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ORIGINAL ARTICLE

Pleckstrin-2-promoted PPM1B degradation plays an important role in transforming growth factor-β-induced breast cancer cell invasion and metastasis

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Abstract

Transforming growth factor-β (TGF-β) is known to promote breast cancer cell migration, invasion, and dissemination; however, the underlying molecular mechanisms are not yet well characterized. Here, we report that TGF-β induces pleckstrin-2 (PLEK2) expression by Smad3 and signal transducer and activator of transcription 3 (STAT3) activating PLEK2 promoter activity. Higher PLEK2 expression is associated with poor prognosis in breast cancer patients. Overexpression and knockout experiments in MDA-MB-231 and MCF-7 breast cancer cells revealed that PLEK2 promotes cell migration, invasion, and dissemination in 2D and 3D cell culture. Moreover, PLEK2 promotes metastasis of breast cancer cells in vivo. Pleckstrin-2 localizes to the cell membrane and cell protrusions following TGF-β treatment. Furthermore, inhibition of PI3K phosphorylation abolishes TGF-β- and PLEK2-induced cell invasion. The carboxyl-terminal PH domain of PLEK2 is critical for TGF-β- and PLEK2-induced Akt activation and plays an important role in cell invasion. Pleckstrin-2 interacts with PPM1B and promotes its ubiquitin-dependent degradation. The PLEK2-PPM1B axis utilizes nuclear factor-κB signaling to promote cell migration and invasion. Our data implicate the TGF-β-STAT3/Smad3-PLEK2-PPM1B signaling cascade in TGF-β-induced breast cancer cell migration and invasion. These findings suggest that PLEK2/PPM1B could represent novel targets for the intervention of breast cancer metastasis.

KEYWORDS
breast cancer cell, invasion, migration, PLEK2, TGF-β

Abbreviations: CA, constitutively activation; CSC, cancer stem cell; DEG, differentially expressed gene; DN, dominant negative; EGFR, epidermal growth factor receptor; EMT, epithelial–mesenchymal transition; GO, Gene Ontology; MS, mass spectrometry; NCSC, noncancer stem cell; NF-κB, nuclear factor-κB; PH, Pleckstrin homology; PIP2, PI (4, 5) P2; PIP3, PI (3–5) P3; PLEK2, Pleckstrin-2; PPM, metal-dependent protein phosphatase; qPCR, quantitative PCR; RRCC, PLEK2 mutant R267C/R268C; STAT3, signal transducer and activator of transcription 3; TGF-β, transforming growth factor-β; TGFBR, transforming growth factor-β receptor.

Our results revealed the TGF-β-STAT3/Smad3-PLEK2-PPM1B signaling cascade in TGF-β-induced breast cancer cell migration and invasion. These findings suggest that PLEK2/PPM1B could represent novel targets for breast cancer metastasis intervention.

1 | INTRODUCTION

Breast cancer is the most commonly diagnosed cancer among women,[1] and metastasis is the leading cause of breast cancer-associated death and poor prognosis.[2] The invasion–metastasis cascade is a multistep process through which cancer cells from the primary tumor disseminate by migration and invasion, colonizing at distant organs.[2,3] The vast majority of patients with advanced metastatic disease are incurable with current therapeutic regimens.

Transforming growth factor-β can function as a tumor suppressor or tumor promoter at different stages of cancer progression.[4] During the early stages, TGF-β inhibits cancer cell proliferation and induces cell apoptosis. By contrast, during the late stages, TGF-β induces EMT[5] and stem cell phenotypes to promote cancer cell migration, invasion, and metastasis.[6] A better understanding of the molecular mechanisms through which TGF-β promotes invasion and migration of breast cancer cells could allow the development of improved therapeutic strategies for cancer patients.

In the present study, we found that TGF-β induced the expression of several genes including *PLEK2*. A high expression level of PLEK2 was related to poor prognosis in breast cancer patients. Pleckstrin-2 is a member of the Pleckstrin family[7] and contains two PH domains at the N- and C-terminus, as well as a Disheveled, Egl-10, and Pleckstrin (DEP) domain in the center.[8] Recent studies have shown that PLEK2 plays an important role in the metastasis and progression of many cancer types.[9–14] Pleckstrin-2 promotes cell migration and invasion in vitro and cancer metastasis in vivo through the EMT program in non-small-cell lung cancer and gallbladder cancer.[14,15] Evidence suggests that PLEK2 exerts its pro-oncogenic function through the PI3K/Akt signaling pathway.[16,17] Although it has been reported that high expression levels of PLEK2 are related to the dissemination of luminal type A breast cancer cells,[18] the exact function and molecular mechanism of PLEK2 in breast cancer progression has not yet been well characterized.

Here, we focus on the molecular mechanisms underlying the TGF-β-induced expression of PLEK2 and its regulation of cell invasion and migration in breast cancer. We show that Smad3/STAT3 signaling is involved in TGF-β-induced PLEK2 expression. Pleckstrin-2 plays an important role in TGF-β-induced cell migration and invasion in 2D cell culture, tumor organoid invasion and cell dissemination in 3D cell culture, and lung metastasis in a mouse xenograft model of breast cancer. The C-terminal PH domain of PLEK2 is required for PLEK2-induced breast cancer cell invasion, which involves the PI3K/Akt signaling pathway. Furthermore, PLEK2 interacts with PPM1B to promote its ubiquitin-dependent degradation. PPM1B suppresses the TGF-β- and PLEK2-mediated phosphorylation of p65. Together, our data reveal that PLEK2 plays an essential role in TGF-β-induced breast cancer metastasis, suggesting that PLEK2/PPM1B could represent a novel target for the intervention of breast cancer metastasis.

2 | MATERIALS AND METHODS

2.1 | Cell lines, reagents, and Abs

All cell lines were obtained from the ATCC and cultured as described previously.[19] Detailed information regarding the reagents and Abs is provided in Table S2.

2.2 | Statistical analysis

All experiments were repeated at least three times. Student's *t*-test was used to analyze differences between two groups. Statistical analysis of more than two groups was carried out by ANOVA test. Statistical analysis was undertaken using SigmaPlot (version 9.0, Systat Software Inc.) or GraphPad Prism (version 8.4.0, Graphpad Software Inc.). The threshold for statistical significance was $p < 0.05$. Results in the bar graphs are expressed as the mean ± SD.

2.3 | Supplementary materials and methods

Detailed materials and methods are provided in Data S1.

3 | RESULTS

3.1 | PLEK2 gene expression is induced by TGF-β in breast cancer cells

MDA-MB-231 and MCF-7 cells were sorted into MDA-MB-231-CSC, MDA-MB-231-NCSC, MCF-7-CSC, and MCF-7-NCSC subpopulations by FACS. The four cell subpopulations were divided into eight groups and RNA sequencing was carried out (Figure S1A). The gene expression levels in TGF-β-treated cells and the corresponding mock-treated control cells were compared. Genes with a fold-change greater than two were selected as DEGs. An overview of the significant DEGs in the four different subpopulations is provided in Figure S1B. Analysis of the gene expression profiles of the four subpopulations treated with TGF-β identified a set of 16 significant TGF-β-induced genes comprised of 12 upregulated genes, including *PLEK2*, and four downregulated genes (Figures 1A and S1C). The mRNA expression levels of certain significant DEGs in MDA-MB-231 and MCF-7 cells were measured in the presence and absence of TGF-β (Figure S1D). The fold changes in gene expression seen by RNA sequencing were confirmed by RT-qPCR (Figure S1E). Pleckstrin-2 was mainly expressed in triple-negative and luminal A-type breast cancer cells (Figure 1B). Furthermore, TGF-β induced PLEK2 expression in MDA-MB-231 and MCF-7 cells at both the mRNA and protein levels (Figure 1C,D). Transforming growth factor-β also induced the expression of *PLEK2* mRNA in SUM-159 and T47D breast cancer cells (Figure S1F). These findings suggest that PLEK2 gene expression is induced by TGF-β in breast cancer cells.

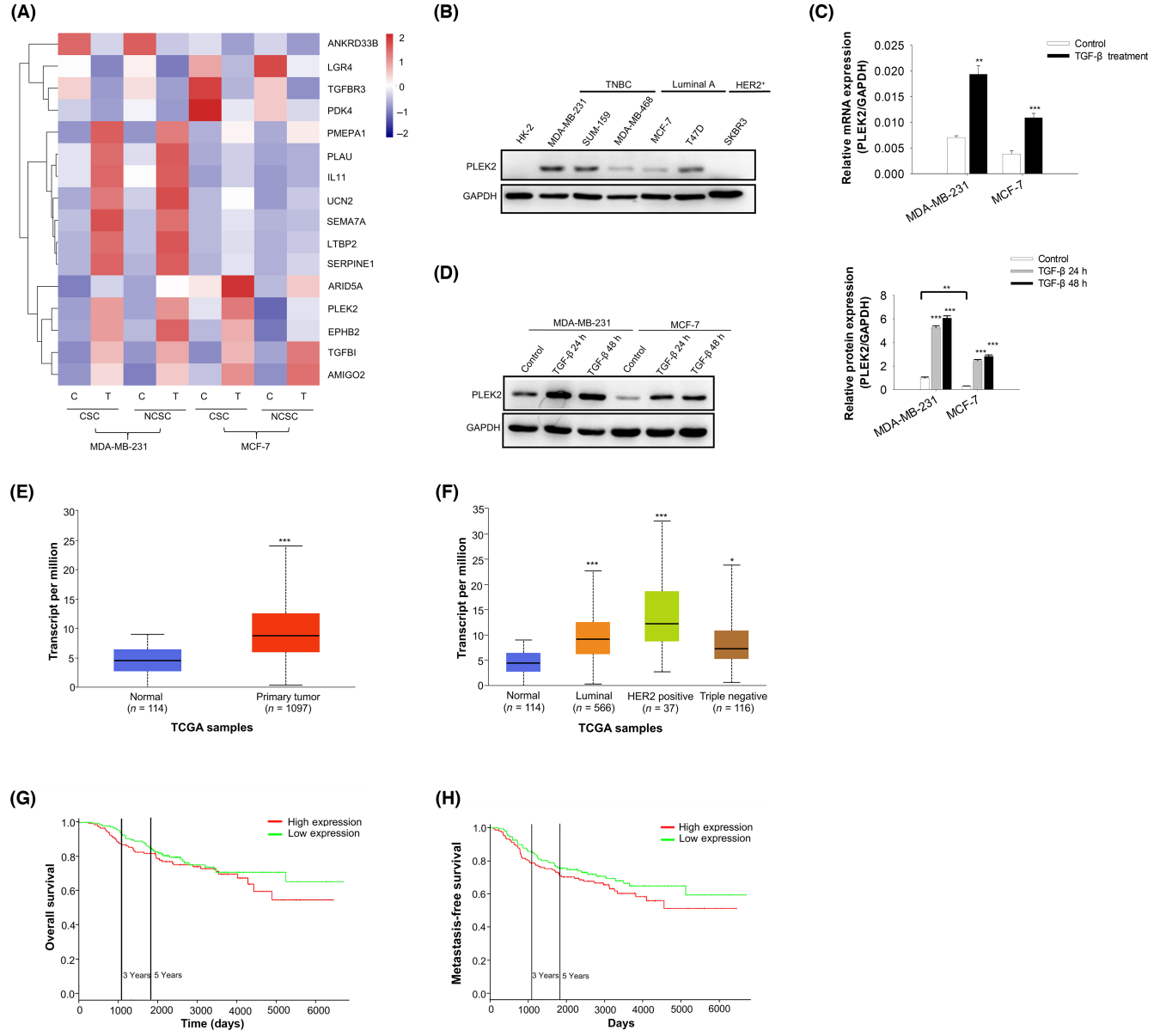


FIGURE 1 Pleckstrin-2 (PLEK2) is highly expressed in human breast cancer and is associated with poor prognosis. (A) Heatmap representation of the RNA sequencing data, as plotted using Omicshare Tools, showing the expression levels of significant transforming growth factor-β (TGF-β)-induced genes in the indicated cells. (B) Protein expression of PLEK2 in HK-2 human renal tubular epithelial cells and different types of breast cancer cells. (C,D) mRNA (C) and protein (D) expression levels of PLEK2 in MDA-MB-231 and MCF-7 cells in the presence and absence of TGF-β as analyzed by RT-quantitative PCR and immunoblotting, respectively. Statistical analysis of data in (D) is shown on the right. GAPDH was used as a loading control. The values of the MDA-MB-231-Control group were set to 1 for normalization. $n = 3$. (E,F) Gene expression profile of PLEK2 in (E) normal tissues and primary tumor samples and (F) different subtypes of breast cancer (obtained from the UALCAN database). (G,H) Kaplan–Meier analysis of (G) overall survival and (H) metastasis-free survival in the NKI dataset using the ProgGene version 2 database. $n = 295$ samples. Hazard ratio (HR) of (G): 1.3 (1.01–1.66), HR of (H): 1.33 (1.07–1.65). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. C, control; CSC, cancer stem cell; HER2, human epidermal growth factor receptor 2; NCSC, non-cancer stem cell; T, TGF-β-treated; TCGA, The Cancer Genome Atlas; TNBC, triple-negative breast cancer.

3.2 | Pleckstrin-2 is highly expressed in human breast cancer and is associated with poor prognosis

Cells migrate by extending plasma membrane protrusions, such as lamellipodia and invadopodia, at the leading edge.[20] Pleckstrin-2 was enriched in the plasma membrane region, cell leading edge, and leading edge membrane GO terms as our GO analysis results (http://www.webgestalt.org/; Table S1). Pleckstrin-2 expression was significantly upregulated in primary tumors and different subtypes of breast cancer tissues in comparison with normal tissues (http://ualcan.path.uab.edu/;[21] Figure 1E, F). A high expression level of PLEK2 was associated with metastasis and decreased overall and metastasis-free survival (http://www.compbio.iupui.edu/proggene/;[22,23] Figure 1G,H).

3.3 | Pleckstrin-2 is necessary for TGF-β-induced cell migration and invasion

Deletion of PLEK2 by CRISPR/Cas9 (PLEK2-KO-MDA-MB-231) (Figure S2A, left) and restoration of PLEK2 expression in PLEK2-KO-MDA-MB-231 cells (Figure S2A, right) were confirmed by immunoblotting. Results of the wound healing assay, single-cell migration tracking, and Transwell invasion assay revealed that deletion of PLEK2 inhibited cell migration, single-cell migration speed, and cell invasion, respectively (Figure 2A–C). Restoration of PLEK2 expression in PLEK2-KO-MDA-MB-231 cells completely rescued their single cell migration ability (Figure 2B) and partially restored cell migration and invasiveness (Figure 2A,C). Stable overexpression of PLEK2 by lentiviral infection in MDA-MB-231 and MCF-7 cells (OE-PLEK2-MDA-MB-231 and OE-PLEK2-MCF-7) was verified by immunoblotting (Figure S2B). Overexpression of PLEK2 increased cell migration (Figure 2D,E) and invasion (Figure 2F).

To evaluate the role of PLEK2 in TGF-β-induced cell migration and invasion, PLEK2-WT and PLEK2-KO-MDA-MB-231 cells were treated with 5 ng/mL TGF-β for 24 h. Deletion of PLEK2 partially blocked TGF-β-induced cell migration (Figure 2G) and abrogated TGF-β-induced single-cell migration (Figure 2H) and invasion (Figure 2I). Protein expression levels of TGFBR1 and TGFBR2 were similar in both cell lines (Figure S10A). Furthermore, the TGFBR1/2 inhibitor LY2109761 eliminated TGF-β-induced cell migration and invasion but did not completely block cell migration or invasion induced by PLEK2 (Figure S3A,B). These findings suggest that PLEK2 is necessary for TGF-β-induced cell migration and invasion.

3.4 | Pleckstrin-2 is essential for formation of TGF-β-induced invasive protrusions and cell dissemination of organoids in 3D cell culture

A 3D cell culture system was used to mimic the in vivo tumor microenvironment as previously reported.[24–26] The doubling time of the indicated cells was analyzed using Trypan blue staining and a hemocytometer (Figure S4A,B). Knockout of PLEK2 in MDA-MB-231 cells decreased the number of organoids with invasive protrusions (Figure 3A). Disseminated cells in 3D culture were verified by calcein-AM/propidium iodide double staining (Figure 3B,D,F). Knockout of PLEK2 in MDA-MB-231 cells decreased the number of organoids with disseminated cells (Figure 3B). Restoration of PLEK2 expression rescued the number of organoids with invasive protrusion and dissemination morphologies, producing a phenotype consistent with that of the control (Figure 3A,B). Overexpression of PLEK2 in MDA-MB-231 and MCF-7 cells promoted cell invasion and dissemination in 3D culture (Figure 3C,D). These results indicate that PLEK2 is necessary for the induction of an invasive phenotype and cell dissemination of organoids in a 3D culture system.

Consistent with 2D cell culture results, deletion of PLEK2 partially blocked the TGF-β-induced invasive phenotype and cell dissemination of organoids in 3D culture (Figure 3E,F). Furthermore, the mesenchymal marker vimentin was significantly upregulated in disseminated PLEK2-overexpressing MDA-MB-231 and MCF-7 cells (Figure S5A–C). These results show that PLEK2 plays an important role in TGF-β-induced tumor organoid invasion and dissemination of breast cancer cells.

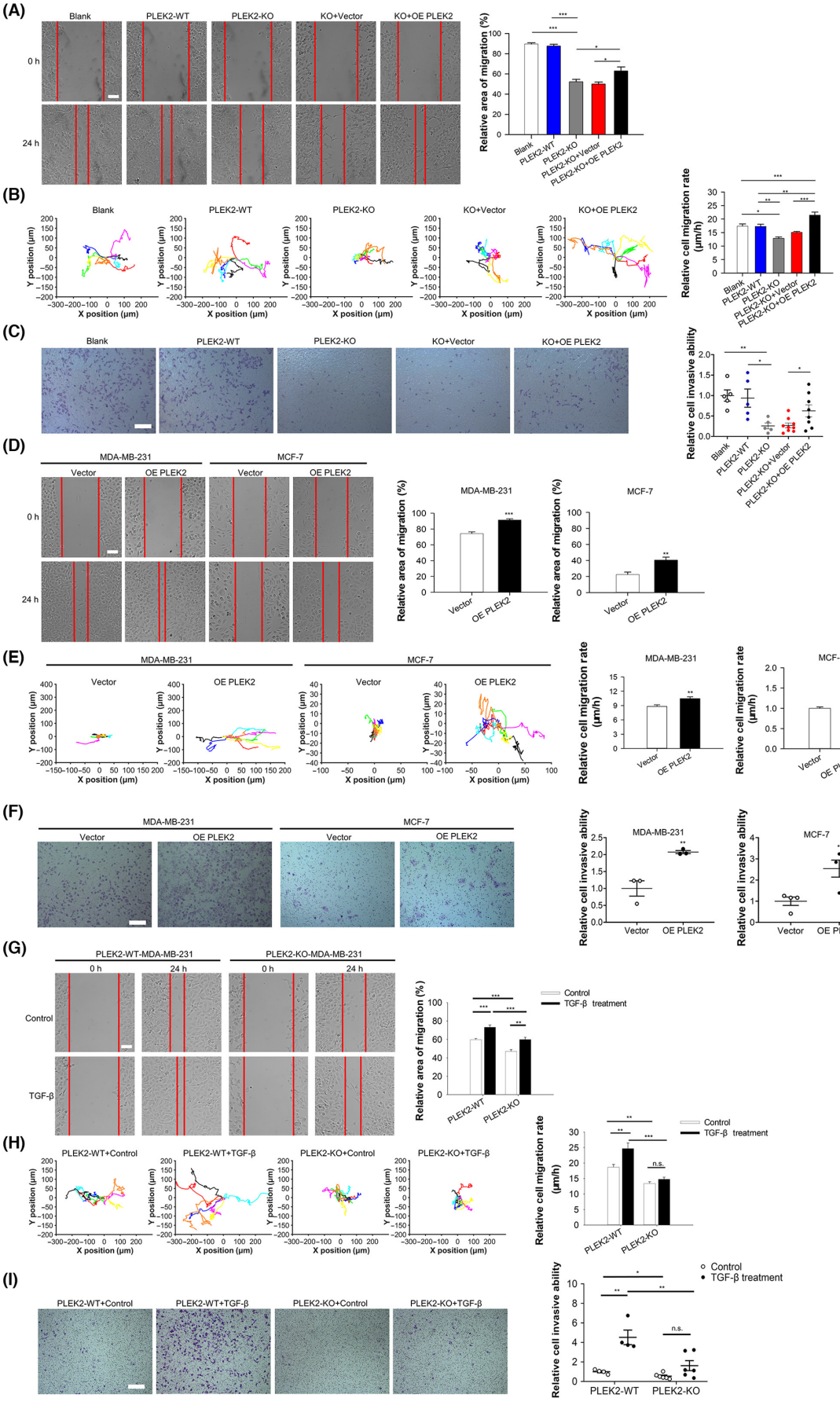
3.5 | Pleckstrin-2 promotes metastasis of breast cancer cells in vivo

The effect of PLEK2 on lung metastasis in xenograft models of breast cancer was evaluated. Control, PLEK2-KO, and OE-PLEK2 MDA-MB-231 cells were injected into the tail vein of BALB/c nude mice as previously described.[27,28] There was no obvious difference in bodyweight or lung weight among the three groups (Figure 4A,B). Staining with H&E showed that PLEK2 KO decreased metastatic lung foci, whereas these foci were increased following PLEK2 overexpression (Figure 4C–E). These results indicate that PLEK2 promotes metastasis of breast cancer cells to the lung.

3.6 | Smad3/STAT3 signaling is involved in TGF-β-induced PLEK2 expression

Considering that TGF-β induced PLEK2 expression at both the mRNA and protein levels, we hypothesized that TGF-β regulates PLEK2 expression at the transcriptional level. Accordingly, a 3 kb region of the PLEK2 promoter was submitted to the JASPAR (http://jaspar.binf.ku.dk/) and GTRD (http://gtrd.biouml.org/) databases with a view to predicting its transcription factors. The search yielded 54 common transcription factors between the JASPAR and GTRD databases (Figure 5A). RNA sequencing data and previously published reports identified four candidate transcription factors of PLEK2: Smad3, STAT3, JunB, and FOXP1. Among these, Smad3 is known to be a transducer of TGF-β signaling; therefore,

FIGURE 2 Pleckstrin-2 (PLEK2) is essential for transforming growth factor-β (TGF-β)-induced cell migration and invasion. (A,D,G) Representative migration images of the indicated cells in the wound healing assay. Scale bar, 100 μm. The graph shows the relative closure rates of the different groups. (B,E,H) Trajectory plots showing the trajectory of the indicated cells obtained by tracking in real time for 24 h. All tracks were set to a common origin using MATLAB. Each line represents the cell migration route and each color represents a specific cell. The graph shows the single-cell migration speeds of the different types of cells (right). (C,F,I) Representative invasion images of the indicated cells in the Transwell invasion assays. Scale bar, 200 μm. The graph shows the relative invasive ability of the different types of cells (right). The values of the (C) Blank group, (F) Vector group, and (I) PLEK2-WT + Control group were set to 1 for normalization. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n.s., not significant; OE, overexpressing.


(A)
Blank
PLEK2-WT
PLEK2-KO
KO+Vector
KO+OE PLEK2
0 h
24 h
Relative area of migration (%)
(B)
Relative cell migration rate (μm/h)
(C)
Relative cell invasive ability
(D)
MDA-MB-231
MCF-7
Vector
OE PLEK2
(E)
(F)
(G)
PLEK2-WT-MDA-MB-231
PLEK2-KO-MDA-MB-231
Control
TGF-β
TGF-β treatment
(H)
PLEK2-WT+Control
PLEK2-WT+TGF-β
PLEK2-KO+Control
PLEK2-KO+TGF-β
n.s.
(I)
X position (μm)
Y position (μm)

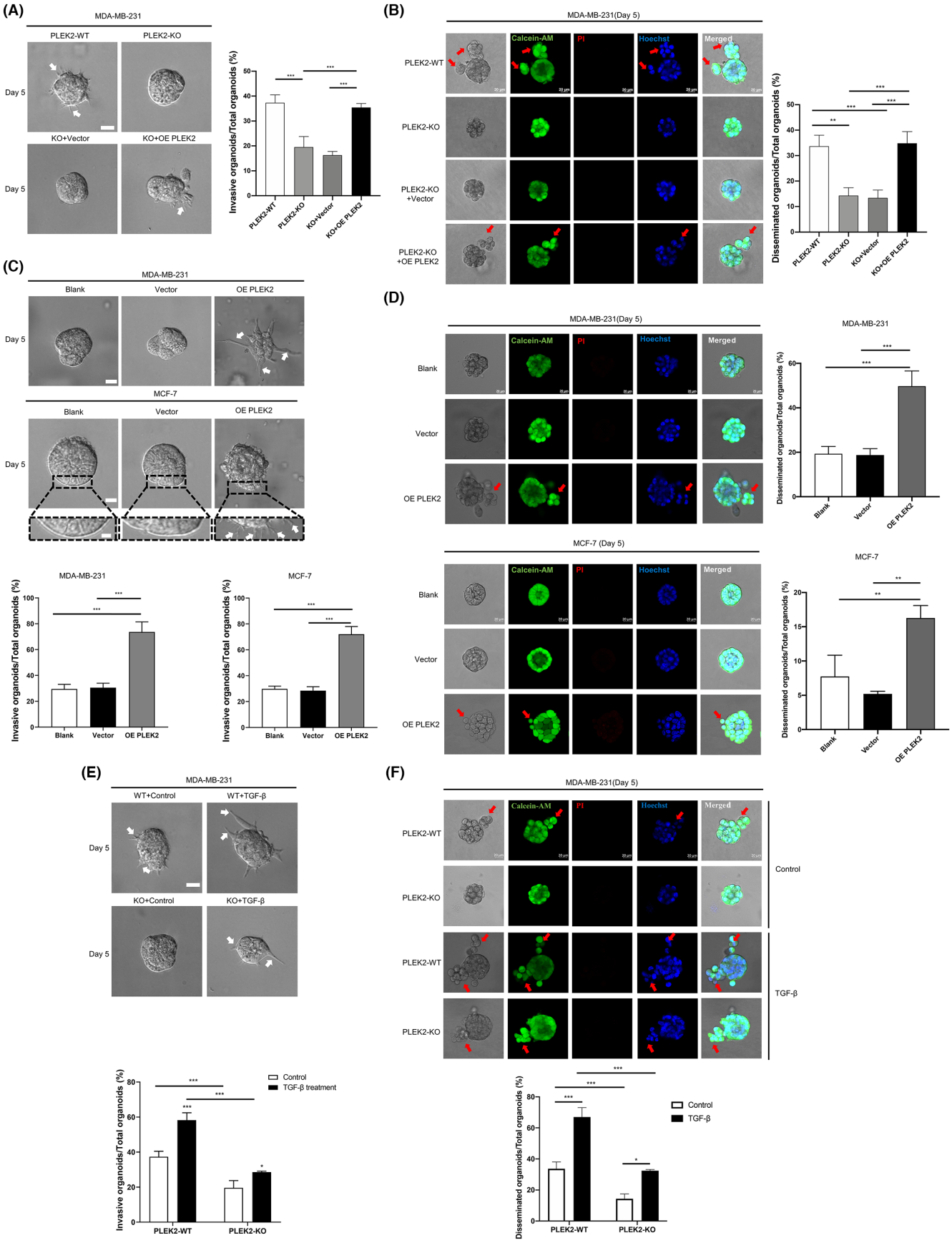

(A)
MDA-MB-231
PLEK2-WT
PLEK2-KO
KO+Vector
KO+OE PLEK2
Day 5
Invasive organoids/Total organoids (%)
(B)
MDA-MB-231(Day 5)
Calcein-AM
PI
Hoechst
Merged
PLEK2-WT
PLEK2-KO
PLEK2-KO +Vector
PLEK2-KO +OE PLEK2
Disseminated organoids/Total organoids (%)
(C)
MDA-MB-231
Blank
Vector
OE PLEK2
MCF-7
Invasive organoids/Total organoids (%)
(D)
MDA-MB-231(Day 5)
MCF-7 (Day 5)
Disseminated organoids/Total organoids (%)
(E)
MDA-MB-231
WT+Control
WT+TGF-β
KO+Control
KO+TGF-β
Control
TGF-β treatment
(F)
MDA-MB-231(Day 5)
Control
TGF-β

FIGURE 3 Pleckstrin-2 (PLEK2) is essential for cell protrusion extension and cell dissemination of organoids in 3D culture. (A,C,E) Representative invasion images of the indicated cells in 3D culture. Scale bar, 30 μm. White arrows indicate cell invasion. The inset of (C) is an enlarged view of the boxed region. Statistical analysis of (A,C,E) is shown. Invasive ability was normalized to the control group. Each group was quantitated using 100–300 cells. (B,D,F) Representative dissemination images of the indicated cells in 3D culture. Scale bar, 20 μm. Red arrows indicate cell dissemination. Statistical analysis of (B,D,F) is shown. The cell dissemination ability was normalized to the control group. Each sample was quantitated using 50–100 cells. Three independent experiments were performed. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. OE, overexpression; TGF-β, transforming growth factor-β.

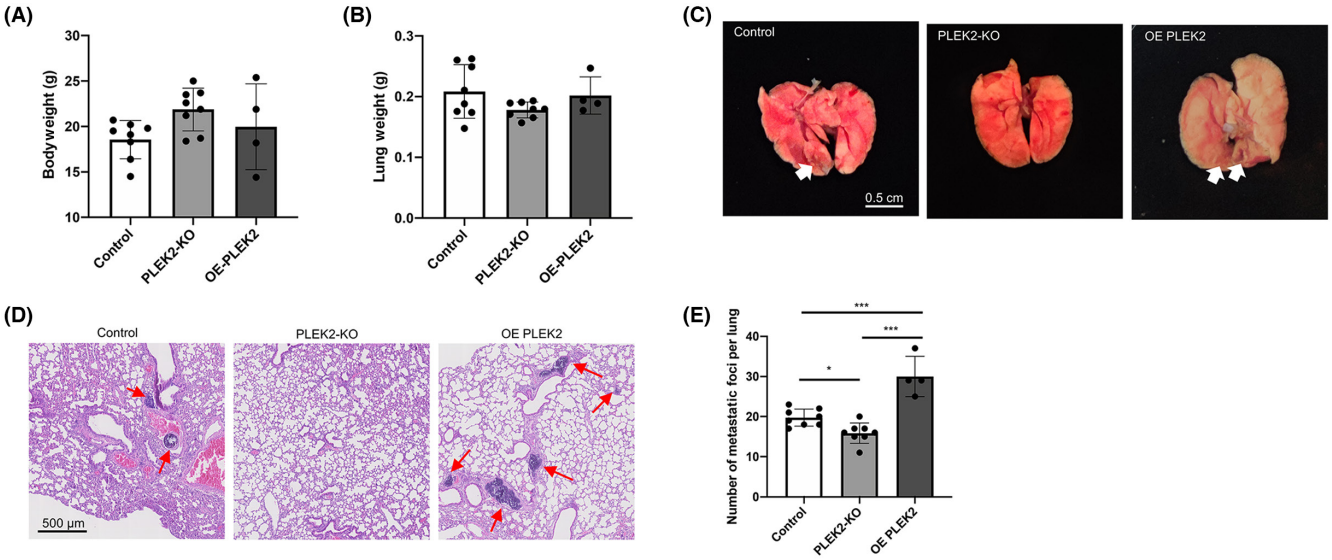


FIGURE 4 Pleckstrin-2 (PLEK2) promotes metastasis of breast cancer cells in vivo. (A) Indicated MDA-MB-231 cells (control, PLEK2-KO, overexpression [OE]-PLEK2) were injected into the tail vein of 6-week-old female BALB/c nude mice. Bodyweight of mice was measured. (B) Lung weight was measured 8 weeks postinjection. (C) Representative lung images of metastasis in the three groups are shown. White arrows indicate lung metastatic foci. Scale bar, 0.5 cm. (D) Representative H&E staining images are shown. Red arrows indicate lung metastatic foci. Scale bar, 500 μm. (E) Statistical analysis of (D) is shown. $n_{(Control)} = 8$, $n_{(PLEK2\text{-}KO)} = 8$, $n_{(OE\ PLEK2)} = 4$. $^{**}p<0.01$, $^{***}p<0.001$.

we aimed to determine whether TGF-β could induce the expression of the other three transcription factors. Transforming growth factor-β increased the mRNA expression levels of *JunB* and *FOXP1* (Figure S6A), and increased the protein expression level of JunB but not that of FOXP1 (Figure S6B). Furthermore, TGF-β increased the phosphorylation (Y^{705}) level of STAT3 in MDA-MB-231 and MCF-7 cells (Figure 5B). The JASPAR database analysis revealed that the PLEK2 promoter contains seven, six, and four putative binding sites for Smad3, STAT3, and JunB, respectively; thus, PLEK2 promoter segments of different lengths were constructed (Figure S6C). Dual luciferase assays show that STAT3 and Smad3 increased the promoter activity of PLEK2 (Figure 5C,D); however, JunB did not regulate PLEK2 promoter activity (Figure S6D). Treatment of MDA-MB-231 and MCF-7 cells with the STAT3-specific inhibitor Stattic or the Smad3-specific inhibitor SIS3 partially blocked TGF-β-induced PLEK2 expression at both the mRNA and protein levels (Figure 5E,F). Nevertheless, STAT3 and Smad3 did not elicit a synergetic effect on the TGF-β-induced upregulation of PLEK2.

To further investigate the importance of STAT3 and Smad3 in the regulation of PLEK2 expression, MDA-MB-231 and MCF-7 cells stably overexpressing STAT3-WT/CA/DN and Smad3-WT/CA/DN were generated by lentiviral infection. A schematic of STAT3 and Smad3 mutation variants is shown in Figure 5G. The mRNA and protein expression levels of PLEK2 were significantly increased in MDA-MB-231 and MCF-7 cells overexpressing STAT3-CA and Smad3-CA as compared with control or those overexpressing STAT3-DN and Smad3-DN (Figure 5H–J). Collectively, these results indicate that Smad3/STAT3 signaling is involved in TGF-β-induced PLEK2 upregulation.

3.7 | Transforming growth factor-β significantly increases PLEK2 localization at cell membrane and protrusions

Previous studies have shown that PLEK2 colocalizes with F-actin, has a high affinity for the PI3K products PIP2 and PIP3, and interacts with Coffilin, Rac1, EGFR, and SHIP2.[14,15,29–31] Surprisingly, many of the proteins and lipids known to interact or colocalize with PLEK2 are associated with invadopodia[32]; therefore, we hypothesized that the intracellular localization of PLEK2 affects the regulation of invadopodia formation and function. Immunofluorescence staining shows that PLEK2 localization at the cell membrane and

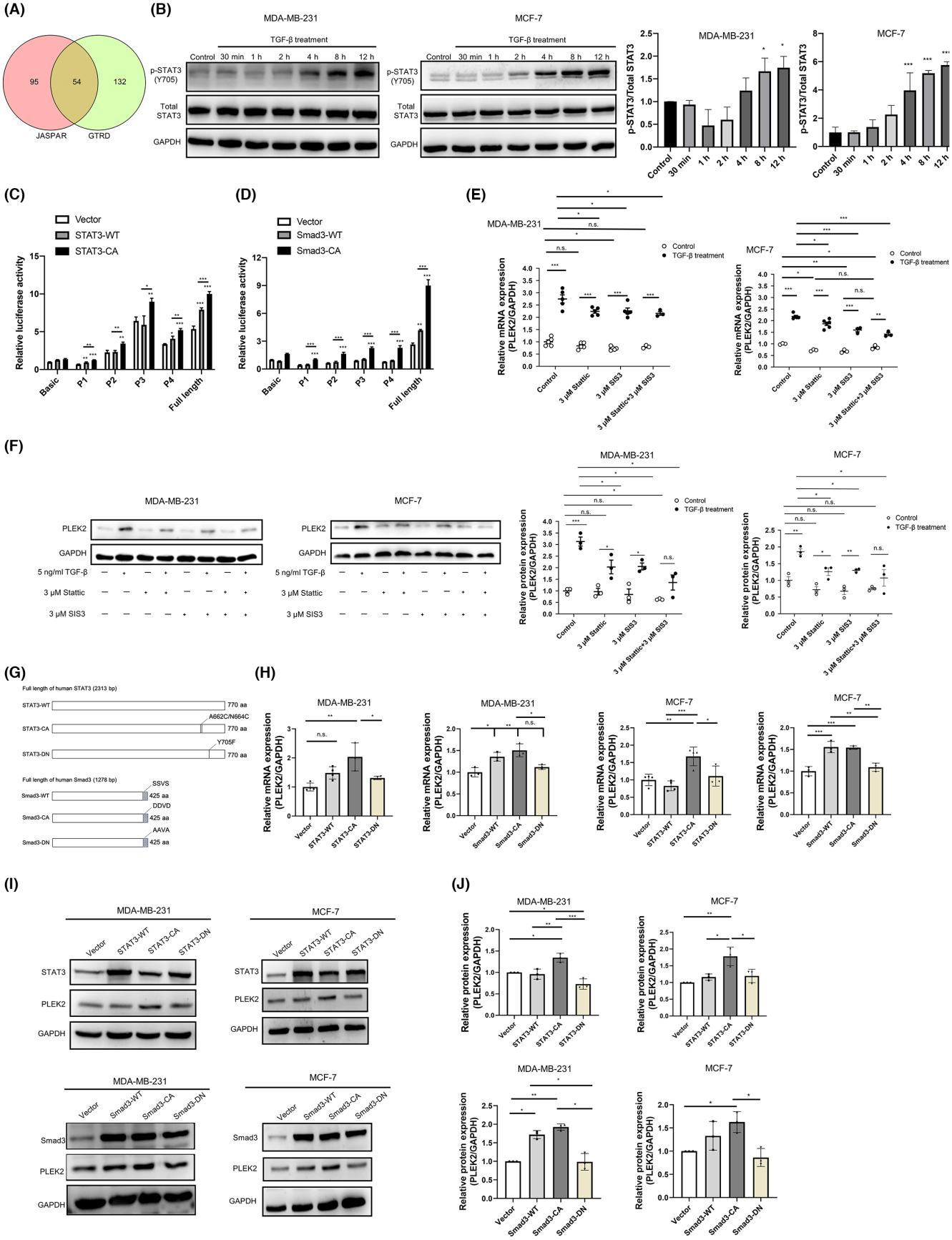

(A)
95
54
132
JASPAR
GTRD
(B)
MDA-MB-231
MCF-7
TGF-β treatment
Control 30 min 1 h 2 h 4 h 8 h 12 h
p-STAT3 (Y705)
Total STAT3
GAPDH
p-STAT3/Total STAT3
(C)
Vector
STAT3-WT
STAT3-CA
Relative luciferase activity
Basic P1 P2 P3 P4 Full length
(D)
Vector
Smad3-WT
Smad3-CA
(E)
Relative mRNA expression (PLEK2/GAPDH)
Control
TGF-β treatment
Control
3 μM Stattic
3 μM SIS3
3 μM Stattic+3 μM SIS3
(F)
PLEK2
GAPDH
5 ng/ml TGF-β
3 μM Stattic
3 μM SIS3
Relative protein expression (PLEK2/GAPDH)
(G)
Full length of human STAT3 (2313 bp)
STAT3-WT 770 aa
STAT3-CA A662C/N664C 770 aa
STAT3-DN Y705F 770 aa
Full length of human Smad3 (1278 bp)
Smad3-WT SSVS 425 aa
Smad3-CA DDVD 425 aa
Smad3-DN AAVA 425 aa
(H)
Relative mRNA expression (PLEK2/GAPDH)
Vector
STAT3-WT
STAT3-CA
STAT3-DN
Smad3-WT
Smad3-CA
Smad3-DN
(I)
STAT3
Smad3
PLEK2
GAPDH
(J)
Relative protein expression (PLEK2/GAPDH)

FIGURE 5 Smad3 and signal transducer and activator of transcription 3 (STAT3) are involved in transforming growth factor-β (TGF-β)-induced Pleckstrin-2 (PLEK2) upregulation. (A) Venn diagram, as plotted with Omicshare Tools, to predict the potential transcription factors that regulate PLEK2 promoter activity using the JASPR and GTRD databases. (B) Representative immunoblots showing the protein expression levels of total STAT3 and the phosphorylation (Y^{705}) of STAT3 in MDA-MB-231 and MCF-7 cells treated with TGF-β for different periods of time. Ratios of phosphorylated STAT3 (p-STAT3) to total STAT3 are shown on the right. Values of the control group were set to 1 for normalization. $n = 3$. (C,D) Relative luciferase activity in HEK293T cells cotransfected with (C) different STAT3 expression vectors or (D) different Smad3 expression vectors and different PLEK2 promoters. Values of the Basic + Vector group were set to 1 for normalization. $n = 3$. (E,F) The (E) mRNA and (F) protein expression levels of PLEK2 in MDA-MB-231 and MCF-7 cells under different treatment conditions were analyzed. GAPDH was used as the internal control. Values of the control group were set to 1 for normalization. (G) Schematic representation of the STAT3/Smad3-WT/CA/DN mutant used in this study. (H–J) The (H) mRNA and (I) protein expression levels of PLEK2 in the indicated cells were analyzed. GAPDH was used as the internal control. Statistical analysis of data in (I) is shown in (J). Values of the Vector group were set to 1 for normalization. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. n.s., not significant. CA, constitutively activation; DN, dominant negative.

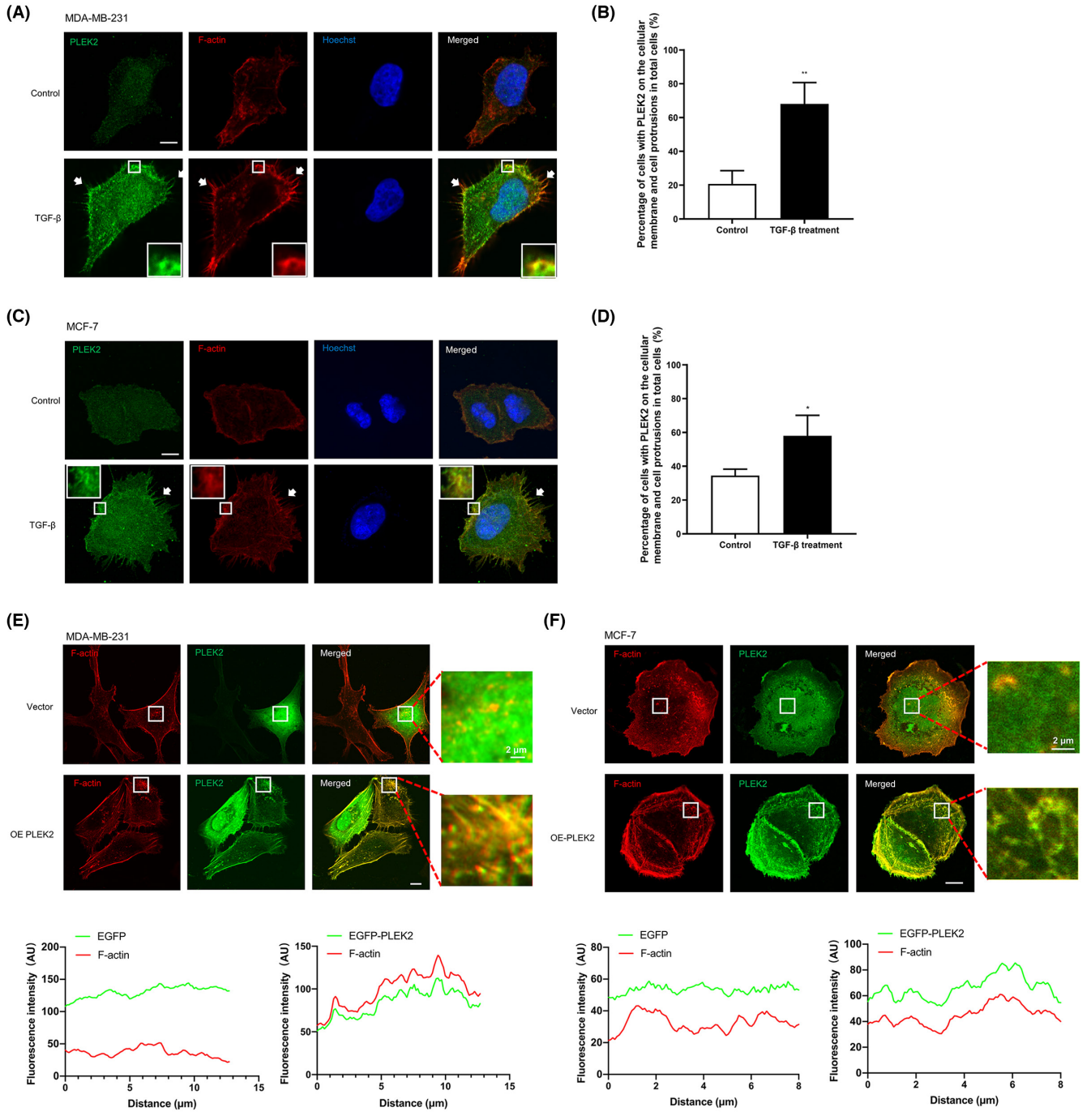


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FIGURE 6 Subcellular localization of Pleckstrin-2 (PLEK2) in breast cancer cells. (A,B) Localization of endogenous PLEK2 and F-actin in MDA-MB-231 and MCF-7 cells in the presence and absence of transforming growth factor-β (TGF-β). White arrows indicate cell protrusions at the cell membrane. The inset is an enlarged view of the boxed region. Scale bar, 10 μm. (C,D) Percentage of cells with PLEK2 localized to the cell membrane and protrusions in the presence and absence of TGF-β. Scale bar, 10 μm. $n = 3$. (E,F) Localization of PLEK2 and F-actin in (E) MDA-MB-231 and (F) MCF-7 cells ectopically expressing pEGFP-C1-long linker-PLEK2. Scale bar, 10 μm. Horizontal axis represents the distance of the indicated point in the box to the left start point. Green lines indicate the fluorescence intensity of pEGFP; red lines indicate the fluorescence intensity of TRITC-Phalloidin dye. OE, overexpression.

protrusions was significantly increased following TGF-β treatment (Figure 6A–D). There also exists clear colocalization of exogenous PLEK2 with F-actin at the leading edge. Moreover, the spot-like pattern of PLEK2 and F-actin colocalization resembled the structure of invadopodia, indicating that this might have been the localization of PLEK2 (Figure 6E,F); however, PLEK2 did not colocalize with Tks5 (Figure S7), a commonly used biomarker of mature invadopodia. These results suggest that PLEK2 colocalizes with F-actin at the cell membrane and protrusions but is not a component of mature invadopodia.

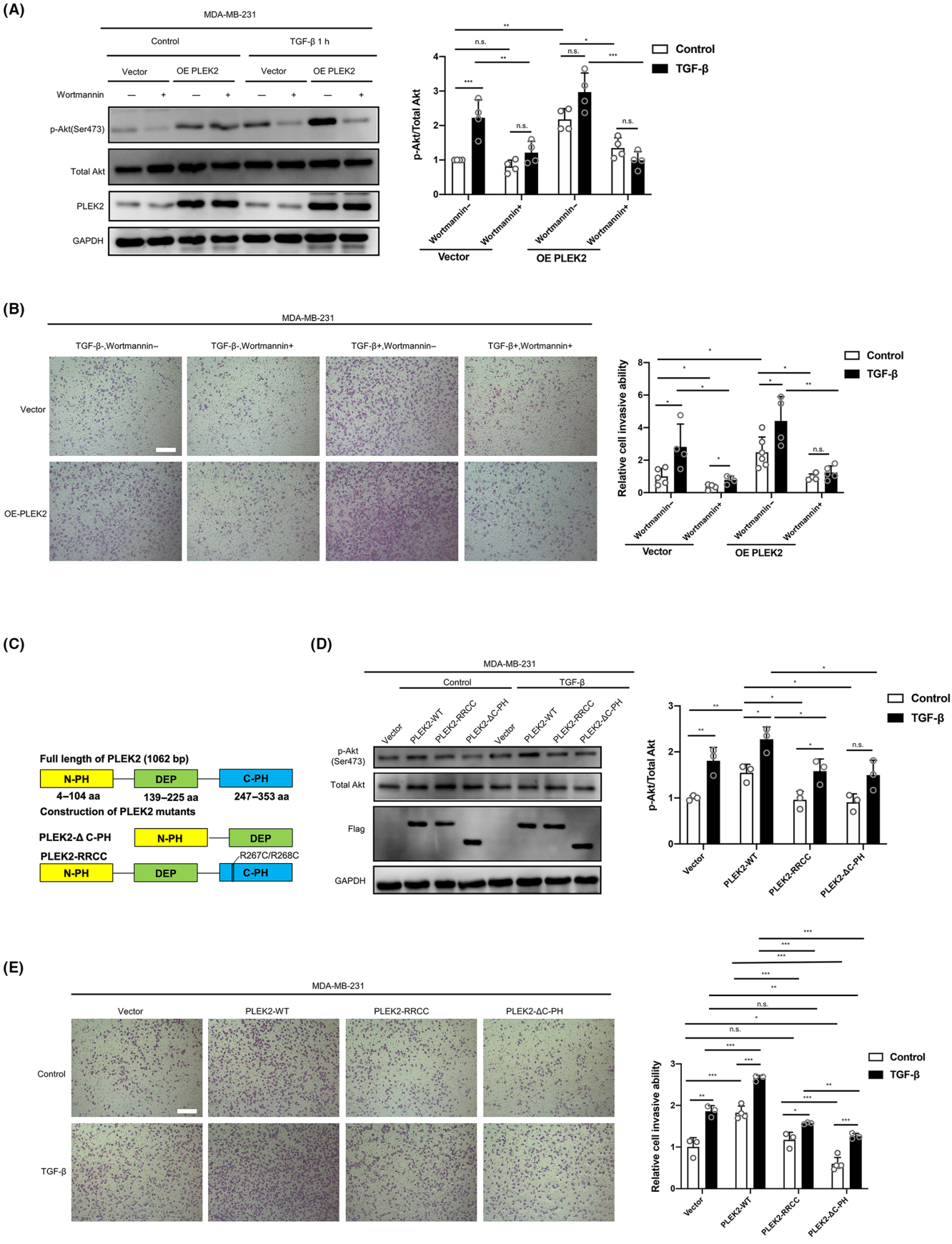
3.8 | The C-terminal PH domain of PLEK2 is important for activation of the PI3K/Akt signaling pathway and is required for breast cancer cell invasion

Inhibiting PI3K with Wortmannin suppresses the membrane association of PLEK2 and PLEK2-induced cell spreading in Jurkat cells.[29] Our data indicates that inhibition of PI3K phosphorylation with Wortmannin abolished TGF-β- and PLEK2-induced phosphorylation of Akt and cell invasion (Figure 7A,B). The C-terminal PH domain of PLEK2 is required for the binding of PI3K-mediated lipid products. The PH domain mutation prevents PLEK2 from binding to the cell membrane and inhibits cell spreading.[29] A PLEK2 mutant, RRCC, has been shown to be incapable of binding to PIP3. Ectopically expressed PLEK2-RRCC did not colocalize with F-actin in COS-1 cell protrusions or regulate actin rearrangement.[30] Here, truncation/mutation variants of PLEK2 (PLEK2-WT/RRCC and PLEK2-ΔC-PH) were constructed to identify the functional domain of PLEK2 (Figure 7C). Protein expression levels of TGFBR1 and TGFBR2 were similar in both cell lines (Figure S10B). Ectopic expression of PLEK2-WT significantly increased the phosphorylation of Akt and promoted cell invasion, while overexpression of PLEK2-RRCC only slightly increased Akt phosphorylation and barely promoted cell invasion. Overexpression of PLEK2-ΔC-PH did not display this capability (Figure 7D,E). These results show that the C-terminal PH domain of PLEK2 is important for PLEK2-induced PI3K/Akt signaling and cell invasion.

3.9 | Pleckstrin-2 interacts with PPM1B and promotes its ubiquitin-dependent degradation

Recent reports suggest that PLEK2 promotes lung and gallbladder cancer metastasis through interactions with protein partners[14,15]; however, there exist no data regarding the role of PLEK2 in breast cancer. Here, PLEK2-interacting proteins were identified by immunoprecipitation–MS analysis (Figure 8A), and 200 specific candidate proteins were selected for GO analysis. The selected proteins were enriched in GO terms related to the NIK/NF-κB signaling pathway, proteasome complex, and focal adhesion (Figure 8B). A search of published works identified PPM1B, a serine/threonine phosphatase of the PPM family, as a suitable candidate for further study. As a tumor suppressor, PPM1B dephosphorylates RhoGDI1 and negatively regulates the motility and invasiveness of breast cancer cells.[33] PPM1B plays a critical role in the dephosphorylation and inhibition of IKKβ, as well as termination of the tumor necrosis factor-α-mediated activation of NF-κB.[34] Co-immunoprecipitation and immunofluorescence staining showed that both endogenous and exogenous PLEK2 interacted with endogenous PPM1B (Figure 8C,D). The protein expression level of PPM1B was significantly decreased in MCF-7 and MDA-MB-231 cells following TGF-β treatment or PLEK2 overexpression (Figure S8B,D), and was increased in PLEK2-KO-MDA-MB-231 cells (Figure S8F); however, the mRNA expression level of *PPM1B* remained unchanged (Figure S8A,C,E). The increased protein expression level of PPM1B was downregulated in PLEK2-KO-MDA-MB-231 cells by restoring PLEK2 expression (Figure S8F). Treatment with TGF-β increased the protein expression level of PLEK2 and decreased that of PPM1B in a time-dependent manner (Figure S8G). These results indicate that PLEK2 could regulate the expression of

FIGURE 7 C-terminal Pleckstrin homology (PH) domain of Pleckstrin-2 (PLEK2) is important for activation of the PI3K/Akt signaling pathway and is required for breast cancer cell invasion. (A) Representative immunoblots showing the phosphorylation level of Akt (Ser^{473}) and the protein expression level of Akt in the indicated cells. Ratios of phosphorylated Akt (p-Akt) to total Akt in the different groups are shown. (B) Representative images showing cell invasion in the Transwell assays. Scale bar, 200 μm. Relative cell invasive ability is shown. (C) Schematic representation of PLEK2 WT, deletion mutations of the C-terminal PH (C-PH) domain, and the point mutation (RRCC). (D) Protein expression levels of the different C-terminally Flag-tagged PLEK2 variants (PLEK2-WT-Flag, PLEK2-RRCC-Flag, and PLEK2-ΔC-PH-Flag) in MDA-MB-231 cells were confirmed by immunoblotting. Representative immunoblots showing the phosphorylation level of Akt (Ser^{473}) and the protein expression level of Akt in the indicated cells. Ratios of p-Akt to total Akt in the different groups are shown (right). (E) Representative images showing cell invasion in Transwell invasion assays. Scale bar, 200 μm. Graph shows the relative cell invasive ability of the different types of cells (right). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. DEP, Disheveled, Egl-10, and Pleckstrin domain; N-PH, N-terminal PH; n.s., not significant; OE, overexpression; TGF-β, transforming growth factor-β.


(A)
MDA-MB-231
Control
TGF-β 1 h
Vector
OE PLEK2
Vector
OE PLEK2
Wortmannin
— + — + — + — +
p-Akt(Ser473)
Total Akt
PLEK2
GAPDH
p-Akt/Total Akt
Control
TGF-β
Wortmannin−
Wortmannin+
Wortmannin−
Wortmannin+
Vector
OE PLEK2
(B)
MDA-MB-231
TGF-β-,Wortmannin−
TGF-β-,Wortmannin+
TGF-β+,Wortmannin−
TGF-β+,Wortmannin+
Vector
OE-PLEK2
Relative cell invasive ability
Control
TGF-β
Wortmannin−
Wortmannin+
Wortmannin−
Wortmannin+
Vector
OE PLEK2
(C)
Full length of PLEK2 (1062 bp)
N-PH
DEP
C-PH
4–104 aa
139–225 aa
247–353 aa
Construction of PLEK2 mutants
PLEK2-Δ C-PH
N-PH
DEP
PLEK2-RRCC
R267C/R268C
N-PH
DEP
C-PH
(D)
MDA-MB-231
Control
TGF-β
Vector
PLEK2-WT
PLEK2-RRCC
PLEK2-ΔC-PH
Vector
PLEK2-WT
PLEK2-RRCC
PLEK2-ΔC-PH
p-Akt (Ser473)
Total Akt
Flag
GAPDH
p-Akt/Total Akt
Control
TGF-β
Vector
PLEK2-WT
PLEK2-RRCC
PLEK2-ΔC-PH
(E)
MDA-MB-231
Vector
PLEK2-WT
PLEK2-RRCC
PLEK2-ΔC-PH
Control
TGF-β
Relative cell invasive ability
Control
TGF-β
Vector
PLEK2-WT
PLEK2-RRCC
PLEK2-ΔC-PH

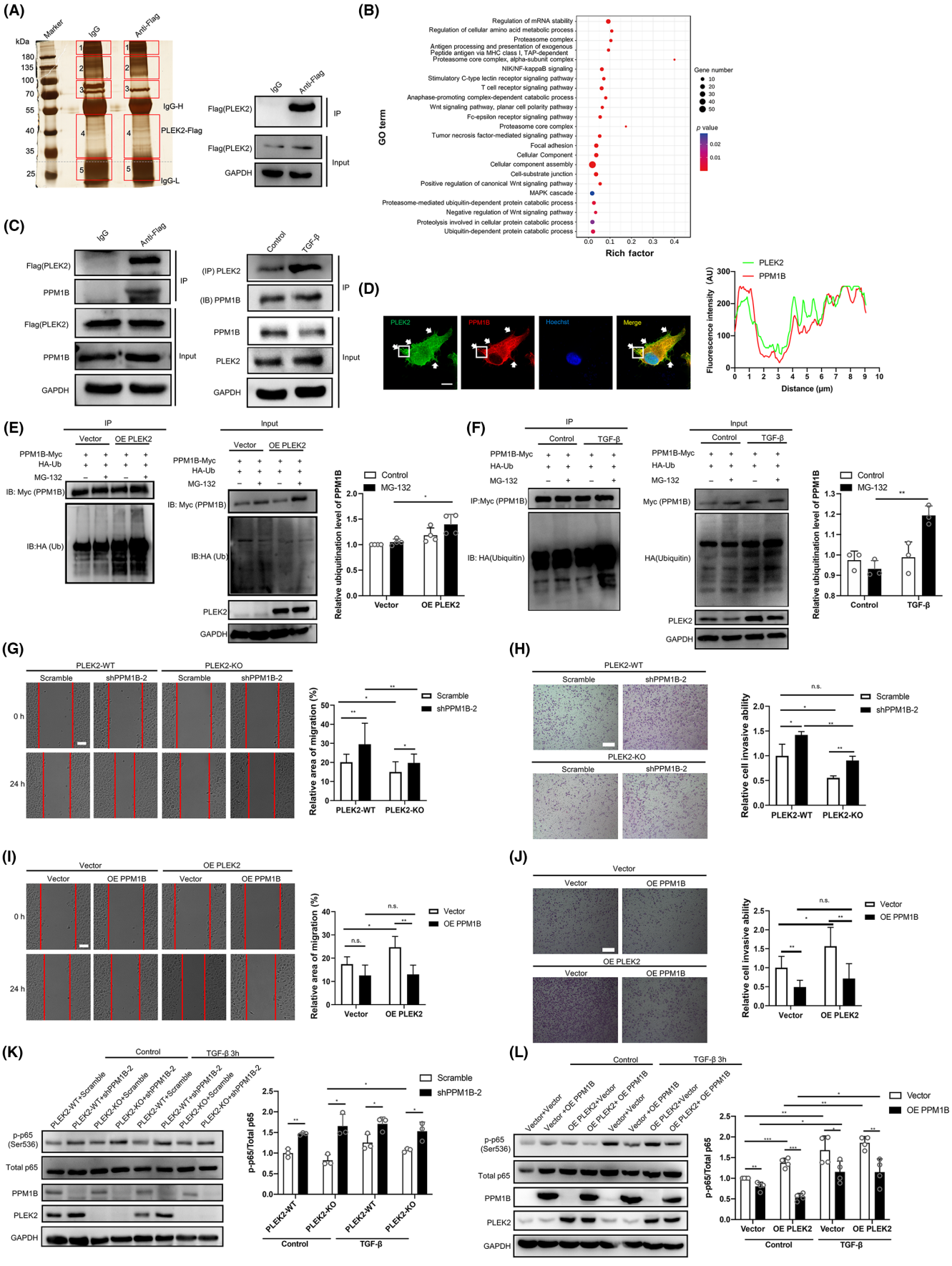

(A)
Marker
IgG
Anti-Flag
kDa
180
135
100
70
55
40
35
25
IgG-H
PLEK2-Flag
IgG-L
Flag(PLEK2)
IP
GAPDH
Input
(B)
Regulation of mRNA stability
Regulation of cellular amino acid metabolic process
Proteasome complex
Antigen processing and presentation of exogenous Peptide antigen via MHC class I, TAP-dependent
Proteasome core complex, alpha-subunit complex
NIK/NF-kappaB signaling
Stimulatory C-type lectin receptor signaling pathway
T cell receptor signaling pathway
Anaphase-promoting complex-dependent catabolic process
Wnt signaling pathway, planar cell polarity pathway
Fc-epsilon receptor signaling pathway
Proteasome core complex
Tumor necrosis factor-mediated signaling pathway
Focal adhesion
Cellular Component
Cellular component assembly
Cell-substrate junction
Positive regulation of canonical Wnt signaling pathway
MAPK cascade
Proteasome-mediated ubiquitin-dependent protein catabolic process
Negative regulation of Wnt signaling pathway
Proteolysis involved in cellular protein catabolic process
Ubiquitin-dependent protein catabolic process
GO term
Rich factor
Gene number
p value
(C)
PPM1B
Control
TGF-β
(IP) PLEK2
(IB) PPM1B
(D)
PLEK2
PPM1B
Hoechst
Merge
Fluorescence intensity (AU)
Distance (μm)
(E)
Vector
OE PLEK2
PPM1B-Myc
HA-Ub
MG-132
IB: Myc (PPM1B)
IB:HA (Ub)
Relative ubiquitination level of PPM1B
(F)
IP:Myc (PPM1B)
IB: HA(Ubiquitin)
Myc (PPM1B)
HA(Ubiquitin)
(G)
PLEK2-WT
PLEK2-KO
Scramble
shPPM1B-2
0 h
24 h
Relative area of migration (%)
(H)
Relative cell invasive ability
n.s.
(I)
OE PPM1B
(J)
(K)
TGF-β 3h
p-p65 (Ser536)
Total p65
p-p65/Total p65
(L)

FIGURE 8 Pleckstrin-2 (PLEK2)-PPM1B axis acts through the nuclear factor-κB signaling pathway to exert its pro-metastatic function. (A) MDA-MB-231 cells overexpressing C-terminally Flag-tagged PLEK2 (PLEK2-Flag) were subjected to immunoprecipitation (IP) with normal mouse IgG (Control) or an anti-Flag Ab. IP samples were subjected to SDS-PAGE and silver staining followed by mass spectrometry (MS) analysis. Red boxes indicate band regions subjected to MS analysis. Immunoblotting confirmed PLEK2 expression (right). (B) Specific candidate proteins with MS data were subjected to Gene Ontology analysis. (C) Co- IP experiments with normal mouse IgG or an anti-Flag Ab and subsequent immunoblotting (IB) (left) were carried out to verify the PLEK2–PPM1B interaction in PLEK2-Flag-MDA-MB-231 cells. Interaction between endogenous PLEK2 and PPM1B in MDA-MB-231 cells in the presence and absence of transforming growth factor-β (TGF-β) was confirmed by Co-IP (right). (D) Immunofluorescence staining and ImageJ analysis showing the colocalization of PLEK2 and PPM1B in MDA-MB-231 cells. Left: green, PLEK2, red, PPM1B, blue, Hoechst. Scale bar, 10 μm. White arrows indicate that PLEK2 and PPM1B were colocalized at protrusions on the cell membrane. Right: horizontal axis represents the distance of the indicated point in the box to the left start point. Green lines indicate the fluorescence intensity of labeled PLEK2; red lines indicate the fluorescence intensity of labeled PPM1B. (E,F) Vector/OE-PLEK2-MDA-MB-231 (E) and MDA-MB-231 cells in the presence or absence of TGF-β (F) were transfected with pcDNA3.1-PPM1B-Myc and pcDNA3.1-HA-ubiquitin (Ub) for 48 h. Cells were subsequently treated with or without 10 μm MG-132 for a further 6 h. Cell lysates underwent IP with an anti-Myc Ab and IB was carried out with the indicated Ab. Statistical analysis is shown on the right. (G,I) Representative images showing migration of the indicated cells in the wound healing assay. Scale bar, 100 μm. Relative closure rates of the different groups are shown on the right. $n = 3$. (H,J) Representative images showing invasion of the indicated cells in the Transwell invasion assay. Scale bar, 200 μm. Relative cell invasive ability is shown on the right. (H) $n = 3$. (J) $n = 6$. (K,L) Representative immunoblots showing the phosphorylation level of p65 (Ser^{536}) and the protein expression levels of total p65, PLEK2, and PPM1B in the indicated cells. Statistical analysis is shown. (M) Schematic illustration of the mechanisms underlying the TGF-β-induced expression of PLEK2 and its regulation of cell invasion and migration in breast cancer. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

PPM1B at the posttranscriptional level. Treatment with TGF-β or PLEK2 overexpression in MDA-MB-231 cells enhanced the ubiquitination of PPM1B, while treatment with MG-132 partially restored the protein expression of PPM1B and suppressed TGF-β- or PLEK2-induced ubiquitination of PPM1B (Figures 8E,F and S8H). These results indicate that the interaction between PLEK2 and PPM1B likely plays an important role in TGF-β-induced breast cancer progression.

3.10 | PLEK2–PPM1B axis utilizes the NF-κB signaling pathway to exert its pro-metastatic function

It has been reported that PPM1B suppresses cancer progression and negatively regulates NF-κB signaling by dephosphorylating IKKβ.[35] Gain-of-function and loss-of-function experiments were carried out to investigate the role of PPM1B in breast cancer cells. The protein expression level of PPM1B was significantly increased in PPM1B-overexpressing MDA-MB-231 and MCF-7 cells (Figure S9A) and was decreased following PPM1B knockdown (Figure S9D). Migration ability and invasiveness were remarkably inhibited in PPM1B-overexpressing cells (Figure S9B,C), whereas PPM1B knockdown resulted in the opposite effects (Figure S9E,F). These results show that PPM1B is a negative regulator of breast cancer cell migration and invasion. Rescue experiments were undertaken to investigate whether PPM1B participates in PLEK2-induced breast cancer cell migration and invasion. The protein expression level of PPM1B was decreased in PLEK2-WT/PLEK2-KO MDA-MB-231 cells following knockdown of PPM1B by shRNA (Figure S2C) and increased in vector/OE-PLEK2-MDA-MB-231 cells following overexpression of PPM1B by lentiviral infection (Figure S2D). Deletion of PLEK2 suppressed migration and invasion, but these characteristics were partially restored in PLEK2-KO MDA-MB-231 cells by knockdown of PPM1B (Figure 8G,H). Ectopic expression of PPM1B in PLEK2-overexpressing MDA-MB-231 cells decreased PLEK2-induced cell migration and invasion (Figure 8I,J). Given the association between PLEK2 and PPM1B, we hypothesized that PLEK2 acts through the PPM1B-NF-κB signaling pathway to promote cell invasion and migration. Knockdown of PPM1B in PLEK2-WT or PLEK2-KO MDA-MB-231 cells increased the phosphorylation level of p65 (Figure 8K). Overexpression of PLEK2 and TGF-β increased the phosphorylation level of p65, while ectopic expression of PPM1B in PLEK2-overexpressing MDA-MB-231 cells decreased TGF-β- and PLEK2-induced phosphorylation of p65 (Figure 8L). Protein expression levels of TGFBR1 and TGFBR2 were similar in both cell lines (Figure S10C). These results show that the PLEK2-PPM1B axis acts through the NF-κB signaling pathway to promote cell migration and invasion.

4 | DISCUSSION

Metastasis is the leading cause of breast cancer-related death, and despite the recent progress in suppressing the metastasis of breast cancer, metastatic disease still lacks effective treatment. To develop effective metastasis prevention and treatment strategies, detailed knowledge of the underlying molecular mechanisms is required.

Transforming growth factor-β is a critical regulator of EMT and TGF-β signaling plays context-dependent roles in cancer,[36,37] with the effects on CSCs and NCSCs being different.[38,39] Transforming growth factor-β signaling is only activated in CSCs in response to TGF-β treatment, regulating EMT in breast cancer[39]; however, the aggressiveness of cancer is related to new CSCs generated from NCSCs. Transforming growth factor-β promotes the transition of NCSCs to CSCs by inducing ZEB1 expression in breast cancer.[38] The common genes expressed in CSCs and NCSCs in response to TGF-β could be the key to cancer aggressiveness. To identify metastasis-related genes induced by TGF-β in breast cancer, gene expression profiles were analyzed by RNA sequencing

(Figures S1A-C). Combining bioinformatics with RT-qPCR uncovered that TGF-β induced the expression of certain genes, including *PLEK2*, in breast cancer cells (Figures S1D,E). Transforming growth factor-β induced PLEK2 expression in MDA-MB-231 and MCF-7 cells at both the mRNA and protein levels (Figure 1C,D). Recent studies of non-small-cell lung cancer and gallbladder cancer have reported involvement of PLEK2 in cancer metastasis[14,15]; however, its role in breast cancer remains obscure. Here, PLEK2 was expressed at a markedly higher level in primary breast carcinoma and different subtypes of breast carcinoma in comparison with normal tissue (Figure 1E,F). A high expression level of PLEK2 was correlated with poor prognosis in breast cancer patients (Figure 1G,H).

Deletion of PLEK2 and treatment with the TGFBR1/2 inhibitor LY2109761 in MDA-MB-231 cells abolished TGF-β-induced migration and invasion; however, LY2109761 treatment did not completely abrogate PLEK2-induced cell migration and invasion (Figures 2G–I and S3A,B). Restoration of PLEK2 expression in PLEK2-KO-MDA-MB-231 cells completely rescued single cell migration ability and partially rescued cell migration and invasion (Figure 2A–C). In addition, overexpression of PLEK2 in MDA-MB-231 and MCF-7 cells also promoted migration and invasion (Figure 2D–F). These results are consistent with previous reports.[14,15] Pleckstrin-2 also plays an important role in TGF-β-induced breast cancer cell migration, invasion, and metastasis in 3D culture systems (Figure 3A–F). Furthermore, PLEK2 promotes metastasis of breast cancer cells to the lung in vivo (Figure 4C–E).

The molecular mechanism by which TGF-β regulates PLEK2 expression in breast cancer cells remains unclear. In the present study, we show for the first time that TGF-β induces the mRNA and protein expression of PLEK2 in breast cancer cells through transcriptional activation by Smad3 and STAT3 (Figure 5C–J).

Previous studies have reported that PLEK2 interacts or colocalizes with certain proteins or lipids, such as F-actin, PIP3, Rac1, Coffilin, EGFR, and SHIP2,[14,15,29–31] which play essential roles in invadopodia formation and function.[40–42] Transforming growth factor-β significantly increased PLEK2 localization at the cell membrane and protrusions (Figure 6A,C). Moreover, the spot-like pattern of PLEK2 and F-actin colocalization was similar to the structure of invadopodia (Figure 6E,F). However, PLEK2 unexpectedly did not colocalize with Tks5, a marker of mature invadopodia (Figure S7), suggesting that PLEK2 has no effect on mature invadopodia formation and function.

The C-terminal PH domain of PLEK2 is required for lipid binding and its mutation prevents PLEK2 from binding to the cell membrane, inhibiting cell spreading.[29] We hypothesized that the C-terminal PH domain of PLEK2 affects the PI3K signaling pathway to regulate PLEK2-induced cell invasion. Inhibition of PI3K phosphorylation with Wortmannin abolished TGF-β- and PLEK2-induced cell invasion (Figure 7B), indicating that the PI3K/Akt signaling pathway is involved in PLEK2-induced cell invasion and metastasis. These results are consistent with previous reports.[15–17] Furthermore, our data show for the first time that the C-terminal PH domain of PLEK2 is important for TGF-β- and PLEK2-induced Akt activation (Figure 7D) and cell invasion (Figure 7E).

Previous studies have shown that PLEK2 interacts with protein partners to exert its pro-metastatic function in lung and gallbladder cancer.[14,15] Pleckstrin-2 can interact with the kinase domain of EGFR to maintain a state of continuous activation by inhibiting its c-CBL-mediated ubiquitination.[14] Additionally, PLEK2 interacts with SHIP2 in non-small-cell lung cancer cells to target this protein for degradation, which leads to activation of the PI3K/Akt signaling pathway.[14,15] Moreover, PLEK2 interacts with c-Myc to prevent its proteasome-mediated degradation in head and neck squamous cell carcinoma.[43] Here, we determined that PLEK2 interacted with PPM1B in breast cancer cells (Figure 8A,C,D). PPM1B inhibited PLEK2-induced cell migration and invasion (Figure 8G–J). Furthermore, PLEK2 induced the ubiquitin-dependent degradation of PPM1B (Figures 8E,F and S8H), which is involved in the NF-κB signaling pathway (Figure 8K,L). These results suggest that the PLEK2-PPM1B axis exerts pro-metastatic functions through the NF-κB signaling pathway.

Considering that TGF-β plays a crucial role in cancer and other diseases, numerous efforts have been made to develop targeted therapeutics.[44] However, systemic anti-TGF-β therapies using TGF-β-neutralizing Abs or receptor kinase inhibitors may cause severe adverse effects due to the spatiotemporal activation pattern of TGF-β signaling during cancer progression.[45–47] More specific drugs that selectively target downstream signaling without affecting other homeostatic functions of TGF-β could be used to improve current therapeutic strategies for breast cancer patients. Here, we revealed that the TGF-β-STAT3/Smad3-PLEK2-PPM1B axis is critical for TGF-β-induced breast cancer cell migration, invasion, and dissemination. We also showed that the NF-κB signaling pathway participates in the TGF-β-STAT3/Smad3-PLEK2-PPM1B axis in breast cancer cells. These findings provide new insights into the function and detailed mechanism of TGF-β-induced cell invasion and metastasis.

AUTHOR CONTRIBUTIONS

L.D. designed and performed the experiments, analyzed the data, and wrote the manuscript. J.L. performed some experiments. Y.T. and L.D. performed the animal experiments. R.F. supervised this study and wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest

ETHICS STATEMENT

Approval of the research protocol by an Institutional Review Board: N/A.

Informed consent: N/A.

Registry and registration no. of the study/trial: N/A.

Animal studies: The experimental procedures on the animals were conducted in accordance with the Principles for the Utilization and Care of Vertebrate Animals and the Guide for the Care and Use of Laboratory Animals and approved by the IACUC of the Center for Experimental Animal Research and Peking University Laboratory Animal Center (IACUC No. LSC-FengRQ-1).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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