overnight with primary antibodies against Act-ITGB1 and CD47 (Detailed information on the antibodies used is provided in Supplementary Table S1). Subsequently, the slides were incubated with secondary antibodies (Alexa Fluor 647-labeled Goat Anti-Mouse IgG and Cy3-labeled Goat Anti-Rabbit IgG, 1:500, Beyotime) at room temperature for 45 min. After washing with PBS, the nuclei were labeled with DAPI for 5 min (#BL105A, Biosharp). Representative images were captured using a Leica DMi8 microscope (Leica microsystems).

Lipid rafts were labeled using the Vybrant Alexa Fluor 555 Lipid Raft Labeling Kit (#V34404, Invitrogen). Briefly, cells were labeled with the fluorescent CT-B conjugate for 10 min at 4°C, and subsequently, CT-B-labeled lipid rafts were crosslinked with the

anti–CT-B antibody for 15 min at 4°C. Cells were washed several times with chilled PBS and fixed in 4% formaldehyde for 15 min at 4°C. After staining with DAPI, representative images were captured using a Leica DMi8 microscope (Leica microsystems). The mean fluorescence intensity of the labeled lipid rafts was measured by flow cytometry (Beckman Coulter). The colocalization rate was quantified by LAS X software (Leica microsystems) applying the formula: colocalization rate (%) = colocalization area/area foreground, and area foreground = area image - area background.

Western blot analysis

Breast cancer cells were lysed in RIPA lysis buffer (#P0013B, Beyotime) containing a protease and phosphatase inhibitor cocktail (#P1045, Beyotime). The protein amounts were determined using an Enhanced BCA Protein Assay Kit (#P0010, Beyotime). The normalized protein amounts were subjected to SDS-PAGE and transferred onto polyvinylidene fluoride membranes for western blotting. The membranes were incubated with specific primary antibodies at 4°C overnight and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Biosharp) for 1h. Proteins were visualized using Clarity Western ECL Substrate (#1705061, Bio-Rad) on a ChemiDoc XRS+ system (Bio-Rad). Activated-ITGB1, ITGB1, and CD47 protein levels were quantified by densitometry using ImageJ software according to three repeated assays and normalized to Flotilin-1 or β-actin levels. Detailed information on the antibodies used is provided in Supplementary Table S1.

Apoptosis detection

For the detachment-induced apoptosis assay, TNBC cells were cultured in the wells of 6-well plates pre-coated with poly-HEMA (#P3932, Sigma-Aldrich), dissolved at 10 mg/ml in ethanol. After 24 h of suspension, cells were harvested for apoptosis analysis using Annexin V-APC/PI kit (#P-CA-207, Procell). Briefly, 5×10^5 cells were collected and incubated with Annexin V-APC and PI staining buffer for 15 min at room temperature, and the apoptosis index was determined by a flow cytometer (Beckman

Coulter). For CTC apoptosis detection, 1×10^6 MDA-MB-231 (GFP-positive; stably expressing shNC, shACSL4#1, and shACSL4#2) were injected via the tail vein of NCG mice. After 24 h post injection, blood was collected and mixed with RBC lysis buffer (#R1010, Solarbio) to retain only the nucleated cell fraction. GFP-positive tumor cells were selected by flow cytometry and stained using the One-step TUNEL In Situ Apoptosis Kit (#E-CK-A325, Elabscience) according to the manufacturer's instructions. The apoptotic rate was determined by the percentage of TUNEL-positive cells among GFP-positive cells.

Lipid raft isolation

Lipid rafts were isolated using the UltraRIPA Kit for Lipid Raft (#F015, BioDynamics Laboratory) according to the manufacturer's instructions. Briefly, cells were harvested in 0.5 ml of the A buffer with protease inhibitor, mixed by vortexing, and incubated on ice for 10 min. Samples were then centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were kept as detergent-soluble (non-raft) fractions. To wash detergent-resistant (insoluble) pellets with the A buffer, pellets were resuspended in 1 ml of the A buffer, rigorously vortexed, and centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatants were discarded. To solubilize proteins in lipid rafts, detergent-resistant pellets were resuspended in 50 μl of B buffer, rigorously vortexed, incubated at room temperature for 5 min, and centrifuged at 12,000 rpm for 10 min at 25°C. Finally, supernatants were collected as lipid raft fractions. Protein concentrations were measured using an Enhanced BCA Protein Assay Kit (#P0010, Beyotime), and equal amounts of protein were subjected to SDS-PAGE and western blotting analysis. Lipid raft proteins were sent to Shanghai Applied Protein Technology Co., Ltd. and identified using LC-MS/MS.

Phospholipid liposomes preparation and treatment

The phospholipids DPPE (PE16:0/16:0), POPE (PE16:0/18:1), and PAPE (PE16:0/20:4) (Avanti Polar Lipids) in chloroform were evaporated under a nitrogen stream and completely dried under high vacuum. The thin lipid film was suspended in HBS buffer

and incubated in 55℃ water bath for 20 min, with occasional vortexing. Lipid suspensions were then loaded into a Mini-Extruder system (Avanti Polar Lipids) and passed through a 0.1 μm pore size polycarbonate membrane to generate 1 mM liposome stocks. 10 μM of PE liposomes and an equal amount of HBS buffer were used to treat cells for 2 days. For tail vein injection, PAPE liposomes were injected twice a week (200 μl, 2 mg/ml).

Cell adhesion assay

96-well plates were coated with 100 μg/ml fibronectin (#86088-83-7, Solarbio). MDA-MB-231 cells were seeded onto plates at a density of 3×10^4 cells/well. The plates were washed with PBS to remove unattached cells after 30 minutes of seeding. The attached cells were fixed in 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet for 10 min. Next, the cells were lysed, and the absorbance of the solution was measured at 590 nm using a plate reader (Biotek).

Centrifugation cell adhesion assay

96-well plates were coated with 100 μg/ml fibronectin and incubated for 1h at room temperature. 5×10^3 cells were added to the well and incubated for 1h to initiate adhesion. Then, 1 ml PBS was gently added to each well, and macro fluorescence photos were taken with a Leica DMi8 microscope (Leica microsystems) to quantify the initial adherent cells. After filling each well with PBS, the plate was sealed with a sealing tape and set upside-down on a centrifuge (BY-200M, Baiyang) and centrifuged at 800 rpm for 5 min to detach weakly adherent cells. After the centrifugation, the detached cells were slowly aspirated, and each well was carefully filled with 200 μl of PBS. Macro fluorescence photos of the plate were taken again. Automatic cell counting was performed with the macro fluorescence photos and ImageJ software.

Fluorescence recovery after photobleaching (FRAP) assay

Cells were stained with DiI (#C1036, Beyotime) for 10 min at 37°C. FRAP images were acquired using a Leica DMi8 microscope and the LAS X software (Leica

microsystems). One pre-bleaching image was collected, and the region of interest was bleached using 80% laser power. The fluorescence intensity was measured every 2 s for 2 min.

Transwell migration and invasion assay

2×10^4 TNBC cells were suspended in 200 μl serum-free medium. For the invasion assay, cells were seeded into the upper well of an 8-μm-pore chamber (#353097, Corning) coated with Matrigel (1:7.5, Corning). For the migration assay, the cells were seeded into the upper well without coating. The medium (500 μl) containing 10% FBS was added to the lower wells. Cells were incubated at 37°C and allowed to invade the lower chamber for 8 hours. The migrated or invaded cells of each chamber were counted under a microscope (Nikon) in five randomly selected views, and the mean value was calculated. The experiments were repeated at least three times.

Lipid analysis-mass spectrometry

Tumor tissues, avoiding non-tumor tissue, were harvested, washed with PBS immediately, placed in liquid nitrogen for 10 s, removed, and stored at -80℃. All tumor tissues were sent to BIOTREE Biotechnology (Shanghai, China) for quantitative targeted lipidomic analysis. For non-targeted lipidomic analysis, MDA-MB-231, BT-549, OVCAR-8 and HCT8 cells transfected with shRNAs were mixed with quenching agent at a 1:5 volume ratio. Cells were centrifuged at 4 °C for 5 min, 1000 g, placed in liquid nitrogen for 30 s and stored at -80°C. Cell samples were sent to BIOTREE Biotechnology (Shanghai, China) for non-targeted lipidomic detection and LipidALL Technologies (Changzhou, China) for fatty acyl-CoA analysis.

In vivo xenograft experiments

4–6 weeks-old pathogen-free severely immunodeficient female NCG (NOD/ShiLtJGpt-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Gpt) mice were purchased from GemPharmatech (Nanjing, China). Five or six mice per group were used in each experiment. MDA-MB-231 cells (1×10^6) or their derivatives were suspended in 100

μl of PBS and then injected into the tail vein of mice, whereas MDA-MB-231 cells (1×10^6) or their derivatives were suspended in 50 μl of PBS and mixed with Matrigel (1:1), and then orthotopically injected into the mouse mammary fat pad. The mice were sacrificed 30 days after the injection. All lungs and livers were harvested for metastasis analysis. For drug treatment, NCG mice weight 20-22 g, aged 6–8 weeks, were selected and intraperitoneally injected with PRGL493 (250 μg/kg, #HY-139180, MCE), cisplatin (5 mg/kg, # HY-17394, MCE), and paclitaxel (5 mg/kg, #HY-B0015, MCE). The tumor volumes were calculated using the formula (width^2 × length)/2. All animal experiments were approved by the Ethics Committee of Chongqing Medical University (Reference Number 2021085).

Statistics analysis

All in vitro experiments were independently performed at least three times, as indicated in the figure legends. Statistical analyses were performed using Prism (version 9.0, GraphPad software). The log-rank test was used for the statistical analysis of survival. Two-tailed Student's t-test was used to calculate the statistical differences between the two groups. One-way ANOVA was used to calculate the statistical differences between any three or more groups. Two-way ANOVA was used to analyze the FRAP fluorescence recovery. Fisher's exact test was employed for comparing categorical variables. *P*-values are shown in the figures and/or legends with asterisks. ***, $P < 0.001$; **, $P < 0.01$, *, $P < 0.05$. *P* values were considered statistically significant at $P < 0.05$.

Data Availability

The breast cancer patient data were acquired from the METABRIC datasets via cBioPortal (http://www.cbioportal.org/), ROC plotter website (https://www.rocplot.org/), UCSC Xena (http://xena.ucsc.edu/) and Kaplan-Meier Plotter (https://kmplot.com/analysis/); Gene expressions of TNBC and non-TNBC cell lines were acquired from CCLE datasets (https://sites.broadinstitute.org/ccle). All other data generated in this study are available upon request from the corresponding author.

Results

1 Phospholipid unsaturation is elevated in metastatic TNBC tumors

Our early study found that lipid metabolism was activated in TNBC. To determine the lipid profile of metastatic TNBC tumors, LC-MS/MS-based lipidomic analysis was performed to detect altered lipid metabolites in four pairs of primary and metastatic tumor tissues in livers from NCG mice injected with MDA-MB-231 cells. A total of 785 lipid metabolites were identified, including 71 that were significantly increased and 70 that were decreased in metastases compared to the tumors in situ (Fig S1A). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) species, which mainly exist in the plasma membrane, are the major and most significantly altered lipids in metastases (Fig. S1B). To further analyze the acyl chain composition of phospholipids (PLs), we found that the abundance of saturated fatty acid-containing phospholipids (SFA-PLs) and monounsaturated fatty acid-containing phospholipids (MUFA-PLs) was markedly decreased, while the abundance of polyunsaturated fatty acid-containing phospholipids (PUFA-PLs) was significantly higher in metastatic tumors than in primary tumors (Fig S1C). After assessing the proportion of phospholipids with different saturation degrees (fatty acyl chain C=C number), we found that metastatic tumors tended to produce more total phospholipids (Fig 1A), PE (Fig 1B), and PC (Fig 1C) with four or more unsaturation and less saturated and monounsaturated phospholipids. In mammalian cells, saturated fatty acids are preferentially linked at the *sn*-1 position of phospholipids, and unsaturated fatty acids at the *sn*-2 position (13). Therefore, unsaturated fatty acids at the *sn*-2 position of the phospholipids were further analyzed. Both lipid chain length and unsaturation of PLs were notably different between metastatic and primary tumors in situ, such as higher levels of polyunsaturated fatty acyl chains (18-22 carbon acyl chains) (Fig 1D-1E) and more polyunsaturated PC and PE were detected in metastatic tumors (Fig 1F-1G). These data indicate a close relationship between polyunsaturated PC, PE, and tumor metastasis. Thus, the abundance of PC- or PE-containing long-chain PUFAs (LC-PUFAs) was further evaluated in metastatic and in situ tumors. As shown in Fig 1H, some representative LC-PUFA-containing phospholipids, such as PE(16:0/22:6), PC(16:0/22:6),

PE(16:0/20:5), and PE(16:0/20:4), were significantly increased in metastatic tumors, which was consistent with the data obtained from metastatic TNBC patients, with higher PUFA-PLs in metastases (Fig. S1D). Taken together, these data suggest that phospholipids with longer PUFAs are metabolic characteristics of metastatic TNBC.

2 The enhanced ACSL4 is correlated with poor prognosis of TNBC and contributes to metastasis

TNBC tumors feature a high metastatic index compared to ER+ BC. ER+ tumors have more PL-MUFAs, while TNBC tumors have more PL-PUFAs, suggesting that primary TNBC tumor possesses unique phospholipid remodeling that may facilitate tumor metastasis (14,15). Previous studies have confirmed that acyl-CoA synthetase, lyso-phospholipid acyltransferase, and phospholipase A2 isotypes play important roles in phospholipid saturation (16). To explore the key enzymes regulating PUFA-phospholipid remodeling in TNBC cells, we first analyzed the differentially expressed genes (DEGs) between TNBC patients and ER-positive (ER+) patients using the METABRIC (Molecular Taxonomy of Breast Cancer International Consortium) dataset. By comparing the DEGs and metabolism-related genes in the KEGG human metabolic gene list in detail, 149 aberrantly expressed metabolic DEGs were identified in the TNBC subtype (Fig S2A). Among these, four candidate genes (ACSL4, PLA2G10, PLA2G16, and PLA2G7) were found to be related to phospholipid remodeling in TNBC (Fig. S2B). The data from the UCSC Xena datasets revealed that ACSL4 was preferentially upregulated and PLA2G10 was slightly downregulated in patients with basal breast cancer compared to those with other subtypes (Fig S2C). Preferential upregulation of ACSL4 in TNBC cells was also observed after analyzing its mRNA levels in 45 breast cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE) datasets (Fig S2D-S2E), which was further confirmed in representative TNBC and non-TNBC cell lines (Fig S2F).

To expand the above findings, ACSL4 protein levels were evaluated by immunohistochemical staining (IHC) in 30 paired tumors and normal tissues from patients with TNBC. Stronger staining of ACSL4 protein was observed in the vast

majority of TNBC tissues (Fig 2A-2B). Next, a tissue microarray (TMA) containing samples from 95 patients with luminal breast cancer, 34 patients with Her2+ breast cancer, and 31 patients with TNBC from a retrospective cohort of patients was employed. Consistently, there was a higher IHC score for ACSL4 in TNBC than in other subtypes, such as luminal or $HER2^{+}$ breast cancer (Fig 2C-2D). High levels of ACSL4 were notably correlated with N stage (Fig 2E) and poor overall survival of breast cancer patients (Fig 2F) according to Kaplan–Meier survival analysis. In our cohort of breast cancer with or without metastasis, ACSL4 expression apparently correlated with distant metastasis (Fig 2G-2H). In our cohort of TNBC tumors, higher mRNA levels of ACSL4 were observed in primary tumors from metastatic TNBC patients than in non-metastatic TNBC patients (Fig 2I). Moreover, the expression of ACSL4 in patients with breast cancer was negatively correlated with distant metastasis-free survival using the KM plot database (Fig S2G). Importantly, higher level of ACSL4 was observed in lung and liver metastases than the paired primary tumors from NCG mice injected with MDA-MB-231 cells. Similarly, ACSL4 expression was increased in metastases compared with that in primary tumors from patients with breast cancer (Fig 2J-2L). To further confirm the role of ACSL4 in TNBC metastasis, shRNA-mediated ACSL4 knockdown cells and ectopic ACSL4 re-expression in ACSL4-deficient cancer cells were established (Fig S3A). Remarkably, ACSL4 knockdown impaired the migration and invasion abilities of TNBC cells, which could be rescued by ectopic ACSL4 (rACSL4) (Fig S3B-S3E). In the NCG mouse model, loss of ACSL4 in MDA-MB-231 markedly induced circulating tumor cell (CTC) apoptosis (Fig 2M), and decreased the metastatic burden in the lung and liver, accompanied by reduced Ki-67 positive cells in the metastatic lesions (Fig 2N-2O). Collectively, these data demonstrate that ACSL4 is preferentially expressed in TNBC patients and promotes TNBC metastasis by facilitating cell invasion, anoikis resistance, and proliferation of cells seeded in distant tissues. Enhanced ACSL4 serves as a biomarker for malignant TNBC patients.

3 ACSL4 promotes PUFA-PLs production in TNBC cells

As the main components of the membrane, PLs are synthesized de novo through the

Kennedy process and then undergo deacylation-reacylation remodeling via the Lands cycle (17). ACSL4 has been known to activate PUFA to produce PUFA-derived acyl-CoAs, which are esterified into phospholipids through the lysophospholipid acyltransferase-mediated reacylation process (18) (Fig 3A). Indeed, most PUFA-CoA productions, such as 20:4-CoA and 22:5-CoA, decreased (Fig S4A), whereas most SFA-CoAs and MUFA-CoAs increased in ACSL4-deficient cells (Fig S4B), as detected by a non-targeted tandem mass spectrometry assay. We investigated whether PCs and PEs were altered in ACSL4-silenced cells. As expected, a series of PUFA-PCs and PUFA-PEs was reduced by the loss of ACSL4 (Fig S4C). After further analysis of phospholipid saturation, we noticed that the percentage of total phospholipids (Fig S4D), PEs (Fig 3B), and PCs (Fig 3C) with 4+ saturation degree were markedly decreased, and the percentage of phospholipids with one saturation degree was increased in both ACSL4-knockdown MDA-MB-231 and BT-549 cells. Among these LC-PUFA-containing PLs, PE(18:0/20:4) and PE(16:0/20:4) were significantly decreased after ACSL4 knockdown (Fig 3D). Similar results were observed in other ACSL4-high-expressing cancer cells (OVCAR-8 and HCT8), with ACSL4 knockdown decreasing LC-PUFA-containing PLs (Fig S4E). It has been reported that phospholipid composition and saturation influence membrane fluidity and function (19). We propose that ACSL4-mediated phospholipid metabolites potentially affect cellular membrane features. Checked by fluorescence recovery after photobleaching (FRAP) assay using the lipid phase-sensitive fluorescent probe DiI (red fluorescence), cellular membrane fluidity was remarkably decreased in ACSL4-deficient MDA-MB-231 cells (Fig 3E-3G), suggesting that the loss of ACSL4 enhances membrane phospholipid saturation of tumor cells. These data demonstrate that ACSL4-mediated phospholipid metabolism has apparent effects on the phospholipid composition and biological features in the cell membrane of TNBC cells.

Next, we investigated whether the ACSL4-mediated alteration of PUFA-PLs affected TNBC metastasis. Thus, ACSL4-knockdown tumor cells were treated with representative SFA-PE (PE 16:0/16:0, 1, 2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine, DPPE), MUFA-PE (PE 16:0/18:1, 1-palmitoyl-2-oleoyl-sn-

glycero-3-phosphatidylethanolamine, POPE), and PUFA-PL (PE 16:0/20:4, 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylethanolamine, PAPE), and cell metastatic ability was assessed. As expected, treatment of ACSL4-deficient tumor cells with exogenous PAPE liposomes (Fig S4F) successfully rescued the invasion (Fig 3H, Fig S4G), anoikis resistance (Fig 3I-3J) of ACSL4-knockdown cells. PAPE liposome administration also increased metastatic burden of ACSL4-deficient cells, accompanied by more Ki-67 positive cells in metastases (Fig 3K-3L). PUFA-phospholipids can be catalyzed into PUFAs (e.g. Arachidonic acid, AA; eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) and lysophospholipids (e.g., Lysophosphatidic Acid, LPA) by PLA2; and these phospholipid-derived metabolites may potentially have tumor-promoting (e.g., AA, LPA) or tumor-suppressing (e.g., EPA, DHA) effects (20-23). To determine whether exogenous PAPE is degraded into AA and LPA within 2 days of phospholipid supplementation, we measured the AA content in tumor cells after phospholipid supplementation. We found that free AA was not notably increased (Fig. S4H). Furthermore, cell invasion and anoikis resistance were assessed in ACSL4-knockdown breast cancer cells in the presence of PAPE with or without giripladib (PLA2 inhibitor). Administration of tumor cells with PAPE and giripladib had no significant effects on the invasion ability (Fig 3H, Fig S4G) and anoikis resistance (Fig 3I-3J) of ACSL4-knockdown cells compared to those treated with PAPE alone, suggesting that PAPE, but not PAPE-derived metabolites, plays an essential compensatory role in ACSL4-silenced breast cancer cell invasion and anoikis resistance. Collectively, ACSL4 acts as a regulator of membrane phospholipid composition and unsaturation, thereby contributing to breast cancer cell invasion, anoikis resistance, and metastatic growth.

4 Phospholipid remodeling facilitates the location and interaction of ITGB1 and CD47 in lipid rafts

To explore the mechanism underlying ACSL4-mediated cell metastasis, we analyzed the ACSL4-associated signaling networks. Gene expression profiles in breast tumors from TCGA database revealed that ACSL4 expression was correlated with signaling

pathways involved in tumor metastasis, such as ECM-receptor interaction and focal adhesion (Fig S5A). Membrane phospholipid saturation has been reported to affect the localization and activation of oncogenic receptors in lipid rafts (24), and nystatin treatment, which was used to disrupt lipid rafts, decreased cell invasion of wild-type TNBC cells (Fig S5B). Therefore, we hypothesized that ACSL4-increased phospholipid unsaturation may activate pro-metastatic signaling molecules, including receptors and proteins, in lipid rafts to support metastasis-associated signaling pathways. Raft proteins were first analyzed by LC-MS/MS in the control and ACSL4-knockdown MDA-MB-231 cells. In total, 255 proteins were significantly upregulated and 423 were downregulated in ACSL4-deficient MDA-MB-231 cells. These ACSL4-asscoiated proteins, including the well-known membrane proteins CD44, CD47, and integrin β1 (ITGB1), were enriched in the ECM-receptor interaction and focal adhesion signaling pathways (Fig S5C). In addition, the activation of focal adhesion kinase (FAK) (an essential downstream element of ECM-receptor interaction and focal adhesion signaling pathway) was diminished in ACSL4-deficient TNBC cells (Fig S5D) and nystatin-treated control or ACSL4-knockdown MDA-MB-231 cells (Fig S5E). These data suggested that phospholipid remodeling may affect the localization of CD44, CD47, and integrin β1 in rafts, contributing to the activation of FAK signaling. Indeed, further experiments verified that CD47 and ITGB1 in lipid rafts were notably reduced after ACSL4 knockdown without significant changes in whole cell lysates (Fig 4A). Treatment with exogenous PAPE liposomes almost completely restored the protein levels of CD47 and ITGB1 in the lipid rafts of ACSL4-deficient TNBC cells (Fig 4B-4D). Subsequently, we explored whether unsaturated phospholipids affect the size and number of lipid rafts. By staining ganglioside GM1, a lipid raft marker, using the Cholera Toxin B subunit (CTB) antibody that specifically recognizes the pentasaccharide chain of plasma membrane GM1, we found that there was almost no change in ganglioside GM1 staining between ACSL4-knockdown TNBC cells and control cells (Fig 4E-4F), indicating that lipid raft formation is not affected by ACSL4.

CD47 is an integrin-associated protein (25), and we investigated whether membrane lipid remodeling influences the interaction between CD47 and ITGB1 in lipid rafts. IF

staining and IP-WB revealed remarkable colocalization of ITGB1 and CD47 in lipid rafts (Fig S5F) and their interaction (Fig S5G) in ACSL4 wild-type TNBC cells. The loss of ACSL4 caused a significant decrease in the interaction between ITGB1 and CD47, which could be rescued by supplementation with PAPE liposomes. However, the lipid raft inhibitor MβCD reduced the interaction between ITGB1 and CD47 in the PAPE-treated ACSL4-knockdown cells (Fig 4G). Collectively, ACSL4-mediated membrane phospholipid unsaturation and phospholipid remodeling facilitate the localization of CD47 and ITGB1 and their interactions in lipid rafts.

5 CD47 is essential for unsaturated phospholipid-mediated activation of integrin β1/FAK signaling

Activated integrin is crucial for downstream FAK signaling activation (26). However, it remains unclear whether integrin β1 activity is associated with its lipid raft location and/or affected by ACSL4-mediated membrane phospholipid remodeling. Thus, we first detected the location of activated integrin β1 (Act-ITGB1) in the lipid rafts. Using IF staining, we confirmed that the loss of ACSL4 reduced Act-ITGB1 localization in lipid rafts, which could be efficiently restored by exogenous PAPE liposome treatment (Fig 5A-5B). ACSL4 knockdown led to a significant decrease in Act-ITGB1 protein levels in lipid rafts and whole lysates of TNBC cells, which could also be restored by administrating cells with PAPE; interestingly, the Act-ITGB1 levels in non-rafts fraction were not affected by ACSL4 and exogenous PAPE (Fig 5C-5D). Knockdown of caveolin-1 (CAV1, another lipid raft marker) to disrupt the lipid raft structure caused a marked decrease in activated integrin β1 in the lipid rafts of control TNBC cells and abrogated the ability of PAPE to restore integrin β1 activation in ACSL4-deficient TNBC cells (Fig S6A). Second, we sought to determine whether Act-ITGB1 in lipid rafts activates p-FAK signaling and contributes to unsaturated phospholipid-induced tumor cell invasion. PAPE treatment effectively increased p-FAK levels in ACSL4-knockdown TNBC cells, which was blunted by CAV1 silencing (Fig. S6A). Loss of ITGB1 impaired FAK signaling (Fig S6B) and cell adhesion to fibronectin (Fig S6C-S6D) in PAPE-treated ACSL4-deficient tumor cells. Similarly, using the FAK inhibitor,

defactinib, to block FAK phosphorylation (Fig S6E) substantially stalled PAPE-induced cell migration and invasion (Fig S6F-S6G) as well as aniokis resistance (Fig S6H-S6I) in ACSL4-knockdown TNBC cells. These data suggest that membrane phospholipid remodeling, resulting in the localization of ITGB1 in lipid rafts, is responsible for ITGB1/p-FAK signaling activation and cell invasion.

It has been reported that the interaction of integrin β1 with other proteins in lipid rafts contributes to ITGB1-mediated cell invasion (27). We investigated whether CD47 was required to activate integrin β1/FAK signaling. As shown in Figure 5E, silencing of ACSL4 resulted in decreased colocalization of Act-ITGB1 and CD47 in the TNBC cell membrane. In contract, exogenous PAPE treatment restored the colocalization of Act-ITGB1 and CD47 in ACSL4-deficient TNBC cell, which was disturbed by CD47 knockdown. Furthermore, CD47 silencing decreased ITGB1/p-FAK signaling activation and cell invasion in TNBC cells, and CD47 deletion also significantly impaired the ability of exogenous PAPE to restore ITGB1/p-FAK signaling (Fig 5F), cell invasion (Fig 5G-5H), and anoikis resistance (Fig 5I-5J) in ACSL4-deficient TNBC cells. Taken together, our data demonstrated that CD47 is essential for membrane phospholipid remodeling-mediated integrin β1/FAK signaling activation and TNBC cell invasion.

To prove the association between ACSL4 expression and activation of ITGB1/p-FAK signaling *in vivo*, we analyzed ACSL4, ITGB1, and p-FAK protein levels in TNBC tumor tissues. ACSL4-high-expressing tumors had enhanced the expression and localization of Act-ITGB1 in lipid rafts (Fig 6A-6B), significantly related to increased activation of FAK signaling (Fig 6C-6D).

6 Targeting ACSL4 increases TNBC sensitivity to chemotherapy and suppresses TNBC growth and metastasis

As ACSL4 is highly expressed in TNBC and is correlated with enhanced distant metastasis, we further explored the potential clinical value of targeting ACSL4. Using the ROC Plotter, we found that TNBC patients with lower ACSL4 levels were sensitive to paclitaxel (PAC) (responders), whereas patients with higher ACSL4 levels were non-

responders (Fig 7A). Similar results were observed in our cohort of 40 patients treated with paclitaxel, in which most non-responder tumors had higher ACSL4 protein levels than responders (Fig 7B-7C). Given that cisplatin (Cisp) alone or combined with PAC has been demonstrated effective against TNBC in a neoadjuvant and metastatic setting (28), we evaluated the effects of chemotherapy (PAC and Cisp) in combination with PRGL493 (an inhibitor targeting ACSL4) on TNBC tumor response in NCG mice (Fig 7D). As shown in Figure 7E-7G, treating the tumor burden mice with PRGL493 combined with PAC and Cisp remarkably decreased tumor growth compared to PAC in conjunction with cisplatin, PAC, cisplatin, and PRGL493 treatment alone. Similar results were obtained for the lung and liver metastatic lesions in each group of experimental mice (Fig 7H-7J). In all cases, we observed no apparent effect on the body weight of the mice (Fig S7A). Interestingly, PRGL493 administration led to a considerably smaller tumor volume of control TNBC cells, and PRGL493 administration didn't affect the tumor size of ACSL4-deficient cells (Fig S7B-S7C). An accompanying decrease in LC-PUFAs-containing phospholipids was observed, with PRGL493 treatment reducing the levels of PC(16:0/20:4), PE(18:0/20:4), and PE (16:0/20:4) (Fig S7D). Taken together, these data demonstrate that targeting ACSL4 increases the sensitivity of TNBC tumors to chemotherapy.

Discussion

TNBC patients have the worst prognosis because of their aggressive and metastatic phenotypes, high relapse rates, and lack of effective therapeutic targets (29). Although accumulating evidence demonstrates that lipid metabolism plays an essential role in cancer progression and metastasis (30), the underlying mechanism by which abnormal lipids drive TNBC metastasis remains unclear. Here, we showed that phospholipid unsaturation was significantly increased in metastatic TNBC cells than in primary cells. Long-chain acyl-coenzyme A synthetase 4 (ACSL4), a determinant enzyme in PUFA activation, is upregulated in TNBC cells and induces the incorporation of PUFA into phospholipids, resulting in membrane phospholipid remodeling. Moreover, ACSL4-mediated phospholipid remodeling facilitated the enrichment of integrin β1 and CD47 in lipid rafts, activating integrin β1/FAK signaling in a CD47-dependent manner to promote TNBC metastasis (Fig 7K). Notably, targeting ACSL4 improves TNBC sensitivity to neoadjuvant chemotherapy in vivo, providing a potential therapeutic strategy for patients with TNBC.

The metabolic characteristics of metastatic TNBC include increased levels of polyunsaturated phospholipids and phospholipid unsaturation. Altered lipid metabolism is a central hallmark of cancer. The phospholipid composition and saturation determine the biophysical properties of membranes and influence various cellular processes. Increased phospholipid saturation has been reported in clear renal cell carcinoma (31), hepatoma (32), and Apc-mutant intestinal tumor (33). In contrast, ovarian cancer stem cells have high levels of unsaturated lipids that maintain cancer stemness and tumor initiation capacity (34). The maintenance of glioblastoma stem cells depends on polyunsaturated fatty acid synthesis to control the membrane phospholipid composition and structure (11). Thus, the effects of phospholipid saturation on cancer progression are likely to be complex and cell type-specific. In this study, we observed that PE and PC were the most altered lipid species and that polyunsaturated PCs and PEs were significantly increased in metastatic TNBC tumors, resulting in a noticeable increase in phospholipid unsaturation. Recently, it's reported that the lipogenic subtype of TNBC was enriched with high levels of gene expression

associated with *de novo* lipid synthesis, such as FASN and SCD, supporting higher levels of unsaturated fatty acids in TNBC tumors (35). These findings demonstrate that the increased biosynthesis of unsaturated fatty acids and polyunsaturated phospholipids may play critical roles in TNBC progression.

ACSL4 drives membrane phospholipid remodeling to promote TNBC metastasis. Long-chain acyl-coenzyme A synthetases (ACSLs) are a family of enzymes that convert free long-chain fatty acids into acyl-CoA forms. Among these, ACSL4 mainly facilitates the incorporation of intracellular long-chain PUFAs into phospholipids (36). ACSL4 is known to enrich cellular membranes with PUFAs and determine cell sensitivity to ferroptosis (37); the impact of phospholipid remodeling on the progression of ACSL4-high expressing cancer cells is unclear. The current study demonstrated that ACSL4 was preferentially expressed in tumors from patients with TNBC, and the loss of ACSL4 downregulated the proportion of unsaturated PE and PC. These alterations in membrane phospholipid composition promote TNBC metastasis, indicating that phospholipid composition and unsaturation mediated by ACSL4 play a critical tumor-promoting role in TNBC. Similarly, a high-fat diet augmented hepatic lysophosphatidylcholine acyltransferase 3 (LPCAT3) expression and increased phospholipid unsaturation, contributing to insulin resistance in the liver (38). FABP3 overexpression in newly formed muscles decreases polyunsaturated phospholipids, resulting in limited muscle recovery and aging (39). These studies highlight that abnormal polyunsaturated phospholipid metabolism is closely associated with disease.

Lipid rafts play critical roles in the pro-metastatic signaling mediated by the ACSL4-polyunsaturated PE axis in TNBC cells. Lipid rafts are cholesterol- and sphingolipid-enriched microdomains within the plasma membrane that act as scaffolds to enhance intracellular signaling cascades in metastatic cancer cells (7). For example, in metastatic prostate cancer, the CXCL12/CXCR4 chemokine axis specifically activates EGFR, HER2, and Src within the lipid raft microdomains to promote cell migration (40). In addition, CD44 interacts with C1QBP in lipid rafts to activate PI3K/MAPK downstream, a signaling pathway that facilitates the pro-metastatic phenotypes of pancreatic cancer (41). Here, we demonstrated that ACSL4-mediated phospholipid

remodeling induced the enrichment and interaction of integrin β1 and CD47 in lipid rafts, activating integrin β1/FAK signaling, and promoting TNBC metastasis. In parallel, increasing membrane saturation has been shown to affect compartmentalization and activity of membrane-bound c-Src kinase by recruiting proteins to lipid rafts of adipocytes (42). Supplementation with DHA remodeled phospholipid composition and membrane phenotypes in human mesenchymal stem cells, which increased Akt activation in raft microdomains, thereby promoted osteogenic differentiation (43). Herein, our work and previous studies support that membrane phospholipid composition and degree of saturation are important regulators of protein–protein interactions and oncogenic molecule activation in lipid rafts. Another interesting finding of the current study is that CD47 is crucial for integrin β1 function, and CD47 deficiency impairs the enrichment and activation of integrin β1 in lipid rafts, thus mitigating ACSL4-polyunsaturated PE axis-mediated FAK phosphorylation as well as TNBC invasion and anoikis resistance. Tumor metastasis is a multiple-step process, almost every step of which has been regulated by integrins (44). The interaction of integrin with other proteins, such as nucleolin (45), CD44 (46), and Annexin (47), plays critical roles in integrin signaling pathways. Thus, our work and other studies support the hypothesis that the interaction of integrin with other proteins is necessary for lipid-raft-dependent integrin β1 activation and tumor metastasis.

Combination therapy consisting of an ACSL4 inhibitor, paclitaxel, and cisplatin represses TNBC metastasis. Chemotherapy plays an irreplaceable role in cancer treatment. Relapse and drug tolerance often occur in the later stages of chemotherapy (48), and exploring new molecular targets to overcome resistance and increase chemotherapy sensitivity in TNBC is crucial for improving patient prognosis. Strategies for targeting aberrant lipid metabolism in cancer therapy have been explored. In glioblastoma, ELOVL2 and FADS2 are two key enzymes involved in LC-PUFA synthesis and phospholipid composition, and inhibition of ELOVL2 or FADS2 could augment tumor cell apoptosis by the EGFR inhibitor, lapatinib (11). In chemoresistant TNBC tumors, silencing ACSL4 inhibited catabolism of fatty acids and reduced phospholipid content, accompanied by decreased mitochondrial membrane

phospholipids, which effectively sensitize these tumors to paclitaxel (49). These studies provide evidence that targeting the biosynthesis or catabolism of fatty acids to impair the structural components of the cell membrane is a promising combinatorial target to improve chemotherapy sensitivity. Here, we confirmed that PRGL493, combined with paclitaxel and cisplatin, could maximally inhibit TNBC growth and metastasis by directly targeting ACSL4 and decreasing cellular unsaturated phospholipids. Our results further strengthen the potential benefits of targeting fatty acid catabolism to enhance antitumor responses in clinical settings.

In summary, this study demonstrates that upregulated phospholipid unsaturation is a critical feature of metastatic TNBC. We identified ACSL4 as a driver of TNBC metastasis, both in vitro and in vivo. Mechanistically, ACSL4-mediated phospholipid remodeling contributes to the enrichment and interaction of integrin β1 and CD47 in lipid rafts, thus facilitating TNBC invasion by activating integrin β1/FAK signaling in a CD47-dependent manner. Targeting ACSL4 increases TNBC sensitivity to paclitaxel/cisplatin. This finding may facilitate the development of a selective treatment strategy to prevent metastasis in patients with TNBC.

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Authors' Contributions

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Figure Legends

Figure 1. Phospholipid unsaturation is elevated in metastatic TNBC tumors.

A-H, Lipidomic analysis of primary tumors and paired liver metastases harvested 30 days after mammary fat pad injection with MDA-MB-231 cells (n=4). The percentage of phospholipids (PLs) (**A**), phosphatidylethanolamine (PE) (**B**), and Phosphatidylcholine (PC) (**C**) with different unsaturations in total PLs, PC, or PE. The percentage of PE (**D**) or PC (**E**) with less than C18 and C18-22 acyl chains at *sn*-2 in total PE or PC. The percentage of PE (**F**) or PC (**G**) with saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) at *sn*-2 in total PE or PC. **H,** Relative abundance of representative PUFA-PLs in primary and metastatic tumors. The data are shown as the normalized fold change of metastatic tumors compared to primary tumors. All data represent mean ± SD. Student's *t*-test was utilized: $^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P< 0.001$, ns, no significance.

Figure 2. The enhanced ACSL4 correlates with poor TNBC prognosis and contributes to metastasis.

A and **B,** Representative images (**A**) and quantification (**B**) of ACSL4 IHC staining in TNBC tumor samples (n=30) and paired adjacent normal tissues (n=30). **C-F,** ACSL4 IHC staining in a tissue microarray (TMA-1) containing primary tumor samples from patients with breast cancer. Representative images (**C**) and quantification (**D**) of ACSL4 IHC staining in breast cancer tissues from patients with different subtypes. (**E**) IHC scores of ACSL4 in breast cancer tissues from patients with different N stages. (**F**) Kaplan-Meier analysis of the overall survival of patients with breast cancer in the TMA-1 cohort. **G** and **H**, Representative images (**G**) and quantification (**H**) of ACSL4 IHC staining in primary breast tumor tissues from patients with (M+) or without (M-) distant metastasis. **I,** Relative mRNA levels of ACSL4 in the primary TNBC tumor tissues from patients with (M+) or without (M-) metastasis. The data are shown as the normalized fold change of TNBC M+ compared to TNBC M-. **J** and **K,** Representative IHC staining images (**J**) and quantification (**K**) of TMA-2 cohort ACSL4 expression in the primary and metastatic breast tumors. Primary and metastatic tumor tissues from

NCG mice injected with MDA-MB-231 cells were shown in the upper panel; unpaired primary and metastases from the TMA-2 cohort containing primary and metastatic breast cancer tissues from patients were shown in the lower panel. **L,** IHC staining images of ACSL4 expression in the paired primary and metastases tumors from 2 patients with TNBC. **M,** Representative images of TUNEL staining and quantification of TUNEL-positive circulating MDA-MB-231 cells (CTCs) to evaluate anoikis resistance of CTCs. CTCs (GFP-positive) were isolated from mouse blood and selected by flow cytometry. **N-O,** MDA-MD-231 cells expressing two independent, non-overlapping shRNA against ACSL4 (shACSL4#1, shACSL4#2) or non-targeting shRNA (shNC) were injected into the tail vein of NCG mice. Lungs and livers were harvested at day 30 after injection. (**N**) Representative images of lung and liver and Ki-67 IHC staining in metastases (n=6/group). (**O**) Quantification of metastatic nodules of lung surfaces (left), metastatic foci of liver sections (middle), and Ki-67 levels in lung or liver metastatic areas (right). For Ki-67 quantification, 9 random microscopic fields from 3 mice of each group were captured. All data represent mean ± SD. Fisher's exact test (**B**), Student's *t*-test (**E, H**, **I**, **M,** and **O**), one-way ANOVA (**D** and **K**) and log-rank test (**F**) were utilized; $^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P< 0.001$.

Figure 3. ACSL4 promotes PUFA-PL production in TNBC cells.

A, Schematic diagram of ACSL4 catalyzes LC-PUFA into LC-PUFA-phospholipids. **B-D,** Lipidomic analysis of control (shNC) and ACSL4-knockdown (shACSL4#1) TNBC cells (n=3). Percentage of PEs (**B**) or PCs (**C**) with different unsaturations in total PEs or PCs. (**D)** Relative abundance of representative PUFA-PLs. The data are shown as the normalized fold change of ACSL4-knockdown cells compared to the control cells. **E-G,** Representative images of fluorescence recovery after photobleaching (FRAP) in the control and ACSL4-knockdown MDA-MB-231 cells (**E**). The plasma membrane was labeled by DiI (red fluorescence). FRAP curves (**F**) and quantification of total fluorescence recovery (**G**) following photobleaching (n=18/group). **H-J**, the control, and ACSL4-knockdown TNBC cells were treated with indicated phospholipid liposomes alone or combined with giripladib. Representative images of invasion (**H**)

and flow cytometry (Annexin V-APC/PI staining) (**I**). Before Annexin V-APC/PI staining, cells were detached culture for 24 h. PI, Propidium iodide. Quantification of apoptotic rate (**J**) in (**I**). **K** and **L**, the control and ACSL4-knockdown MDA-MB-231 cells were injected into the tail vein of NCG mice, and PAPE liposomes were intravenously injected twice a week. Lungs and livers were harvested at day 30 after injection. Representative images (**K**) and quantification (**L**) of H&E staining and Ki-67 IHC staining in lung and liver metastasis (n=6 mice/group). For Ki-67 quantification, 9 random microscopic fields from 3 mice of each group were captured. All data represent mean ± SD. One-way ANOVA (**J** and **L**), two-way ANOVA (**F**), and Student's *t*-test (**B, C, D**, and **G**) were utilized; $^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P< 0.001$, ns, no significance.

Figure 4. Phospholipid remodeling facilitates the location and interaction of ITGB1 and CD47 in lipid rafts

A, Western blot was used to detect CD44, CD47, and integrin β1 (ITGB1) protein levels in total cell lysates (Total) and lipid raft fractions (Rafts) derived from the control (shNC) and ACSL4-knockdown (shACSL4#1) TNBC cells (n=3). Flotillin-1 was a marker for lipid rafts. β-actin was used as a loading control for total cell lysates. **B,** Western blot analysis of CD47 and ITGB1 in total cell lysates (Total), lipid rafts (Rafts), and non-lipid rafts (Non-rafts) of the control and ACSL4-knockdown TNBC cells treated with or without PAPE (n=3). **C** and **D,** Densitometry analysis of protein levels of ITGB1 (**C**) and CD47 (**D**) in (**B**). The data are shown as the normalized fold change of ACSL4-knockdown cells with or without PAPE treatment compared to the control cells. **E,** Representative IF staining images of lipid rafts (CTB-labeled, red) in the control and ACSL4-knockdown TNBC cells. **F,** Quantification of mean fluorescence intensity of CTB-labeled lipid rafts in (**E**). The data are shown as the normalized fold change of ACSL4-knockdown cells compared to the control cells. **G,** Co-IP analysis of the interaction between ITGB1 and CD47 in TNBC cells with indicated treatments (n=3). MβCD was used to disrupt lipid raft. All data represent mean ± SD. One-way ANOVA (**C** and **D**) and Student's *t*-test (**F**) were utilized; $^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P< 0.001$, ns,

no significance.

Figure 5. CD47 is essential for unsaturated phospholipid-mediated activation of integrin β1/FAK signaling

A, Representative IF staining images of lipid rafts (CTB-labeled, red) and activated integrin β1 (Act-ITGB1, green) in the control (shNC) and ACSL4-knockdown (shACSL4#1) TNBC cells with or without PAPE treatment. The colocalization of Act-ITGB1 with rafts shown in yellow. **B,** Quantification of colocalization rate in (**A**)**.** The colocalization rate was determined using LAS X software. **C,** Western blot of Act-ITGB1 and ITGB1 in total cell lysates (Total), lipid rafts (Rafts), and non-lipid rafts (Non-rafts) of the control and ACSL4-knockdown TNBC cells treated with or without PAPE (n=3). Flotillin-1 was a marker for lipid rafts. β-actin was used as a loading control for total cell lysates. **D,** Quantification of Act-ITGB1 protein levels in (B). The data are shown as the normalized fold change of ACSL4-knockdown cells with or without PAPE treatment compared to the control cells. **E,** Representative IF staining images of colocalization of Act-ITGB1 with CD47 in the control and ACSL4-knockdown TNBC cells transfected with siRNAs against CD47 (si-CD47) in the presence of PAPE. The colocalization of Act-ITGB1 with rafts shown in yellow. **F,** Western blot analysis of Act-ITGB1 and ITGB1 in lipid rafts (Rafts), as well as p-FAK and CD47 in total cell lysates (Total) of TNBC cells with indicated treatments (n=3). Flotillin-1 was a marker for lipid rafts. β-actin was used as a loading control for total cell lysates. **G,** Representative images of cell invasion in TNBC cells with indicated treatments. **H**, Quantification of invaded cells in (**G**). **I,** Representative images of flow cytometry (Annexin V-APC/PI staining) in TNBC cells with indicated treatments. **J,** Quantification of cell apoptotic rate in (**I**). All data represent mean ± SD. One-way ANOVA (**B** and **D**) and Student's *t*-test (**H** and **J**) were utilized; ** $P< 0.01$, *** $P< 0.001$.

Figure 6. ACSL4 expression level positively correlates with ITGB1 and FAK activation status in TNBC samples

A, Representative images of ACSL4 IHC staining and IF staining of lipid rafts (CTB-

labeled, red) and activated integrin β1 (Act-ITGB1, green) in TNBC tissues with high (n=13) or low (n=28) ACSL4 expression. The colocalization of Act-ITGB1 with rafts shown in yellow. **B,** Quantification of colocalization rate of Act-ITGB1 with lipid rafts in (**A**) **C,** Representative IHC staining images of ACSL4, Act-ITGB1, and p-FAK in TNBC tissues with high or low ACSL4 expression. **D,** Proportions of samples with low or high expression of Act-ITGB1 (left) or p-FAK (right) classified by ACSL4 expression in (**C**). Student's *t*-test (**B**) and Fisher's exact test (**D**) were utilized. $^{*}P< 0.05$, $^{***}P< 0.001$.

Figure 7. Targeting ACSL4 increases TNBC sensitivity to chemotherapy and suppresses TNBC growth and metastasis

A, Online analysis by ROC plotter website (https://www.rocplot.org/) of ACSL4 expression levels in TNBC with or without paclitaxel (PAC) response. **B,** Representative IHC staining images of ACSL4 in PAC-response or non-response TNBC tissues (n=20/group). **C,** Proportion of TNBC patients with high or low ACSL4 expression in non-responders and responders. **D,** Drug treatment schedule for administering PRGL493, cisplatin (Cisp), and paclitaxel (PAC) to NCG mice grafted with MDA-MB-231 cells. Orthotopic mammary fat pad injection (i.m.f.p.). n=6 per group. **E-G,** Images of primary tumors (**E**) from the NCG mice injected with indicated MDA-MB-231 cells including control (Ctrl), PRGL493, PAC, Cisp, PAC+Cisp, and PRGL493+PAC+Cisp at day 30, quantifications of tumor volume (**F**) and weight (**G**) were shown. **H-J**, Representative lungs and H&E staining images of the lung and liver sections from the indicated mice group (**H**), quantification of metastatic nodules on the lung surfaces (**I**), and metastatic foci of liver sections (**J**) were shown. **K,** Graphical abstract: ACSL4 activates PUFAs to produce PUFA-CoAs which are esterified into phospholipids and contribute to membrane phospholipid remodeling. ACSL4-mediated membrane phospholipid remodeling induces integrin β1 and CD47 enriched in lipid rafts, thus facilitating integrin β1/FAK signaling activation and TNBC metastasis. Graphical abstract was created by Figdraw. All data represent mean ± SD. Welch's *t*-test (**A**), Fisher's exact test (**C**), and one-way ANOVA (**F**, **G**, **I**, and **J**) were utilized:

$^{*}P< 0.05$, $^{**}P< 0.01$, $^{***}P< 0.001$.