

Original Article

PLXNB2 promotes breast cancer progression and lung metastasis through the AMPK signaling pathway

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Abstract: Breast cancer is one of the most common malignancies among women worldwide, and metastasis remains the leading cause of breast cancer-related mortality. Although PLXNB2 regulates cell migration and intracellular signaling, its expression pattern and mechanistic role in breast cancer progression and lung metastasis remain unclear. This study investigated PLXNB2 expression in breast cancer (BRCA) and examined its contribution to tumor progression and pulmonary metastasis. PLXNB2 expression in tumor and adjacent normal tissues was analyzed using The Cancer Genome Atlas (TCGA) data. Weighted gene co-expression network analysis (WGCNA), functional enrichment analysis, single-cell transcriptomics, and spatial transcriptomics were used to identify PLXNB2-associated pathways and cellular contexts. Associations with tumor grade and survival were evaluated in clinical cohorts, and the effects of lentiviral PLXNB2 knockdown on proliferation, migration, and invasion were assessed in breast cancer cell lines. Tumorigenicity and metastasis were further examined in vivo. PLXNB2 was significantly upregulated in breast cancer and was enriched predominantly in malignant epithelial cells. High PLXNB2 expression independently predicted poor overall survival in the GSE20711 cohort (HR = 1.610; 95% CI, 1.100-2.357; P = 0.013) and was positively associated with tumor grade (P < 0.05). WGCNA and transcriptomic analyses identified AMPK signaling as a key downstream pathway. PLXNB2 knockdown reduced p-AMPK and p-ACC1 levels without altering total AMPK or ACC1 expression, and reciprocal endogenous co-immunoprecipitation detected AMPK in PLXNB2 immunoprecipitates and PLXNB2 in AMPK immunoprecipitates, supporting an association between the two proteins. In vivo, PLXNB2 depletion reduced tumor growth, lung metastasis, and p-AMPK and p-ACC1 expression. These findings indicate that PLXNB2 promotes breast cancer progression and lung metastasis by activating AMPK signaling and may serve as a diagnostic, prognostic, and therapeutic biomarker.

Keywords: PLXNB2, breast cancer, AMPK, lung metastasis

Introduction

PLXNB2 is a single-pass transmembrane receptor of the class B plexin family that transduces extracellular ligand signals into multiple intracellular signaling pathways. Initially characterized in the nervous system, PLXNB2 contributes to neuronal migration and axon guid-

ance. Subsequent studies have implicated PLXNB2 in angiogenesis, immune-cell regulation, and tissue repair [1-3], consistent with broader roles in cell migration, adhesion, and tissue remodeling. Increasing evidence also identifies PLXNB2 as a regulator of tumor progression. As a functional receptor for angiogenin (ANG), PLXNB2 promotes rRNA transcrip-

tion and activates AKT/ERK prosurvival signaling, thereby supporting tumor growth and angiogenesis. Conversely, disruption of PLXNB2 or its interaction with ANG markedly inhibits tumor growth in several *in vivo* models [4-6]. PLXNB2 may also promote immune evasion and invasive phenotypes by modulating tumor-associated immune responses.

Studies of metastatic disease further indicate that PLXNB2 enhances tumor-cell invasion and dissemination by regulating cytoskeletal remodeling, cell adhesion, and migration. In glioma and cancers of unknown primary origin, aberrant PLXNB2 activation sustains invasive phenotypes and facilitates tumor-cell migration [7-10]. These observations suggest that PLXNB2 functions not only as a growth-promoting factor but also as a signaling node that integrates migratory and invasive programs during metastasis.

In breast cancer, high PLXNB2 expression is associated with shorter distant metastasis-free survival and overall survival, and its prometastatic activity has been linked to the formation of circulating tumor cell (CTC) clusters. PLXNB2 promotes heterogeneous CTC clustering and increases the potential for lung metastasis through interactions with ligands expressed by immune cells, whereas PLXNB2 knockout markedly reduces cluster formation and spontaneous metastasis [11]. However, the intracellular mechanisms through which PLXNB2 promotes breast cancer metastasis remain poorly defined, particularly its relationship with pathways involved in fatty acid metabolism. This knowledge gap provided the rationale for the present study.

Methods

TCGA bulk RNA-sequencing analysis of PLXNB2 in BRCA

PLXNB2 expression data for the BRCA cohort were obtained from The Cancer Genome Atlas (TCGA) as RSEM batch-corrected, $\log_2(\text{value} + 1)$ -transformed values. Tumor and adjacent normal tissue samples were included. To minimize technical artifacts, the data were normalized within the cancer type using z scores, defined as $z = (x - \mu)/\sigma$, where μ and σ denote the cancer-specific mean and standard deviation, respectively. Samples with $|z \text{ score}| > 3$

were excluded as extreme outliers. PLXNB2 expression in tumor and normal tissues was compared using a two-sided Wilcoxon rank-sum test (Mann-Whitney U test), with $P < 0.05$ considered statistically significant. This non-parametric test was selected because it is robust to deviations from normality and homogeneity of variance. Because only one gene-cancer comparison was performed, no correction for multiple testing was applied.

Single-cell visualization and differential analysis of PLXNB2 expression using UMAP

Single-cell transcriptomic data from the BRCA_GSE148673 dataset were retrieved from the Tumor Immune Single-Cell Hub 2 (TISCH2) database. TISCH2 applies standardized quality control, principal component analysis, UMAP embedding, and cell-type annotation to each curated dataset. Using the pre-computed UMAP coordinates and TISCH2 annotations, cells were assigned to major lineage classes and color-coded accordingly. Each point in the UMAP plot represents one cell; colors indicate cell types, and labels are positioned at the center of each cell cluster.

PLXNB2 expression data were extracted from the BRCA_GSE148673 dataset in TISCH2. Cells with a raw PLXNB2 count > 0 were classified as PLXNB2-positive, whereas cells with a raw count of 0 were classified as PLXNB2-negative. This binary classification follows the biological framework of stochastic gene silencing [12]. For each cell type, the proportions of PLXNB2-positive and PLXNB2-negative cells were calculated. Separate one-way bar plots showed the cell-type composition of each expression group, and a two-way bar plot directly compared cell-type composition between the two groups.

Spatial heterogeneity of PLXNB2 and identification of tumor boundaries in the breast cancer microenvironment

To evaluate spatial heterogeneity in PLXNB2 expression within the tumor microenvironment, tissue microspots were classified according to the proportion of malignant cells estimated by deconvolution. Microspots with a malignant-cell proportion of 1 were defined as malignant regions, those with a proportion of 0 as normal regions, and the remaining microspots as

mixed malignant regions. If fewer than 30 microspots had a malignant-cell proportion of 1, an adjusted classification was used: proportions > 0.5 defined high-malignancy regions (hMal), a proportion of 0 defined nonmalignant regions (nMal), and the remaining microspots defined low-malignancy regions (lMal). Differences in PLXNB2 expression among groups were evaluated using the wilcox.test function in R. Bar plots were used to display mean PLXNB2 expression across spatial groups.

Spatial transcriptomic analysis of breast cancer tissue sections was performed using the Sparkle database (<https://www.grswsci.top>). Tumor regions were delineated in two steps. First, the Cottrazm package was used to cluster morphology-adjusted expression matrices and integrate high-copy-number-variation features of tumor cells to identify core tumor microspots. Second, neighboring microspots were extended outward from the tumor core on a hexagonal grid, and UMAP distances from the tumor centroid were calculated to distinguish tumor and boundary (Bdy) regions and define the interface between malignant and nonmalignant cells (Cottrazm-boundarydefine). After the core and boundary regions had been delineated, mean PLXNB2 expression in each group was displayed using the dplot5 function in the fromto package. Differences among groups were evaluated using the Wilcoxon rank-sum test implemented with wilcox.test in R.

Weighted gene co-expression network construction and key-module analysis

Genes with similar expression patterns are often co-regulated, functionally related, or involved in the same biological pathways. Weighted gene co-expression network analysis (WGCNA) was therefore used to group genes into expression modules, identify hub genes, and evaluate associations between co-expression networks and phenotypic traits. A weighted network approximating scale-free topology was constructed using the WGCNA package in R. Modules were identified from the topological overlap matrix (TOM) using the dynamic tree-cut algorithm, and modules with a dissimilarity < 0.25 were merged. Gene significance (GS) and module membership (MM) were calculated to quantify gene-trait and

gene-module associations, respectively. Hub genes were selected using thresholds of |GS| > 0.3 and |MM| > 0.5.

Materials and instruments

Fetal bovine serum (FBS) was purchased from Lanzhou Rongye Biotechnology Co., Ltd., and cell-culture medium was obtained from Wuhan Punosai Life Science Co., Ltd. Reverse-transcription and RT-qPCR kits were purchased from Suzhou Nearshore Protein Technology Co., Ltd. Lentiviral vectors were synthesized by Shanghai Gema Pharmaceutical Technology Co., Ltd., and Transwell inserts were purchased from Corning (USA). The ultra-sensitive multifunctional imager was obtained from Cytiva (China). The inverted fluorescence microscope and laser-scanning confocal microscope were obtained from Nikon (Japan). Quantitative PCR instruments, microplate readers, and cell-culture incubators were obtained from Thermo Fisher Scientific (USA).

Cell lines and culture conditions

The human breast cancer cell lines MDA-MB-231 and MCF7 and the murine breast cancer cell line 4T1 were obtained from the Shanghai Institute of Life Sciences, Chinese Academy of Sciences. All cell lines tested negative for mycoplasma contamination before use. Cells were cultured in high-glucose DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂ and were passaged at an appropriate level of confluence.

Animals

Ten BALB/c mice (4-5 weeks old; 15-18 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. Mice were housed under standard conditions (22-26°C, 45-65% relative humidity, and a 12-h light/dark cycle) with free access to food and water. The animals were acclimatized for 1 week before experimentation. All procedures were approved by the institutional ethics committee. Tumors were monitored throughout the experiment, and the maximum tumor diameter did not exceed 2.0 cm at any time, in accordance with animal-welfare requirements.

Table 1. RNA interference sequences

Item	Sequences (5'-3')
human-PLXNB2-shNC	TTCTCCGAACGTGTCACGT
human-PLXNB2-sh1	GAAGCGCGTCAACACCCTTAT
human-PLXNB2-sh2	AGGCTGAGATGACTGACAATG
human-PLXNB2-sh3	TCAAGTCTTACGGCAAGAATA
mouse-PLXNB2-shNC	TTCTCCGAACGTGTCACGT
mouse-PLXNB2-sh1	AGTTCTCCAACTGCTTAATA
mouse-PLXNB2-sh2	CGCAGCCTTTGAGGATGATTT
mouse-PLXNB2-sh3	CTGTGTGTTCCCGCTAGATAA

Immunofluorescence

Tumor tissue sections were washed, fixed, permeabilized, and blocked according to standard protocols. The sections were incubated sequentially with primary antibodies against phospho-AMPK α 1 (Selleck, F3294, 1:100) and phospho-ACC1 (Proteintech, 80281-2-RR, 1:250), followed by an S-vision polymer secondary antibody (ready-to-use goat anti-rabbit; Servicebio, G1301) and DAPI counterstaining. Fluorescence images were acquired using a laser-scanning confocal microscope.

Lentiviral transduction

MDA-MB-231, MCF7, and 4T1 cells were seeded and cultured to 70-80% confluence before transduction with PLXNB2-knockdown or control lentiviral vectors. After 6 h, the transduction medium was replaced with complete culture medium. Three days later, stable cell lines were selected in medium containing 2 μ g/mL puromycin. The PLXNB2-targeting sequences are listed in **Table 1**.

Quantitative reverse-transcription PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), and its concentration and purity were measured with a NanoDrop 2000 spectrophotometer (A260/A280 ratio, 1.8-2.0). One μ g of total RNA was reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (TaKaRa). Quantitative PCR was performed on a QuantStudio 3 system (Applied Biosystems) using SYBR Green PCR Master Mix. Each 20- μ L reaction contained 10 μ L of SYBR Green Mix, 0.8 μ L each of forward and reverse primers (10 μ M), 2 μ L of cDNA template, and RNase-free water. Cycling conditions were

95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. Amplification specificity was verified by melting-curve analysis. Relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with GAPDH as the internal control. Reactions were performed in technical triplicate, and each experiment was independently repeated three times. Primer sequences are listed in **Table 2**.

Protein-expression analysis

Western blotting was used to measure PLXNB2 and proteins in the associated signaling pathway. Tumor tissues (50 mg) were lysed in RIPA buffer containing PMSF protease inhibitor (100 μ L of RIPA buffer per 10 mg of tissue; RIPA:PMSF = 100:1). Lysates were centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatants were collected. Protein concentrations were determined using a BCA assay. Samples were mixed with 5 $\times$ SDS-PAGE loading buffer at a 1:4 ratio, heated at 95°C for 5 min, cooled to room temperature, and loaded at 10 μ L per well. After SDS-PAGE, proteins were transferred to PVDF membranes by wet transfer. Membranes were blocked with 5% BSA for 1.5 h and incubated overnight at 4°C with antibodies against PLXNB2 (Proteintech, 10602-1-AP, 1:1,000), AMPK (Selleck, F2393, 1:1,000), p-AMPK (Selleck, F1561, 1:10,000), ACC1 (Proteintech, 21923-1-AP, 1:8,000), and p-ACC1 (Abways, CY5094, 1:2,000). After four 5-min washes with TBST, the membranes were incubated with the appropriate secondary antibody for 1 h at room temperature. GAPDH (60004-1-Ig, 1:500,000) served as the loading control. Signals were detected using an ECL substrate and imaged with a gel-documentation system.

EdU proliferation assay

Exponentially growing stable breast cancer cells were seeded in 96-well plates at 5×10^3 cells per well and assigned to the shNC or shPLXNB2 group. EdU solution (100 μ L) was added to each well. After 2 h, cells were fixed with 4% paraformaldehyde for 30 min and incubated with 100 μ L of Click-iT reaction solution for 30 min. The cells were then stained with 100 μ L of Hoechst solution for 30 min in the dark and imaged by fluorescence microscopy.

Table 2. Primer sequences

Item	Sequences
PLXNB2 (human)	For 5'-AGCCTCTTCAAGGGCATCTG-3' Rev 5'-GCCACGAAAGACTTCTCCCC-3'
PLXNB2 (Mouse)	For 5'-CAGAAGACAGCCAACAGGACTTG-3' Rev 5'-CGCTTGGACTTGCCTTCATCTAC-3'
GAPDH (human)	For 5'-TCAAGAAGGTGGTGAAGCAGG-3' Rev 5'-TCAAAGGTGGAGGAGTGGGT-3'
GAPDH (Mouse)	For 5'-GCAATTCAACGGCACAGTCAAG-3' Rev 5'-TCGCTCCTGGAAGATGGTGATG-3'

Colony-formation assay

Exponentially growing stable breast cancer cells were seeded in 6-well plates at 1,000 cells per well, with three replicate wells per group. Cells were cultured in complete DMEM for 14 days, and the medium was replaced every 2 days. The cells were then washed three times with PBS, fixed with 4% paraformaldehyde for 20 min, and stained with 0.1% crystal violet for 20 min. After washing and air-drying, colonies were counted microscopically. A cluster containing > 50 cells was defined as one colony. The colony-formation rate was calculated as follows: colony-formation rate (%) = number of colonies/number of seeded cells × 100%.

Wound-healing assay

Cells were seeded in 6-well plates at 2×10^6 cells per well. After a confluent monolayer had formed, a linear wound was created using a sterile 200- μ L pipette tip. Plates were incubated at 37°C, and five randomly selected fields per well were imaged at 0 and 12 or 24 h using an inverted microscope. Wound closure was quantified using ImageJ.

Transwell migration and invasion assays

Stable cell lines were suspended in 200 μ L of serum-free medium and seeded in the upper chambers of Transwell inserts (migration assay, 4×10^4 cells per well; invasion assay, 8×10^4 cells per well). The lower chambers contained 600 μ L of complete medium. After incubation for 24 h at 37°C, cells on the lower surface of the membrane were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 20 min. Cells remaining on the upper surface were removed, and

migrated or invaded cells were counted in five randomly selected fields under an inverted microscope. For invasion assays, the upper chambers were coated with 50 μ L of Matrigel and incubated for 1 h before cell seeding.

Animal grouping and tumor model

Ten BALB/c mice were randomly assigned to the shNC (n = 5) or shPLXNB2 (n = 5) group. 4T1 cells were cultured in DMEM containing 10% FBS to 80-90% confluence, washed with PBS, and detached with 0.25% trypsin. Cells were collected, centrifuged at 1,000 rpm for 3 min, and resuspended at 5×10^6 cells/mL. Each mouse received a subcutaneous axillary injection of 200 μ L of the cell suspension. Body weight and tumor volume [$\text{length} \times \text{width}^2/2$] were measured every 3 days, and lung metastasis was monitored weekly by micro-CT. Once lung metastases were detected, mice were euthanized by intraperitoneal injection of pentobarbital (150 mg/kg), followed by cervical dislocation to confirm death, in accordance with the AVMA Guidelines for the Euthanasia of Animals (2020 edition). Tumor and lung tissues were collected and photographed.

Micro-CT imaging

Micro-CT imaging was performed using a Hiscan XM system (Hiscan, Suzhou, China) with the following settings: voltage, 60 kV; current, 133 μ A; exposure time, 1.2 s; resolution, 50 μ m; and rotation increment, 0.5 degrees. Images were reconstructed and analyzed using the manufacturer's proprietary software.

Hematoxylin and eosin staining

Lung tissues were fixed in 4% paraformaldehyde for 24 h, dehydrated, cleared, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin, mounted, and examined microscopically.

Co-immunoprecipitation

Reciprocal endogenous co-immunoprecipitation was performed in MCF7 and MDA-MB-231 cells. For each reaction, 50 μ L of magnetic beads were washed two or three times with

PBST and resuspended in 150 μ L of PBST containing 4 μ g of anti-PLXNB2 antibody (Proteintech, 10602-1-AP), anti-AMPK antibody (Selleck, F2393), or an equivalent amount of species-matched IgG as a negative control. The beads were incubated overnight at 4°C with rotation. After three washes with PBST, protein lysates were added and incubated overnight at 4°C. The beads were then washed three times with isotonic lysis buffer, eluted in 1 $\times$ loading buffer, boiled at 100°C for 10 min, and analyzed by immunoblotting. For the PLXNB2 pulldown, precipitates were immunoblotted with anti-AMPK (Selleck, F2393, 1:1,000) and anti-PLXNB2 (Proteintech, 10602-1-AP, 1:1,000). For the reciprocal AMPK pulldown, precipitates were immunoblotted with anti-PLXNB2 (Proteintech, 10602-1-AP, 1:1,000) and anti-AMPK (Selleck, F2393, 1:1,000). GAPDH (Proteintech, 60004-1-Ig, 1:500,000) was assessed as a nonspecific precipitation control.

Statistical analysis

Statistical analyses were performed using SPSS version 23.0. Continuous data are presented as the mean $\pm$ standard deviation (SD). Normality and homogeneity of variance were assessed before analysis. Normally distributed data with equal variances were compared using an independent-samples t test for two groups or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple groups. When variances were unequal, Welch's ANOVA followed by the Games-Howell post hoc test was used. Nonparametric tests were applied to data that were not normally distributed. Repeated-measures ANOVA was used for comparisons involving multiple groups and a time variable. All tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Dysregulated PLXNB2 expression in breast cancer

PLXNB2 expression was analyzed in the TCGA BRCA cohort using RSEM batch-corrected, log₂(value + 1)-transformed data. The analysis included 1,096 tumor samples and 111 adjacent normal tissue samples. After within-cancer-type z-score normalization, the groups were compared using the Wilcoxon rank-sum test.

PLXNB2 expression was significantly higher in tumor tissues than in adjacent normal tissues ($P = 1.05 \times 10^{-8}$), with median z scores of 0.115 and -0.274, respectively (**Figure 1A**). Single-cell transcriptomic analysis showed that the proportion of malignant epithelial cells (66.1%) was higher than that in PLXNB2-negative cells (41.8%) among PLXNB2-positive cells. This difference reflects variation in cellular composition and does not measure the overall malignancy of the tissue (**Figures 1B-D, S1**). The proportions of conventional CD4⁺ T cells and CD8⁺ T cells were lower in the PLXNB2-positive cell population than in the PLXNB2-negative cell population (0.2% vs. 12.2% and 0.1% vs. 8.9%). These percentages describe the cell-type composition of the two cell populations and do not directly indicate differences in immune-cell infiltration between tumors with high and low PLXNB2 expression. Spatial analysis further showed higher PLXNB2 expression in tumor cores, tumor-normal interfaces, and high-malignancy regions than in adjacent normal tissue ($P < 0.05$; **Figure 1E-H**), supporting a potential role in breast cancer progression.

High PLXNB2 expression is associated with poor prognosis

PLXNB2 expression differed significantly across breast cancer stages and increased with advancing disease (**Figure 2A**). Expression was higher in patients with metastatic disease (M1) than in those without metastasis (M0) (**Figure 2B**), and immunohistochemical staining showed progressively stronger PLXNB2 expression with increasing breast cancer stage (**Figure 2C**). High PLXNB2 expression was also associated with a greater risk of progression during the progression-free interval (HR = 1.343; 95% CI, 1.020-1.767; **Figure 2D**).

Validation in independent cohorts supported these findings. In GSE20711 and GSE9893, high PLXNB2 expression was associated with shorter overall survival (OS) (HR = 1.610, $P = 0.014$; and HR = 1.651, $P < 0.001$, respectively). In GSE21653 and GSE9893, high expression was associated with shorter disease-free survival (DFS) and relapse-free survival (RFS), respectively (HR = 1.275, $P = 0.031$; and HR = 1.763, $P = 0.001$) (**Figure 2E**). Kaplan-Meier analyses similarly showed shorter OS and RFS

PLXNB2 promotes breast cancer progression

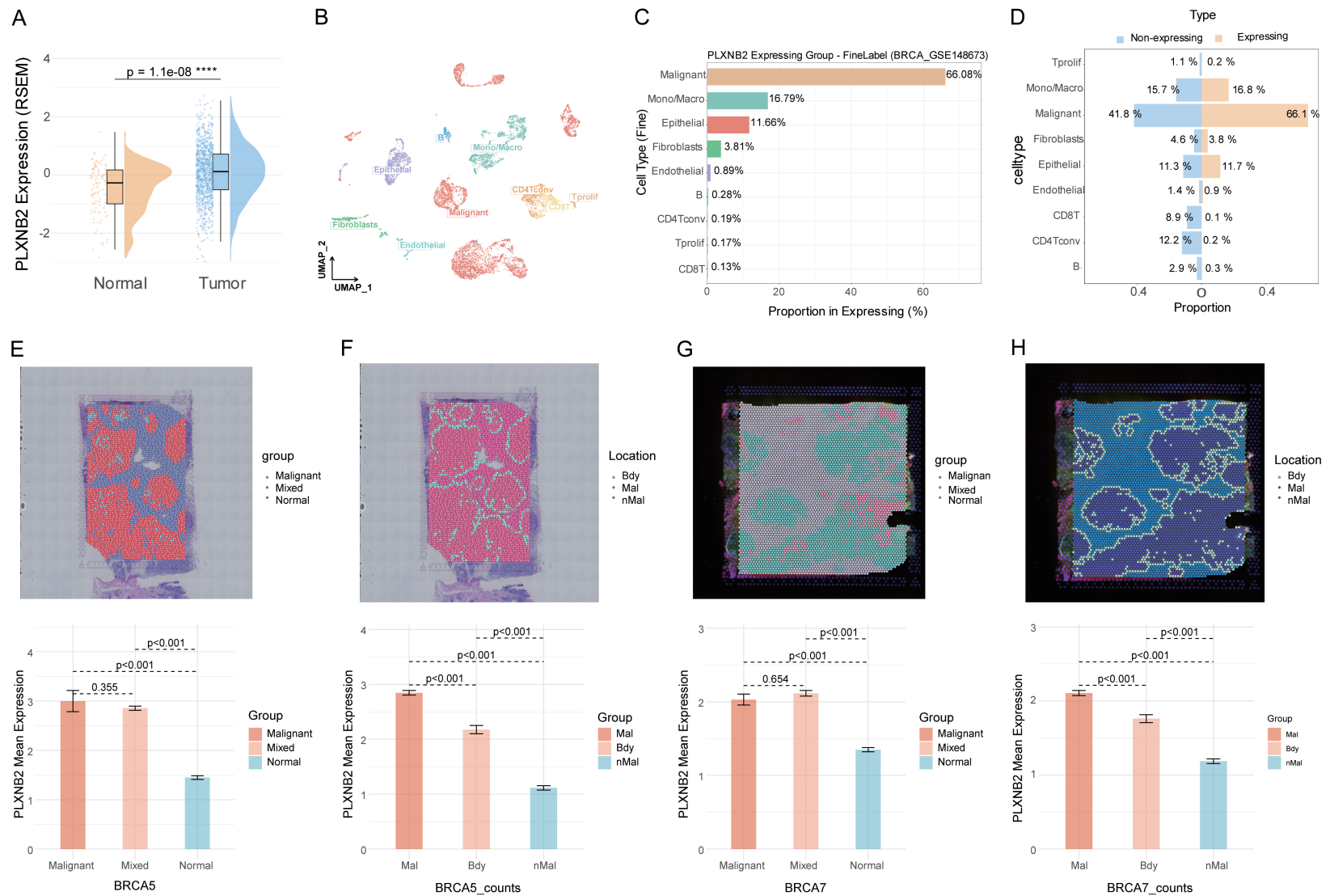


Figure 1. Dysregulated PLXNB2 expression in breast cancer. A. Raincloud plot of PLXNB2 expression in tumor and adjacent normal tissues from the TCGA BRCA cohort. The plot combines a half-violin density plot, a box plot showing the median and interquartile range, and points representing individual samples. The P value from the Wilcoxon rank-sum test is shown above the plot. B. UMAP plot of major cell lineages in the BRCA_GSE148673 dataset. Each point represents one cell,

colors indicate TISCH2-annotated lineages, and labels mark the center of each cluster. C. Mean PLXNB2 expression by FineLabel cell type; error bars indicate the standard error. D. Cell-type composition of the PLXNB2-positive and PLXNB2-negative groups. The 66.1% and 41.8% values represent the proportions of malignant epithelial cells within the PLXNB2-positive and PLXNB2-negative cell populations, respectively; they do not indicate the overall malignancy of PLXNB2-high versus PLXNB2-low tumors. Blue indicates PLXNB2-negative cells, and yellow indicates PLXNB2-positive cells. E, F. Mean PLXNB2 expression across spatial groups, including malignant and normal regions. Differences were evaluated using the Wilcoxon rank-sum test ($P < 0.05$). G, H. Spatial PLXNB2 expression in malignant, tumor-boundary, and normal regions. Differences were evaluated using the Wilcoxon rank-sum test ($P < 0.05$). Spatial transcriptomic data for BRCA7 were obtained from Spatial transcriptomics at subspot resolution with BayesSpace, and BRCA5 data were obtained from BRCA_Visium_FreshFrozen_WholeTranscriptome. mal, malignant-cell region; bdy, boundary region; nMal, nonmalignant region containing normal epithelial, immune, stromal, and other nonmalignant cells.

in patients with high PLXNB2 expression than in those with low expression (log-rank $P < 0.05$), consistent with the Cox regression results (Figure 2F and 2I).

In the TCGA cohort, the prognostic association of PLXNB2 was attenuated after adjustment for TNM stage and other clinical variables (HR = 0.920; $P = 0.581$), suggesting that its prognostic effect may overlap with factors related to tumor progression. In the independent GSE20711 cohort, however, high PLXNB2 expression remained independently associated with poor OS after adjustment for age and tumor grade (HR = 1.610; 95% CI, 1.100-2.357; $P = 0.013$), supporting its potential value as a prognostic biomarker (Figure 2G, 2H).

PLXNB2 knockdown inhibits breast cancer cell proliferation, migration, and invasion

RT-qPCR and Western blotting confirmed efficient PLXNB2 knockdown in shPLXNB2-transduced cells ($P < 0.01$; Figure 3A, 3B). The most effective constructs, human shPLXNB2#2 and mouse shPLXNB2#3, were selected for subsequent experiments. EdU-incorporation and colony-formation assays showed significantly lower proliferation in shPLXNB2 cells than in negative-control cells, as indicated by fewer EdU-positive cells, fewer colonies, and lower single-cell survival rates (all $P < 0.05$; Figure 3C-F). Wound-healing assays showed slower migration of shPLXNB2-transduced cells at 24 h than of control cells ($P < 0.01$; Figure 3G, 3H). Transwell assays further confirmed that PLXNB2 knockdown significantly reduced breast cancer cell migration and invasion ($P < 0.01$; Figure 3I-K). Together, these results indicate that PLXNB2 promotes breast cancer cell proliferation, migration, and invasion *in vitro*.

PLXNB2 knockdown suppresses breast cancer progression by inhibiting AMPK signaling

To investigate the mechanisms underlying PLXNB2 function, tumors were divided into quartiles (Q1-Q4) according to PLXNB2 expression and analyzed by WGCNA. Initial sample clustering identified no outliers (Figure 4A). A soft-thresholding power of 13 generated a scale-free co-expression network with appropriate mean connectivity (Figure 4B). Dynamic tree cutting and module merging identified 18 stable co-expression modules (Figure 4C, 4D), and the TOM heatmap visualized intergene co-expression similarity (Figure 4E). Module-trait correlation analysis showed that high PLXNB2 expression was strongly associated with the MEyellow module, whereas low PLXNB2 expression was negatively associated with the MEyellow and MEmagenta modules. The MEyellow module was selected for further analysis, yielding 309 hub genes associated with PLXNB2 expression (Figure 4F). Functional enrichment analysis implicated these hub genes in AMPK-related tumor pathways and other cancer-associated signaling cascades (Figure 5A).

RNA sequencing of shNC and shPLXNB2 cells was performed to validate these predictions. KEGG analysis of differentially expressed genes identified significant enrichment of AMPK signaling, consistent with the pathways identified from the WGCNA hub genes (Figure 5B, 5C). Gene set enrichment analysis further showed that AMPK signaling was downregulated after PLXNB2 knockdown (Figure 5D). Western blotting demonstrated lower p-AMPK and p-ACC1 levels in shPLXNB2 cells, whereas total AMPK and ACC1 levels remained unchanged (Figure 5E). Reciprocal endogenous co-immunoprecipitation detected AMPK in

PLXNB2 promotes breast cancer progression

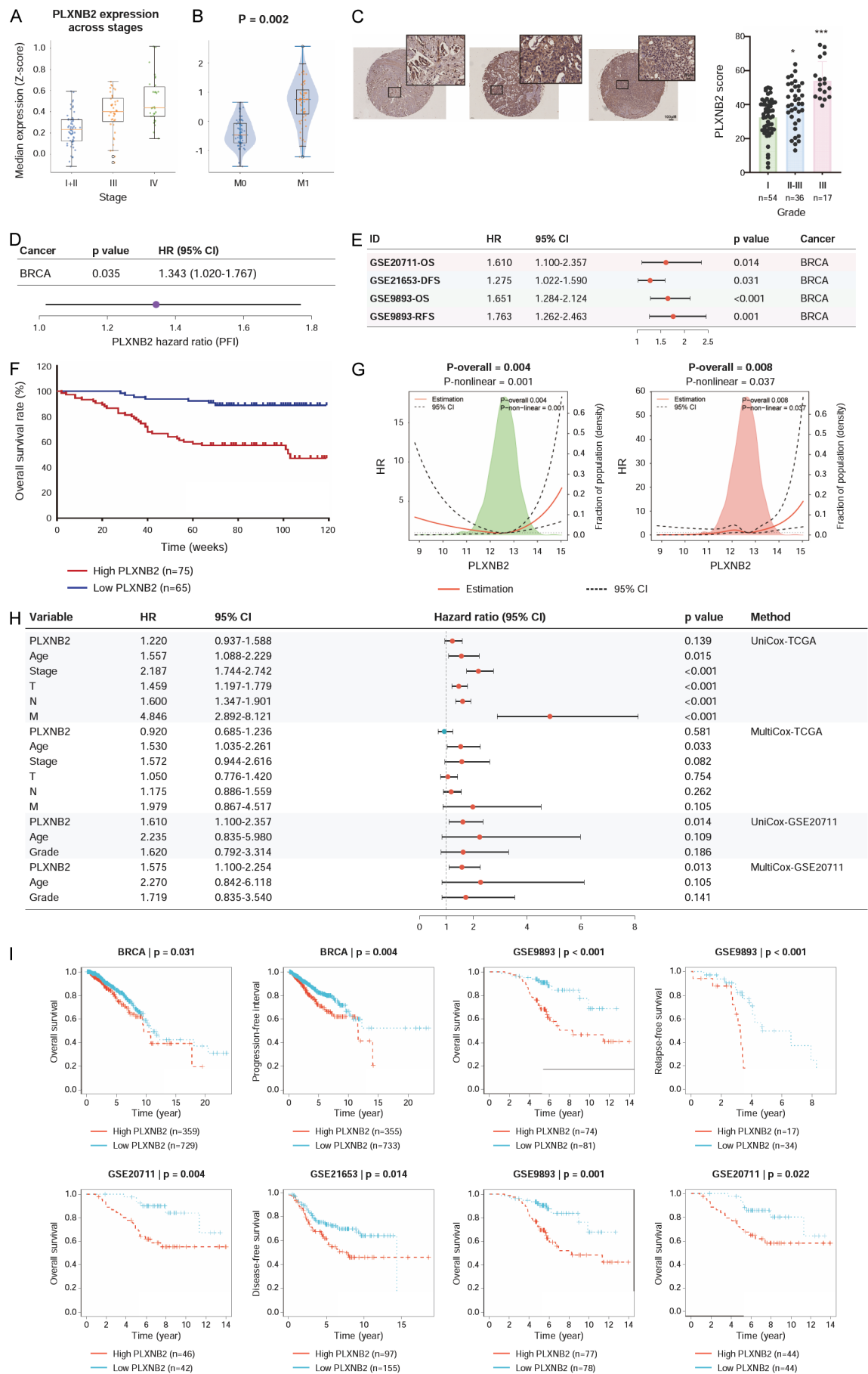


Figure 2. PLXNB2 expression patterns and prognostic significance in breast cancer. A. PLXNB2 expression across tumor stages I-IV in the TCGA breast cancer cohort. B. PLXNB2 expression by metastatic status (M0 vs. M1) in GSE9893. C. Immunohistochemical analysis of PLXNB2 in breast cancer tissues. Scale bars, 20 μ m in the main images (Magnification: 4 $\times$) and 100 μ m in the insets (Magnification: 40 $\times$). Between-group differences were analyzed using Student's t test. * $P < 0.05$; *** $P < 0.001$. D. Univariate Cox regression analysis of the association between PLXNB2 expression and progression-free interval. E. Associations between PLXNB2 expression and survival outcomes (OS, DFS, and RFS) in GSE20711, GSE21653, and GSE9893. F. Kaplan-Meier curves for overall survival according to PLXNB2 expression. G. Restricted cubic spline analysis of PLXNB2-associated prognostic risk in TCGA (left) and GSE20711 (right). H. Univariate and multivariate Cox regression analyses of PLXNB2 expression and clinical variables, including age, stage, TNM classification, and grade, in TCGA and GSE20711. I. Kaplan-Meier analyses of OS, PFS, and RFS in TCGA and the indicated GEO cohorts.

PLXNB2 immunoprecipitates and PLXNB2 in AMPK immunoprecipitates in both MCF7 and MDA-MB-231 cells, supporting an endogenous association between PLXNB2 and AMPK (Figure 5F).

Functional rescue experiments were then performed using the AMPK inhibitor Compound C. PLXNB2 overexpression increased p-AMPK levels, whereas Compound C reduced p-AMPK in PLXNB2-overexpressing cells without substantially altering total AMPK expression (Figure 5G). Cell Counting Kit-8 and wound-healing assays further showed that PLXNB2 overexpression enhanced cell proliferation and migration and that Compound C reversed these effects (Figure 5H-J). These findings indicate that PLXNB2 promotes breast cancer cell proliferation and migration through its interaction with AMPK and activation of AMPK signaling.

PLXNB2 promotes tumor growth and lung metastasis in a mouse model of breast cancer

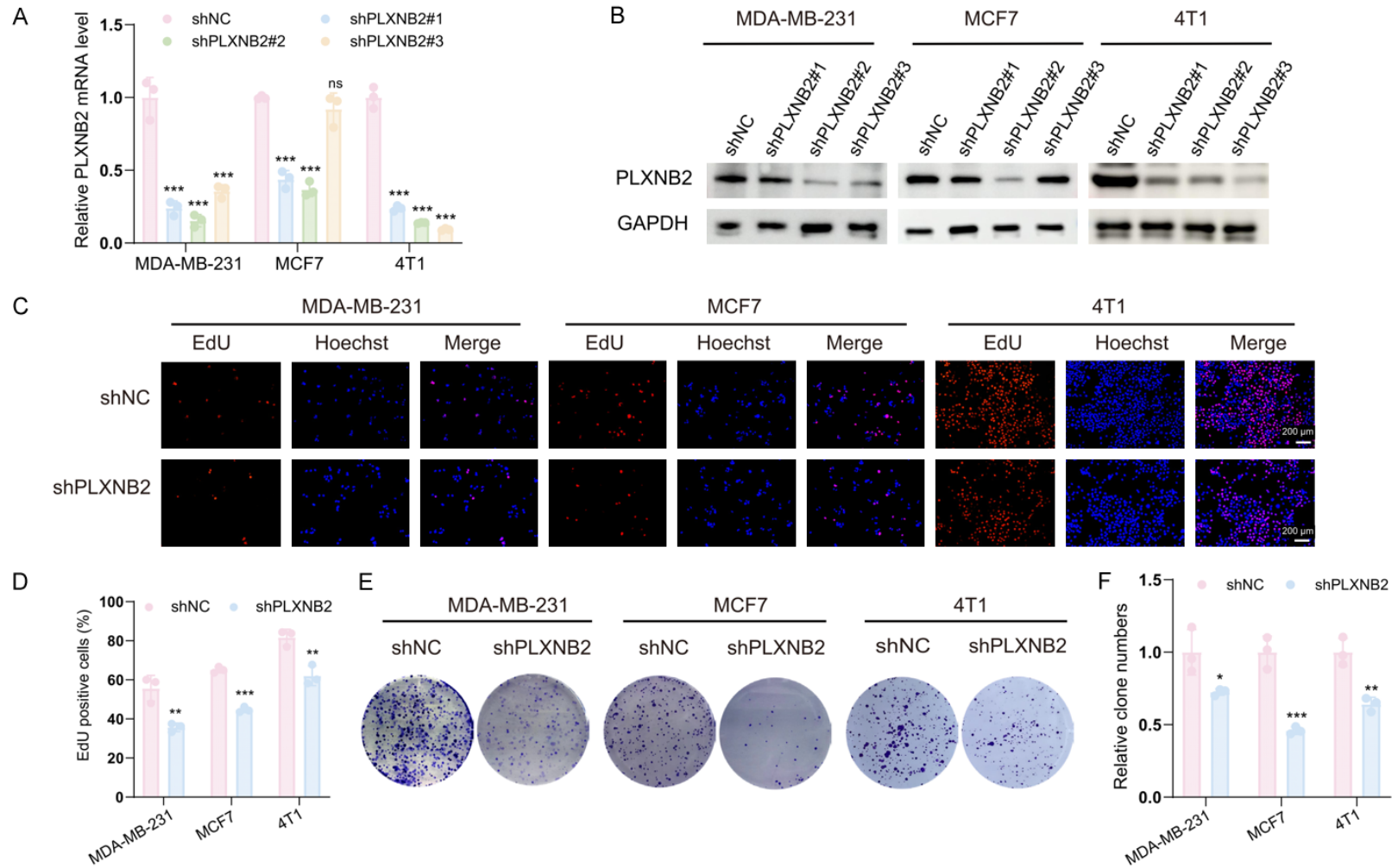
The *in vivo* effects of PLXNB2 were evaluated in a subcutaneous breast cancer model (Figure 6A). Tumors derived from shPLXNB2 cells grew more slowly and reached smaller volumes than control tumors (Figure 6B). Micro-CT showed fewer lung metastases in the shPLXNB2 group (Figure 6C), consistent with gross examination of the tumors and lungs (Figure 6D, 6E). Quantification of pulmonary nodules confirmed a significant reduction in metastatic burden after PLXNB2 knockdown ($P < 0.05$; Figure 6F), and hematoxylin and eosin staining corroborated the reduction in lung metastasis (Figure 6G). Multiplex immunofluorescence showed lower p-AMPK, p-ACC1, PPAR γ , and FASN expression in shPLXNB2 tumors, whereas total AMPK, ACC1, and CPT1A expression remained unchanged (Figure 6H).

These results indicate that PLXNB2 promotes tumor growth and metastatic dissemination *in vivo*, potentially through AMPK-dependent regulation of fatty acid metabolism.

Discussion

This study characterizes the dysregulated expression of PLXNB2 in breast cancer and its role in tumor progression. Integrative multi-omics analyses, survival analyses, single-cell transcriptomics, *in vitro* functional assays, and *in vivo* models consistently linked high PLXNB2 expression to invasive behavior, poor prognosis, and a prometastatic tumor microenvironment. Mechanistically, these effects were associated with activation of AMPK signaling. Previous work showed that PLXNB2 promotes the clustering of circulating tumor cells (CTCs) with monocytes and enhances breast cancer metastasis; high PLXNB2 expression was associated with poor prognosis, whereas PLXNB2 inhibition reduced metastatic dissemination [13]. As a receptor for angiogenin, PLXNB2 also mediates nuclear RNA processing and ribosome biogenesis in several cancer cell types, thereby promoting cell survival and regeneration, while anti-PLXNB2 monoclonal antibodies inhibit tumor growth [14]. In immune cells, PLXNB2 regulates macrophage motility and Rho GTPase activity and may therefore influence immune-cell infiltration into the tumor microenvironment [15]. In models of cancer of unknown primary origin, the PLXNB2 G842C mutant autonomously activates EGFR signaling and sustains cancer stem-cell self-renewal and invasion; these effects can be blocked by EGFR inhibitors [16]. Consistent with these observations, PLXNB2 knockdown in the present study reduced breast cancer cell proliferation, migration, and invasion *in vitro* and suppressed tumor growth and lung metastasis in mice.

PLXNB2 promotes breast cancer progression



PLXNB2 promotes breast cancer progression

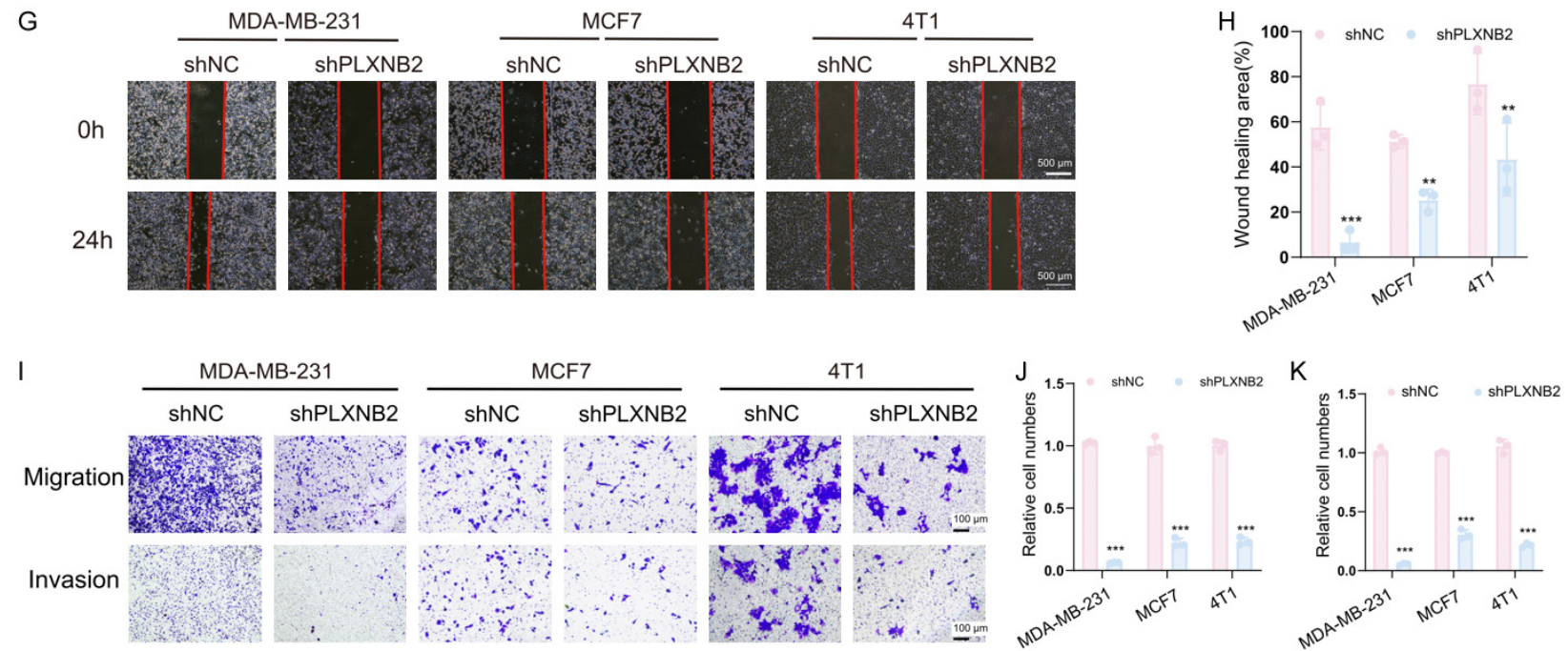


Figure 3. PLXNB2 promotes breast cancer cell proliferation, migration, and invasion. A, B. RT-qPCR and Western blot analyses of PLXNB2 expression in MDA-MB-231, MCF7, and 4T1 cells after lentiviral transduction. C, E. EdU and colony-formation assays showing the effects of PLXNB2 knockdown on cell proliferation. Scale bar, 200 μ m. Magnification: 10 $\times$. D, F. Quantification of the EdU and colony-formation assays. G, H. Wound-healing assays and corresponding quantification showing reduced migration after PLXNB2 knockdown. Scale bar, 500 μ m. Magnification: 4 $\times$. I-K. Transwell assays and corresponding quantification showing reduced migration and invasion after PLXNB2 knockdown. Scale bar, 100 μ m. Magnification: 10 $\times$. Data are presented as the mean $\pm$ SD from three independent experiments. Differences were analyzed using Student's t test for two groups or one-way ANOVA followed by Tukey's post hoc test for multiple groups. * P < 0.05; ** P < 0.01; *** P < 0.001.

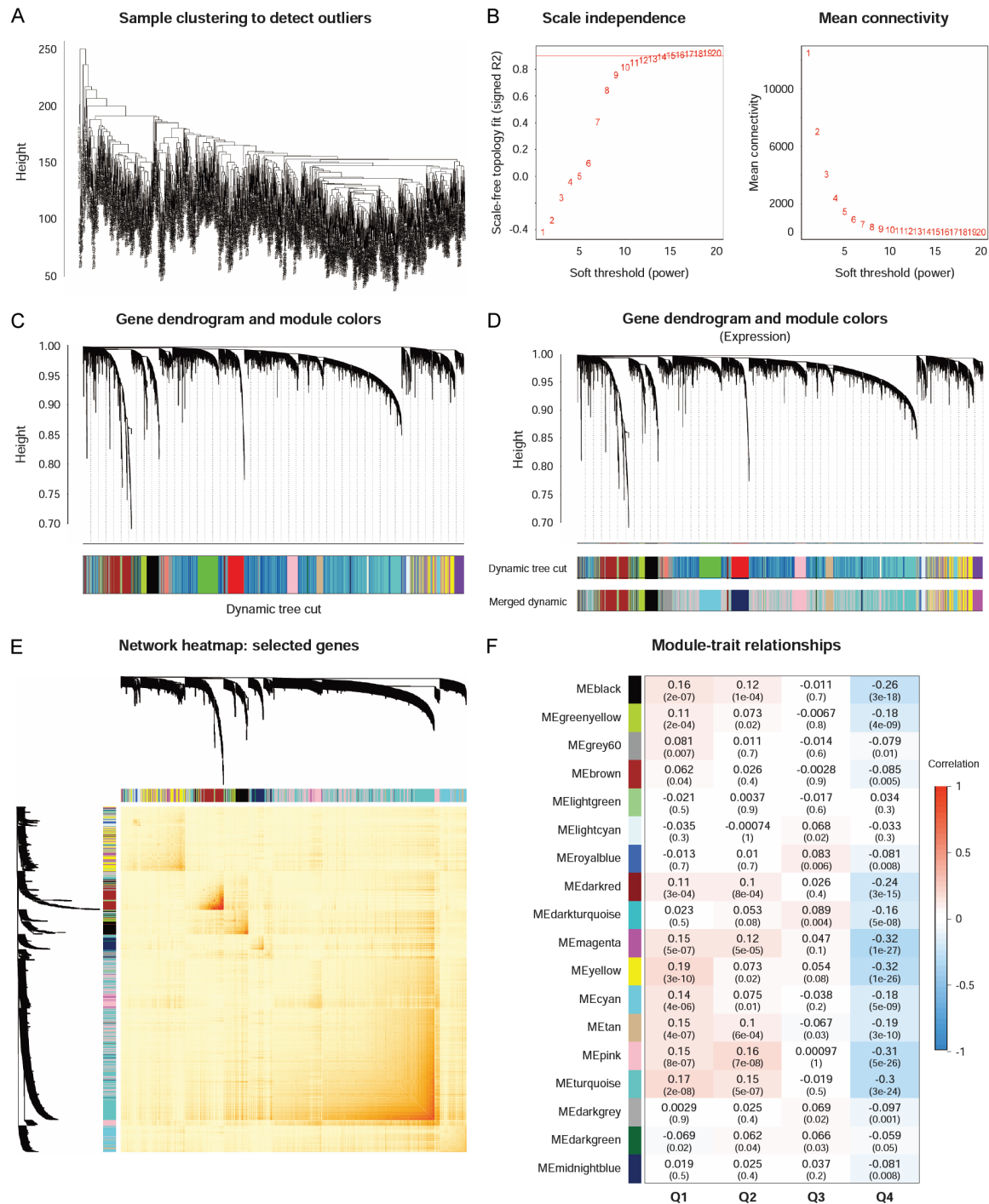
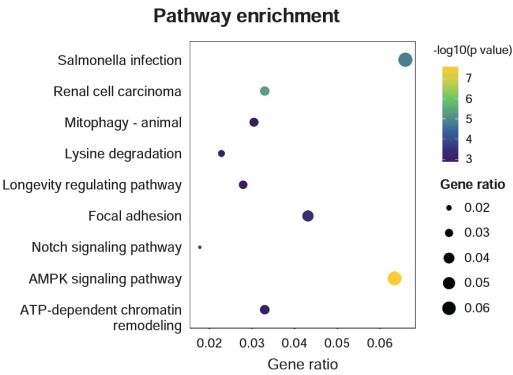


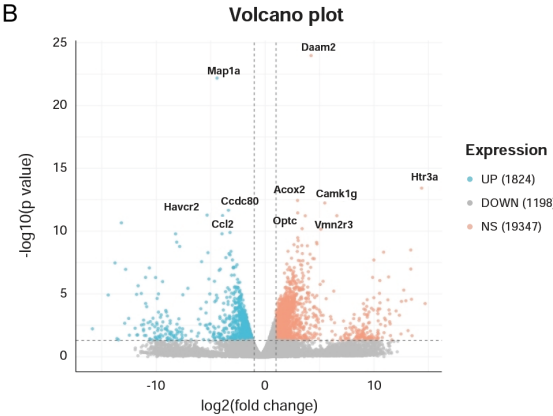
Figure 4. Construction of the weighted gene co-expression network and identification of clinically relevant modules. A. Hierarchical clustering of samples to identify potential outliers; the y axis indicates clustering height. B. Selection of the WGCNA soft-thresholding power. Left, scale-free topology fit index (R^2) across candidate powers; the red line indicates the target threshold ($R^2 = 0.85-0.90$). Right, mean connectivity at each power. C, D. Gene clustering and module identification. C. Gene dendrogram with modules identified by dynamic tree cutting; colors indicate individual modules. D. Modules merged according to module-eigengene similarity. E. Heatmap of the weighted gene co-expression network. Rows and columns represent genes, and color intensity indicates topological overlap; darker diagonal blocks represent modules. Gene dendrograms and module assignments are shown along the left and upper margins. F. Correlations between module eigengenes and PLXNB2-expression quartiles (Q1-Q4). Each cell shows the correlation coefficient, with the P value in parentheses. Red and blue indicate positive and negative correlations, respectively, and color intensity represents correlation strength.

PLXNB2 promotes breast cancer progression

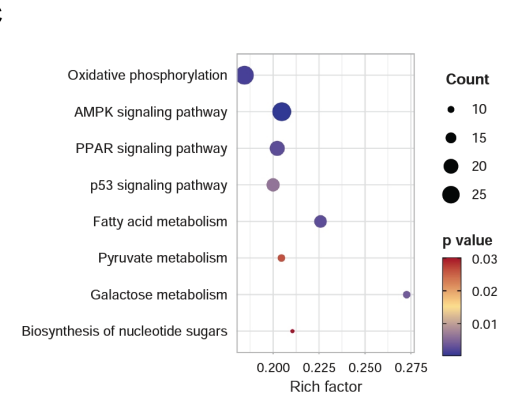
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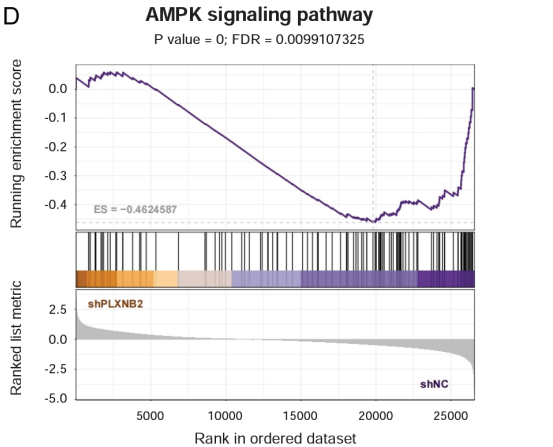
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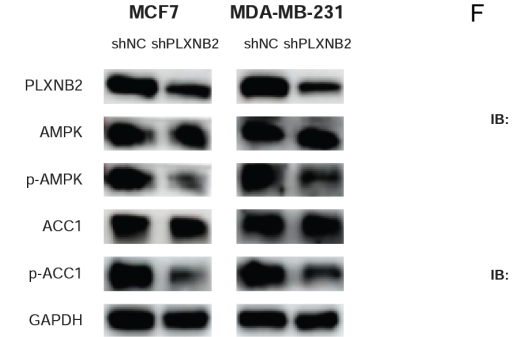
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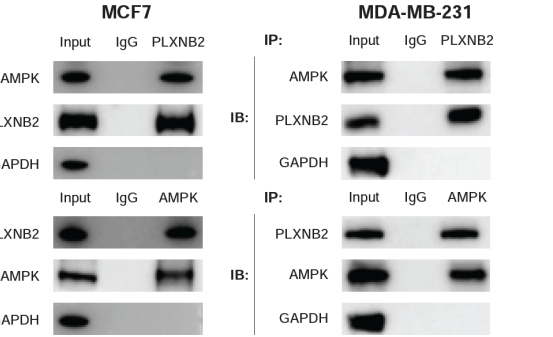
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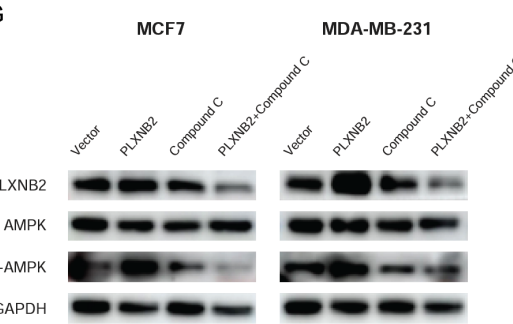
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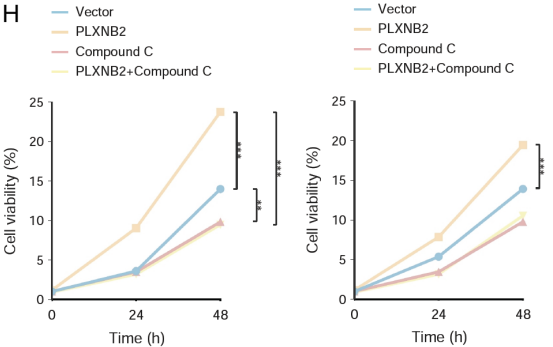
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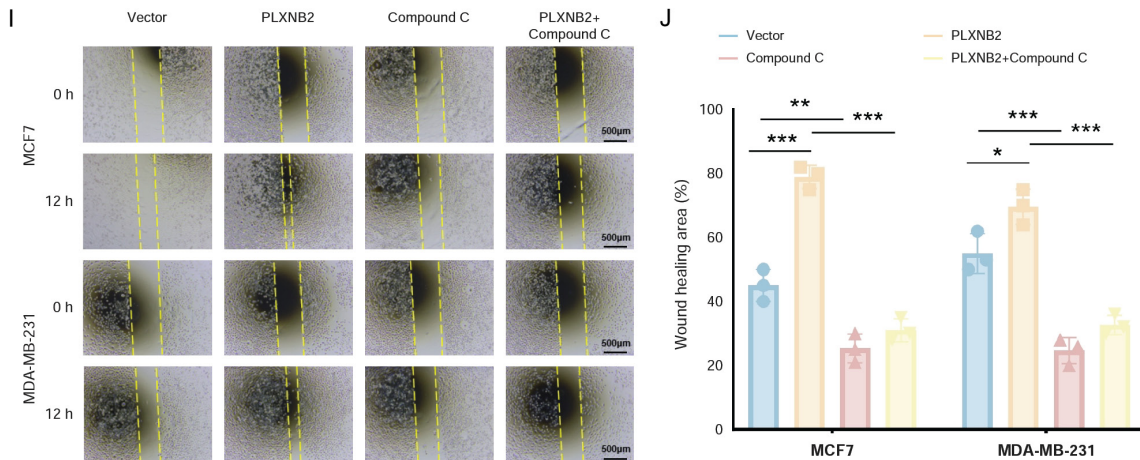


Figure 5. PLXNB2 promotes breast cancer progression through AMPK signaling. A. KEGG enrichment analysis of hub genes in the core module. B. Volcano plot of differentially expressed genes in shNC and shPLXNB2 cells. C. KEGG enrichment analysis of differentially expressed genes. D. Gene set enrichment analysis of pathway changes after PLXNB2 knockdown. E. Expression of AMPK pathway proteins in breast cancer cells. F. Reciprocal endogenous co-immunoprecipitation in MCF7 and MDA-MB-231 cells. AMPK was detected after immunoprecipitation with anti-PLXNB2 (upper panels), and PLXNB2 was detected after reciprocal immunoprecipitation with anti-AMPK (lower panels); species-matched IgG served as the negative control. G. Rescue experiments with the AMPK inhibitor Compound C. H. Cell proliferation measured using the Cell Counting Kit-8 assay. I, J. Cell migration measured using the wound-healing assay. Scale bar, 500 μ m. Magnification: 4 $\times$. Data are presented as the mean $\pm$ SD from three independent experiments. Differences were analyzed using Student's t test for two groups or one-way ANOVA followed by Tukey's post hoc test for multiple groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

As a cell-surface receptor, PLXNB2 can initiate intracellular signaling after binding extracellular ligands and can also regulate downstream pathways through intracellular activation. Several cancer-associated signaling mechanisms have been attributed to PLXNB2. Binding of angiogenin to upregulated PLXNB2 activates autocrine and paracrine signaling and promotes tumor-stroma interactions in colorectal cancer, potentially through RAS or mTOR signaling [17]. In acute myeloid leukemia, PLXNB2 overexpression is associated with poor prognosis and may activate JAK-STAT or NF- κ B signaling, thereby altering immune responses and intercellular communication [18]. Outside cancer, grancalcin binds PLXNB2 on bone marrow mesenchymal stem cells and inhibits the downstream FAK-SRC-YAP pathway, suppressing osteogenic differentiation during age-related skeletal senescence [19]. These context-specific effects underscore the ability of PLXNB2 to engage distinct signaling pathways across cell types.

The present findings indicate that AMPK activation can promote breast cancer progression, which differs from the tumor-suppressive role described in some settings and supports a

context-dependent dual function of AMPK. AMPK can restrict tumor-cell proliferation by inhibiting mTORC1 and suppressing protein and lipid synthesis, and reduced AMPK α 1 expression can disrupt cell-cell adhesion and promote breast cancer metastasis [20, 21]. After tumor establishment, however, hypoxia, nutrient deprivation, and matrix detachment can activate AMPK. This response promotes fatty acid oxidation, autophagy, and energy homeostasis, thereby enabling tumor cells to survive adverse microenvironmental conditions. AMPK has also been reported to maintain breast cancer stem-cell properties and drug resistance and to support the survival of dormant estrogen receptor-positive breast cancer cells during estrogen deprivation [22, 23]. Thus, the protumor effects observed here may reflect AMPK-dependent metabolic adaptation in particular breast cancer subtypes, disease stages, or microenvironmental contexts rather than contradicting its established tumor-suppressive functions.

This study has several limitations. The prognostic analyses relied partly on retrospective public datasets, and the animal experiments included a relatively small cohort; larger pro-

PLXNB2 promotes breast cancer progression

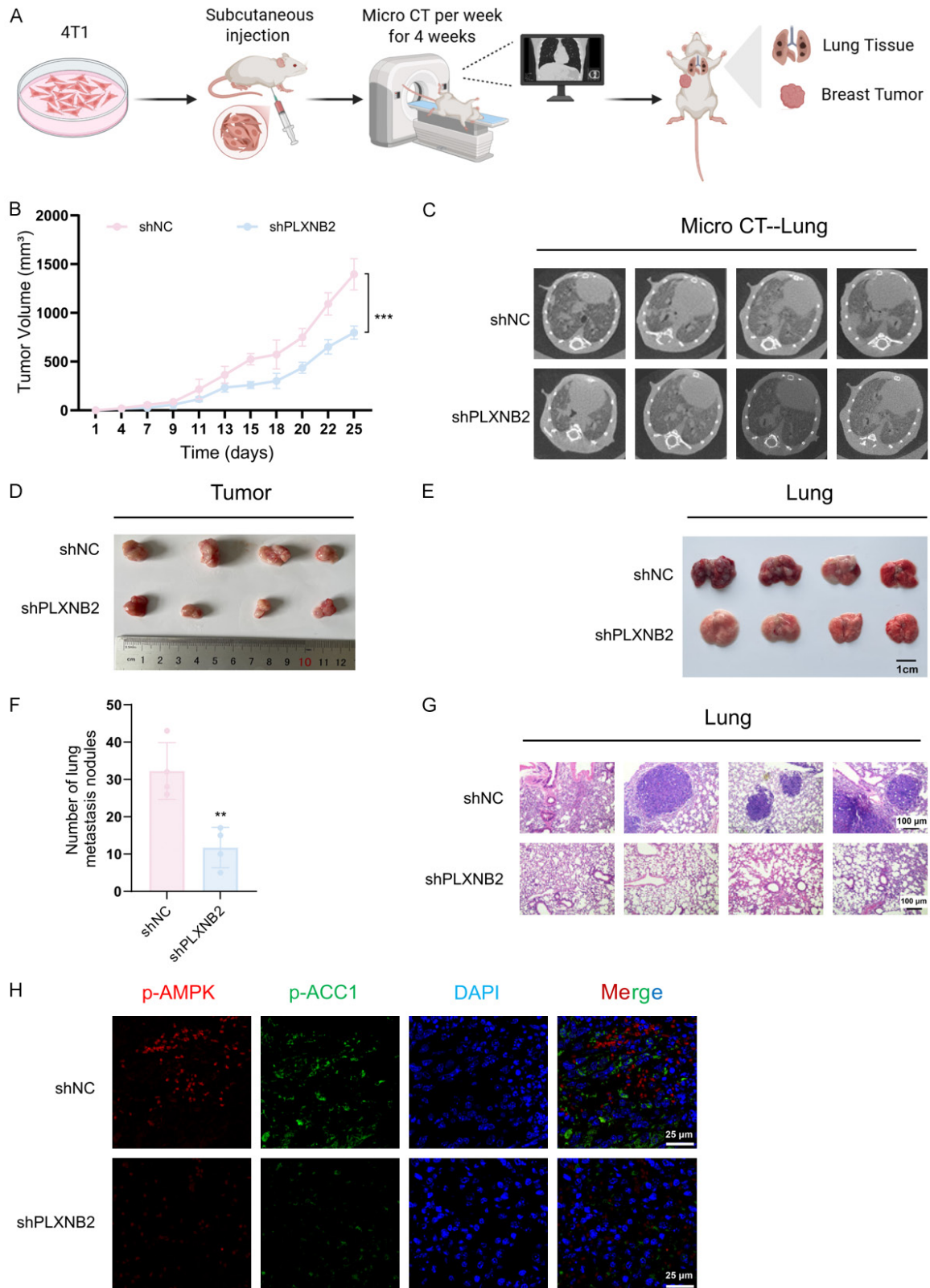


Figure 6. PLXNB2 promotes tumor growth and lung metastasis in vivo. **A.** Experimental workflow for the mouse model. **B.** Growth of subcutaneous tumors in BALB/c mice. **C.** Micro-CT images of lung metastases. **D, E.** Representative gross images of subcutaneous tumors and lungs. Scale bar, 1 cm. **F.** Quantification of lung metastatic nodules. **G.** Hematoxylin and eosin staining of lung tissues. Scale bar, 100 µm. Magnification: 40×. **H.** Multiplex immunofluorescence of tumor tissues. Scale bar, 25 µm. Magnification: 60×. Between-group differences were analyzed using Student's t test. $^{**}P < 0.01$; $^{***}P < 0.001$.

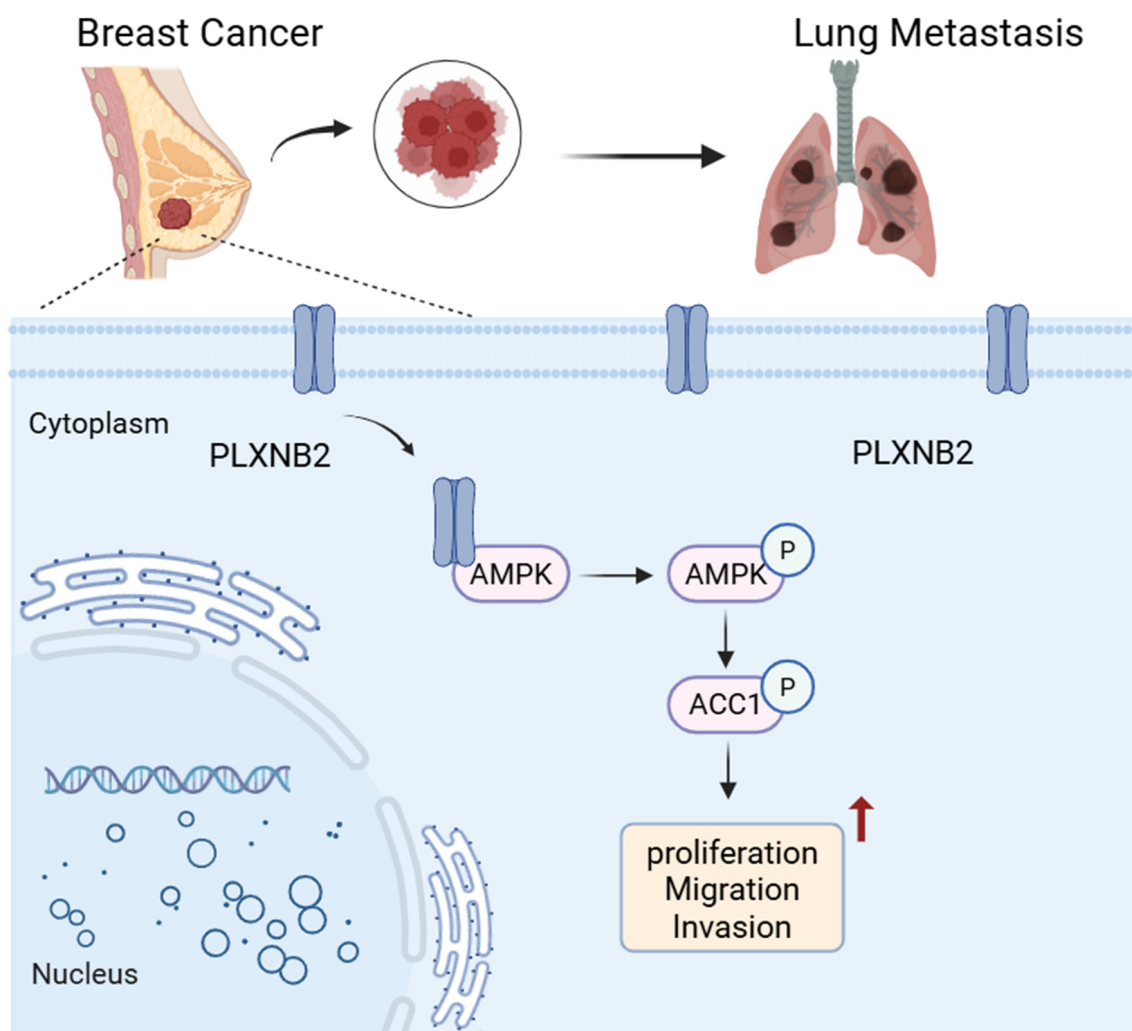


Figure 7. Proposed mechanism. PLXNB2 associates with AMPK and promotes AMPK signaling activation, thereby promoting tumor progression and lung metastasis.

spective clinical studies and additional mechanistic models are therefore needed. Nevertheless, the combined functional, mechanistic, and *in vivo* evidence supports a protumor role for PLXNB2 in breast cancer. PLXNB2 promoted tumor progression and lung metastasis through activation of AMPK signaling. These findings clarify a potential mechanism of breast cancer metastasis and identify PLXNB2 as a candidate therapeutic target for limiting invasion and distant dissemination.

Conclusions

PLXNB2 is upregulated in breast cancer and is associated with advanced disease, unfavorable clinical outcomes, and enrichment in

malignant epithelial cells. Across transcriptomic analyses, cultured breast cancer cells, and a mouse model, PLXNB2 depletion consistently reduced tumor-cell proliferation, migration, invasion, tumor growth, and lung metastasis. These findings identify PLXNB2 as a promoter of breast cancer progression and metastatic dissemination (**Figure 7**).

Reciprocal endogenous co-immunoprecipitation, pathway analyses, phosphorylation changes, and pharmacological rescue with Compound C collectively support a functional association between PLXNB2 and AMPK signaling. Although prospective clinical validation and further definition of the receptor-proximal signaling mechanism are warranted, the PLXNB2-

AMPK axis may provide a basis for developing prognostic biomarkers and therapeutic strategies aimed at limiting breast cancer progression and lung metastasis.

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Disclosure of conflict of interest

None.

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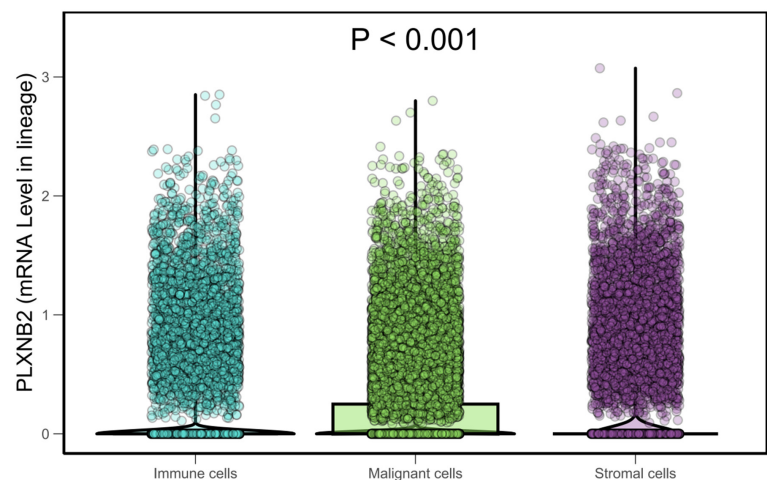


Figure S1. PLXNB2 expression in malignant, immune, and stromal cells.