**ARTICLE**

# LncRNA PVT1 regulates triple-negative breast cancer through KLF5/beta-catenin signaling

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## Abstact

Recent molecularly targeted approach gains advance in breast cancer treatment. However, the estimated 5-year survival rate has not met the desired expectation for improvement, especially for patients with triple-negative breast cancer (TNBC). Here we report that the lncRNA PVT1 promotes KLF5/beta-catenin signaling to drive TNBC tumorigenesis. *PVT1* is upregulated in clinical TNBC tumors. Using genetic approaches targeting *PVT1* in TNBC cells, we found that *PVT1* depletion inhibited cell proliferation, colony formation, and orthotopic xenograft tumor growth. Mechanistically, *PVT1* binds with KLF5 and increases its stability via BAP1, which upregulates beta-catenin signaling, resulting in enhanced TNBC tumorigenesis. *PVT1*, KLF5, and beta-catenin were also revealed to be co-expressed in clinical TNBC samples. Our findings uncover a new singaling pathway to mediate TNBC, and provide *PVT1* as a new target for improving treatment of TNBC.

## Introduction

Breast cancer is the most common malignancy in women [1, 2]. Although recent molecularly targeted approach advances in breast cancer treatment, the estimated 5-year survival rate has not met the desired expectation for improvement, especially for patients with triple-negative breast cancer (TNBC) [3]. Therefore, there is an urgent need to foster a major effort to discover novel therapy targets to treat patients with these tumors. Recent long non-coding RNAs (LncRNAs) are recognized to play critical roles in

TNBC progression [4–6]. However, the mechanisms by which TNBC tumorigenicity is mediated are not well known.

The plasmacytoma variant translocation 1 gene (*PVT1*) is an lncRNA that has oncogenic function in multiple cancers. *PVT1* locates ~50 kb downstream of *MYC* and is frequently co-amplified with *MYC* in colon [7], ovarian [8], lung [9], and breast [8] cancers. *PVT1* was first shown to contribute to carcinogenesis because of its frequent trans-locations in mouse plasmacytomas [10, 11]. Recent *PVT1* has been demonstrated to bind and stabilize Myc [12] in breast cancer and Nop2 [13] in hepatocellular carcinoma. *PVT1* is amplified in TNBC with Rb/p53 deficiency [14]. *PVT1* represses p15 and p16 expression through physical binding with EZH2 in gastric cancer [15]. *PVT1* is also demonstrated as a direct target of HIF in clear cell renal cell carcinoma [16]. Moreover, expression of *PVT1* is correlated with recurrence and/or survival of patients with ovarian [8], lung [9], and colorectal [17] cancers. However, further explorations are required to elucidate the functions of *PVT1* in tumor progression, including breast cancer.

In the present study, we present evidence that *PVT1* promotes proliferation and tumorigenesis in TNBC through stabilizing KLF5 and promoting *CTNNB1* expression. We show that the PVT1–KLF5-β–catenin axis plays important roles in TNBC cell proliferation in vitro and tumor growth in vivo. It is also shown that *PVT1* is upregulated in breast

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**Fig. 1** lncRNA *PVT1* is upregulated in clinical TNBC. **a** Expression levels of *PVT1* mRNA are significantly higher in clinical TNBC samples compared with normal breast tissues. **b** *PVT1* mRNA levels are significantly higher in clinical TNBC specimens compared with the paired peritumoral breast tissues. Expression data of *PVT1* mRNA were downloaded from the Cancer Genome Atlas (TCGA). **c** Expression level of *PVT1* in breast cancers with Her2$^+$, ER$^+$, PR$^+$, and TN (triple-negative) from the TCGA data set. **d** Kaplan–Meier analysis of patients with high *PVT1*-expressing TNBC tumors vs. low *PVT1*-expressing tumors from the TCGA data set. Statistical analysis was performed by log-rank test in a GraphPad Prism version 5.0 for Windows. Median survival (in days): low, undefined; high, 2965. Black bars, censored data. Error bars ± SD. **$P < 0.01$. ***$P < 0.001$. $P$ values were calculated using two-tailed Student's $t$-tests (**a**, **b**) and one-way ANOVA (**c**)
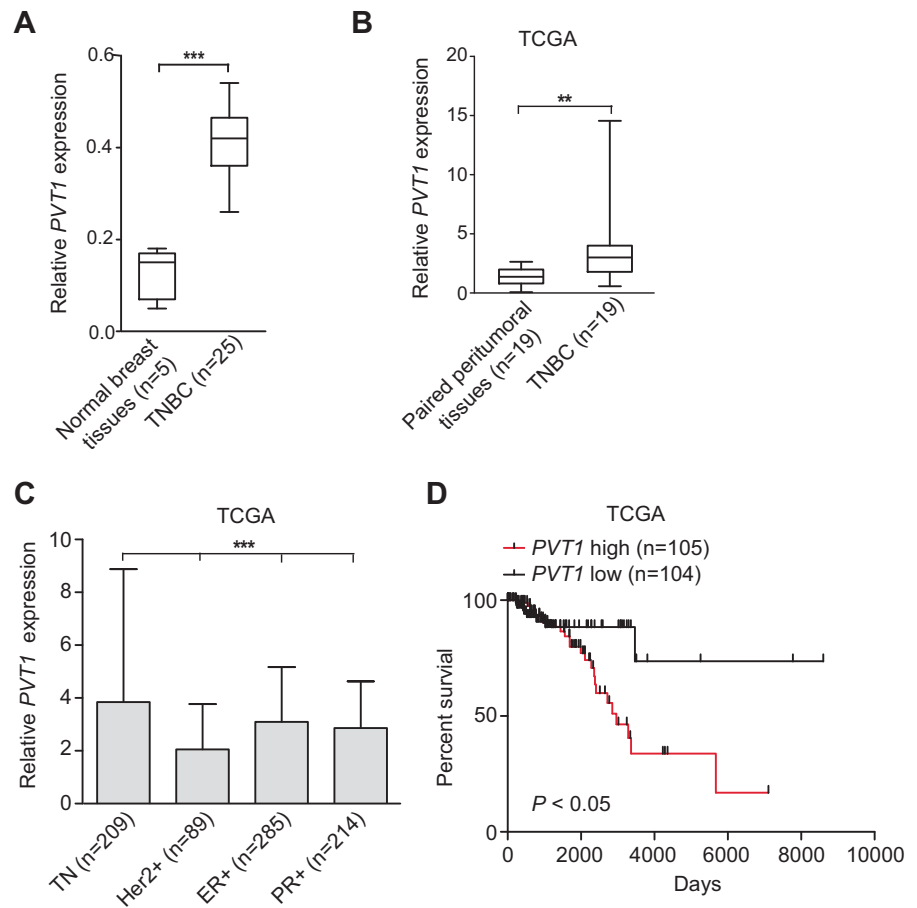


cancer tissues compared with that in normal breast tissues. *PVT1* levels are positively correlated with those of *KLF5* or *CTNNB1* in clinical TNBC tissues. Collectively, our data demonstrate a novel of signaling pathway to regulate TNBC progression.

# Results

## LncRNA *PVT1* is upregulated in clinical TNBC samples

To assess the function of *PVT1* in TNBC, we first assessed the expression of *PVT1* in clinical specimens of patients. As shown in Fig. 1a and Supplementary Table S1, compared to normal breast tissues, *PVT1* was found to be highly expressed in clinical TNBC tumors. To support our finding, we downloaded the Cancer Genome Atlas (TCGA) RNA-seq data set of 1098 clinical invasive breast cancer samples. In this data set, 19 pairs of clinical TNBC tumors and the peritumoral tissues were included. Compared with the paired peritumoral tissues, the expression level of *PVT1* was significantly elevated in TNBC specimens (Fig. 1b and Supplementary Table S2). We examined *PVT1* expression

in clinical breast cancer samples only with Her2$^+$, ER$^+$, PR$^+$, or TN from the TCGA data set. As shown in Fig. 1c and Table S3, *PVT1* was expressed at the highest levels in TNBC tumors compared with other breast cancer subtypes. Moreover, we analyzed the copy numbers of *PVT1* in TCGA breast cancer data set from http://xena.uscs.edu/public-hubs, and found that, compared with normal breast tissues, 55.9% (614/1098) tumors were amplified (log2 > 0.19, tumor vs. normal; Supplementary Figure S1). These data suggest that *PVT1* is overexpressed and amplified in TNBC tumors.

Then, we assessed the relationship of *PVT1* expression and TNBC patient survival by Kaplan–Meier survival analysis using the TCGA data set. As shown in Fig. 1d, patients with high *PVT1* expression (>median level) in the TCGA data set have a statistically significant worse prognosis compared with those with low expression (Fig. 1d and Supplementary Table S4), suggesting that *PVT1* is important for TNBC progression. We also evaluated the clinical relevance of *PVT1* expression with TNBC patient's age, tumor stage, node stage, distant metastasis, and BRCA1 mutations (Supplementary Table 5). However, no significant relation was found.
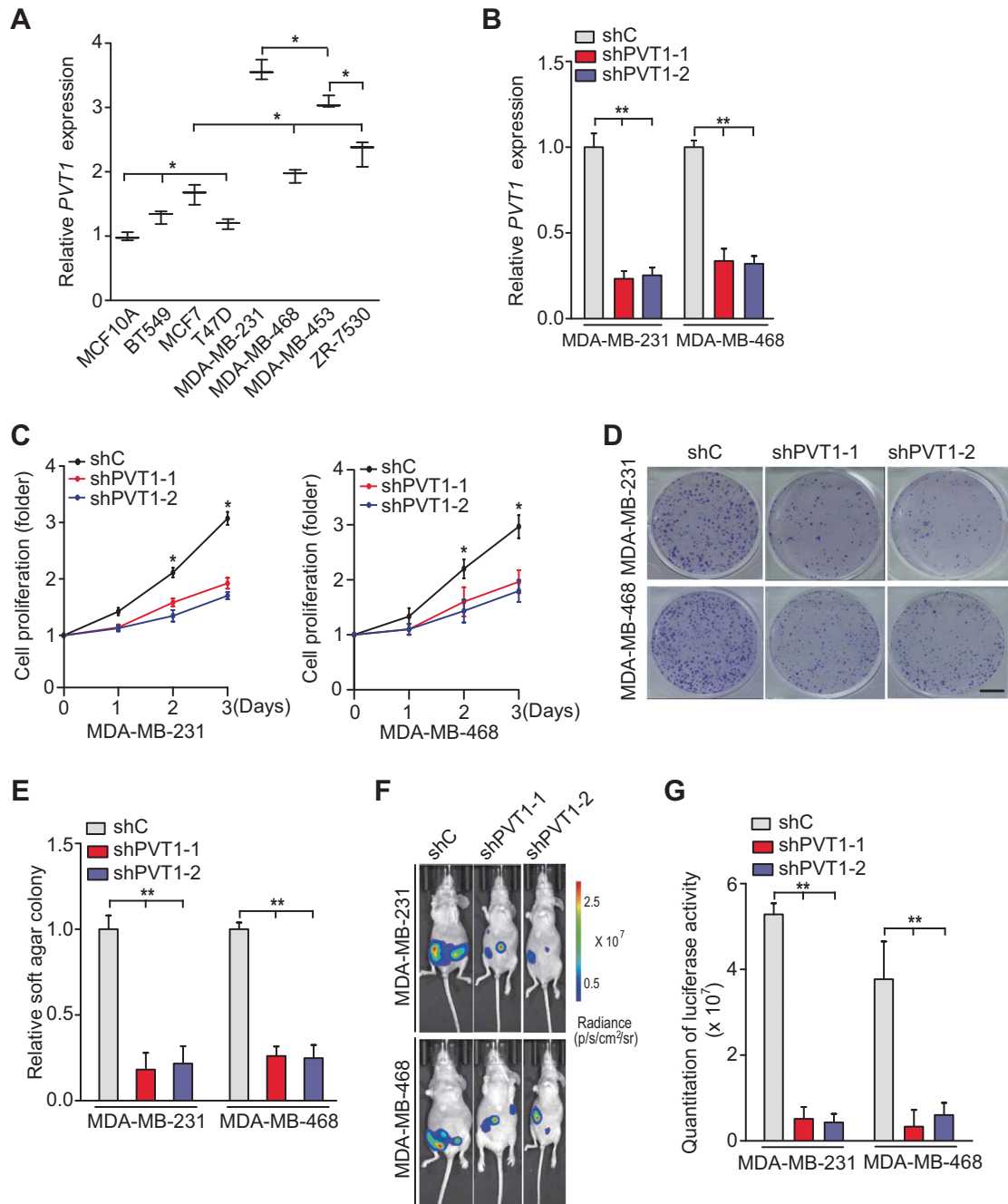
Fig. 2 Knockdown of *PVT1* inhibits TNBC cell proliferation and tumorigenesis. **a** qRT-PCR analysis of *PVT1* mRNA expression in breast normal and cancer cells. *ACTB* was used as a control. **b** Knockdown of *PVT1* using two different shRNAs (shPVT1-1 and shPVT1-2) or a control shRNA (shC) in MDA-MB-231 and MDA-MB-468 TNBC cells. **c, d** Effects of *PVT1* knockdown on breast cancer cell proliferation (**c**) and colony formation in agar (**d**). **e** Quantification of colony formation in (**d**). **f** Representative bioluminescence images of shC- or shPVT1-infected MDA-MB-231 or MDA-MB-468 cells transduced when injected into nude mouse gland fat pads. Mice were imaged at 3–4 weeks after implantation. Data were from two independent experiments with five mice per group. **g** Quantification of the bioluminescence activity in (**f**). Scale bar in (**d**), 4 mm. Error bars ± SD. *$P < 0.05$; **$P < 0.01$. Data are representative from two independent experiments

**Fig. 3** *PVT1* interacts with KLF5. **a** Schematic diagram of putative KLF5-binding sites in *PVT1*. **b** RIP-qPCR assay of the binding of KLF5 with *PVT1* in MDA-MB-231 and MDA-MB-468 cells. **c** Re-expression of shRNA-resistant *PVT1* wild-type (WT), KLF5-binding mutant site 1 (Mut1), site 2 (Mut2), or both sites (Mut1/2). **d** RIP-qPCR assay of effect of re-expression of shRNA-resistant *PVT1* WT or mutants on KLF5 binding. **e, f** WB detection of KLF5 after Flag-MS2bp-MS2bs-based pull-down assay. *EV* empty vector. Error bars ± SD. *$P < 0.05$, ***$P < 0.001$. Data are representative of two or three independent experiments
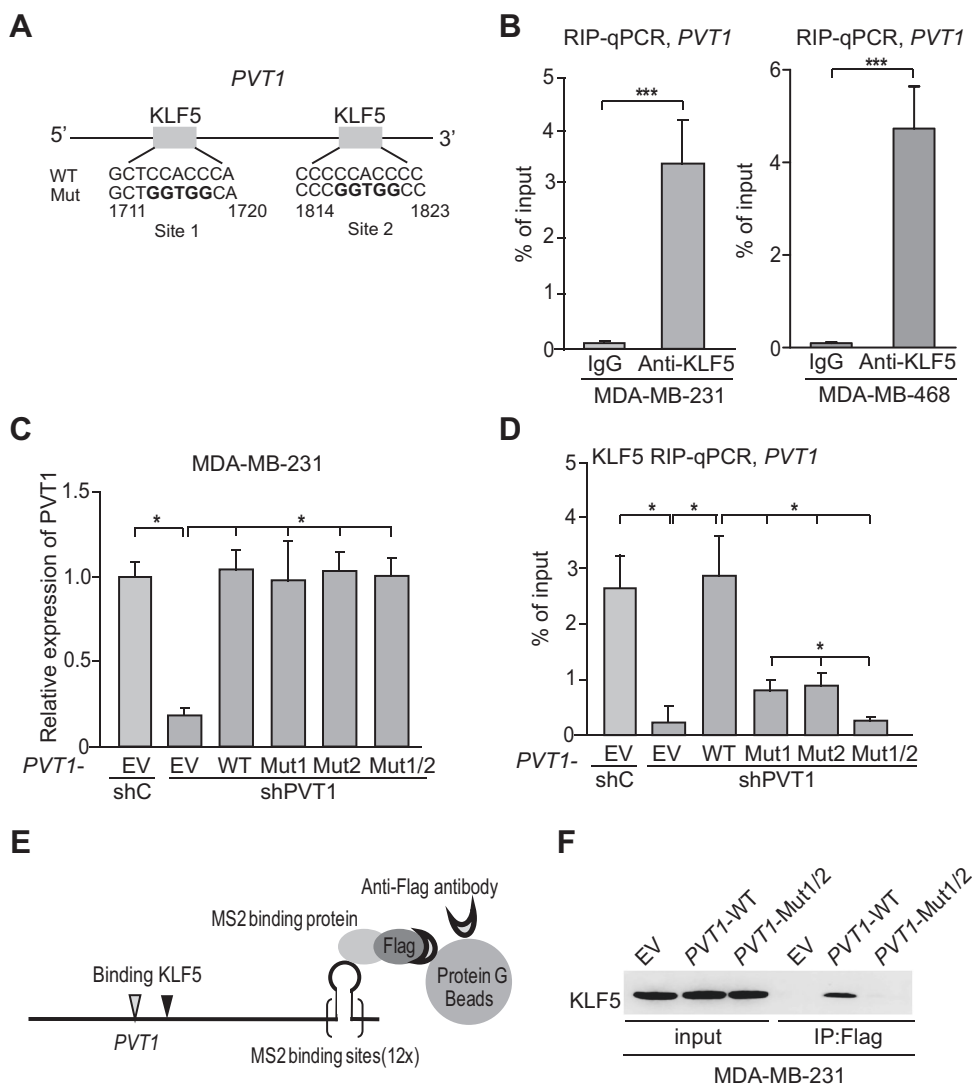


## Depletion of lncRNA *PVT1* inhibits TNBC cell proliferation and tumor growth

To further demonstrate the roles of *PVT1* in TNBC, we examined *PVT1* expression in a normal mammary epithelial cell line and several breast cancer cell lines. As *PVT1-203* (NR_003367) in the 25 splice variants of *PVT1* (Supplementary Figure S2) had been well identified to function in cervical [18], gastric [19], ovarian [8], and hepatocellular [13] cancers, we focused on this variant and designed specific qRT-PCR primers (Supplementary Table S6). Compared with the normal MCF10A cells, the expression level of *PVT1* was significantly high in all breast cancer cells tested (Fig. 2a). Compared with BT549, MCF7, and T47D cell lines, *PVT1* was expressed at relatively higher levels in two TNBC cell lines, MDA-MB-231 and MDA-MB-468.

Next, we used lentivirus-mediated short hairpin RNAs (shRNAs) targeting *PVT1* (shPVT1-1 and shPVT1-2) or a non-silencing control to deplete *PVT1* in MDA-MB-231 and MDA-MB-468 TNBC cells. As shown, shPVT1-1 and shPVT1-2 both targeted PVT1-203 and PVT1-206 variants, and PVT1-206 was an intrasplice variant of PVT1-203 (Supplementary Figure S2). Expression of PVT1-203 and PVT1-206 variants was impaired by both *PVT1* shRNAs (Fig. 2b and Supplementary Figure S3A). Knockdown of endogenous *PVT1* markedly inhibited cell proliferation in MDA-MB-231 and MDA-MB-468 TNBC cells compared with the controls (Fig. 2b, c). *PVT1* knockdown also impaired colony formation in both TNBC cell lines (Fig. 2d, e). Then, we expressed shRNA-resistant PVT1-203* or PVT1-206* variant in shRNA-knockdown cells, and found that PVT1-203* but not PVT1-206* rescued colony formation in both TNBC cells (Supplementary Figures S3B and S3C). These data suggest that PVT1-203 variant is critical for TNBC cell proliferation.

To further demonstrate whether *PVT1* is critical for TNBC tumorigenicity, we employed an orthotopic breast

**Fig. 4** *PVT1* regulates KLF5 protein stability. **a** Knockdown of *PVT1* decreases KLF5 protein expression. **b** qRT-PCR analysis of effects of *PVT1* knockdown on KLF5 mRNA expression. **c** Effects of *PVT1* depletion on KLF5 degradation. Cells were treated with cycloheximide (CHX, 20 mg/ml) for the indicated time. **d** Quantification of KLF5 protein levels in (**c**). **e** Effects of re-expression of shRNA-resistant *PVT1* wild-type (WT) or *PVT1*-KLF5-binding mutant (Mut1/2) on KLF5 degradation. Error bars ± SD. *P < 0.05. Data are representative from two independent experiments
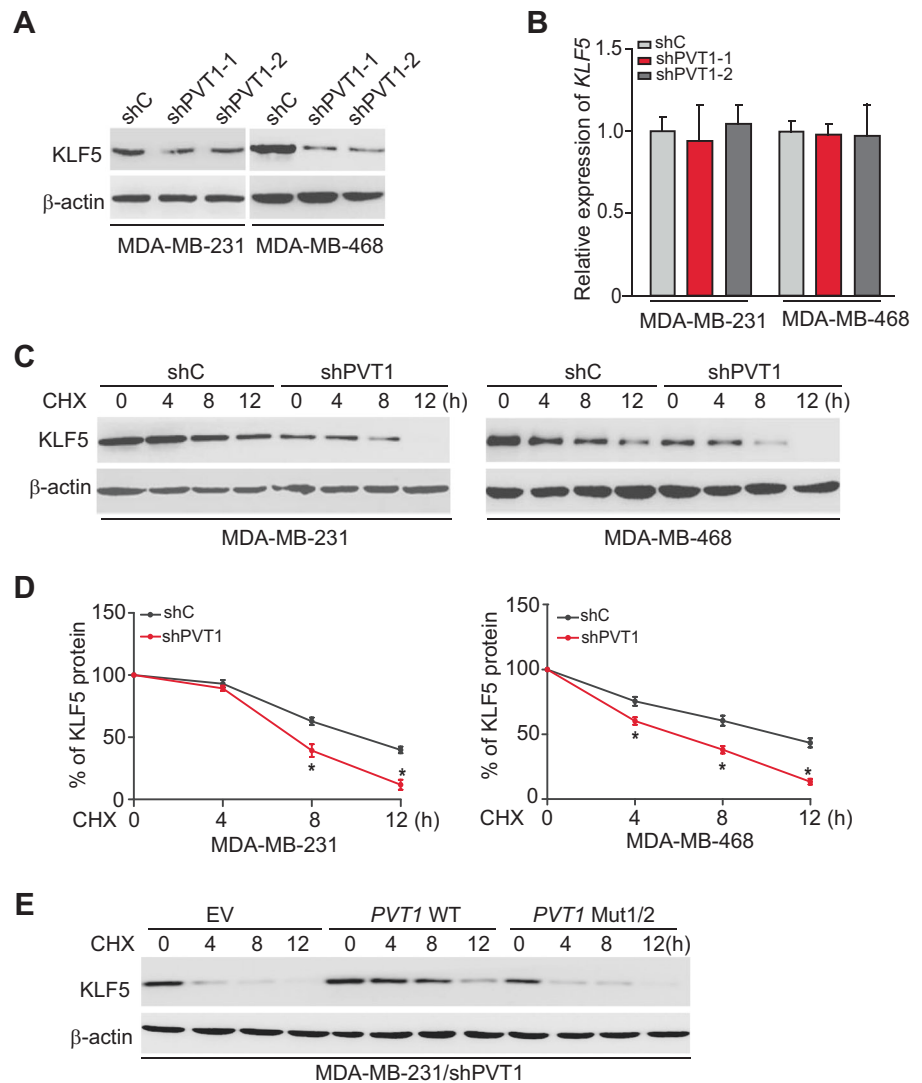


cancer model. MDA-MB-231 or MDA-MB-468 cells transduced with shPVT1-1, shPVT1-2, or shControl (shC) were separately implanted into the mammary gland fat pads of immunocompromised mice. The effects of *PVT1* depletion on TNBC tumorigenesis were then assessed. Compared with the control xenograft models, knockdown of *PVT1* significantly inhibited TNBC tumor growth (Fig. 2f, g). These data support that *PVT1* is critical for cell proliferation and tumor growth in TNBC.

## *PVT1* interacts with KLF5 in TNBC cells

LncRNAs function mainly as sponges to bind functional proteins and regulate their downstream gene expression [19–21]. We hypothesized that *PVT1*-regulated tumor growth depends on its binding proteins. We first performed in silico analysis through the open-assess database of transcription factor-binding profiles [22], and identified that

KLF5 is the most potential one in several *PVT1*-binding transcription factors. As shown in Fig. 3a, it is predicted that KLF5 may bind with *PVT1* at both 1711–1720 and 1814–1823 sites. To assess whether KLF5 binds with *PVT1* in TNBC cells, we performed RNA-binding protein immunoprecipitation (RIP)-real-time PCR (qPCR) using anti-KLF5 antibody in MDA-MB-231 and MDA-MB-468 TNBC cells. As shown in Fig. 3b, we detected the enrichment of *PVT1* with the anti-KLF5 antibody compared with the control.

Next, to support the finding that *PVT1* binds with KLF5, we constructed *PVT1* vectors with mutations at the putative *PVT1*-KLF5-binding site 1 (Mut1), site 2 (Mut2), or both sites (Mut1/2). As shown in Fig. 3c, d, re-expression of shRNA-resistant *PVT1* WT rescued the binding of *PVT1* and KLF5, whereas re-expression of shRNA-resistant *PVT1*-KLF5-binding Mut1, Mut2, or Mut1/2 did not, suggesting that both the sites are important for *PVT1*-KLF5 binding.
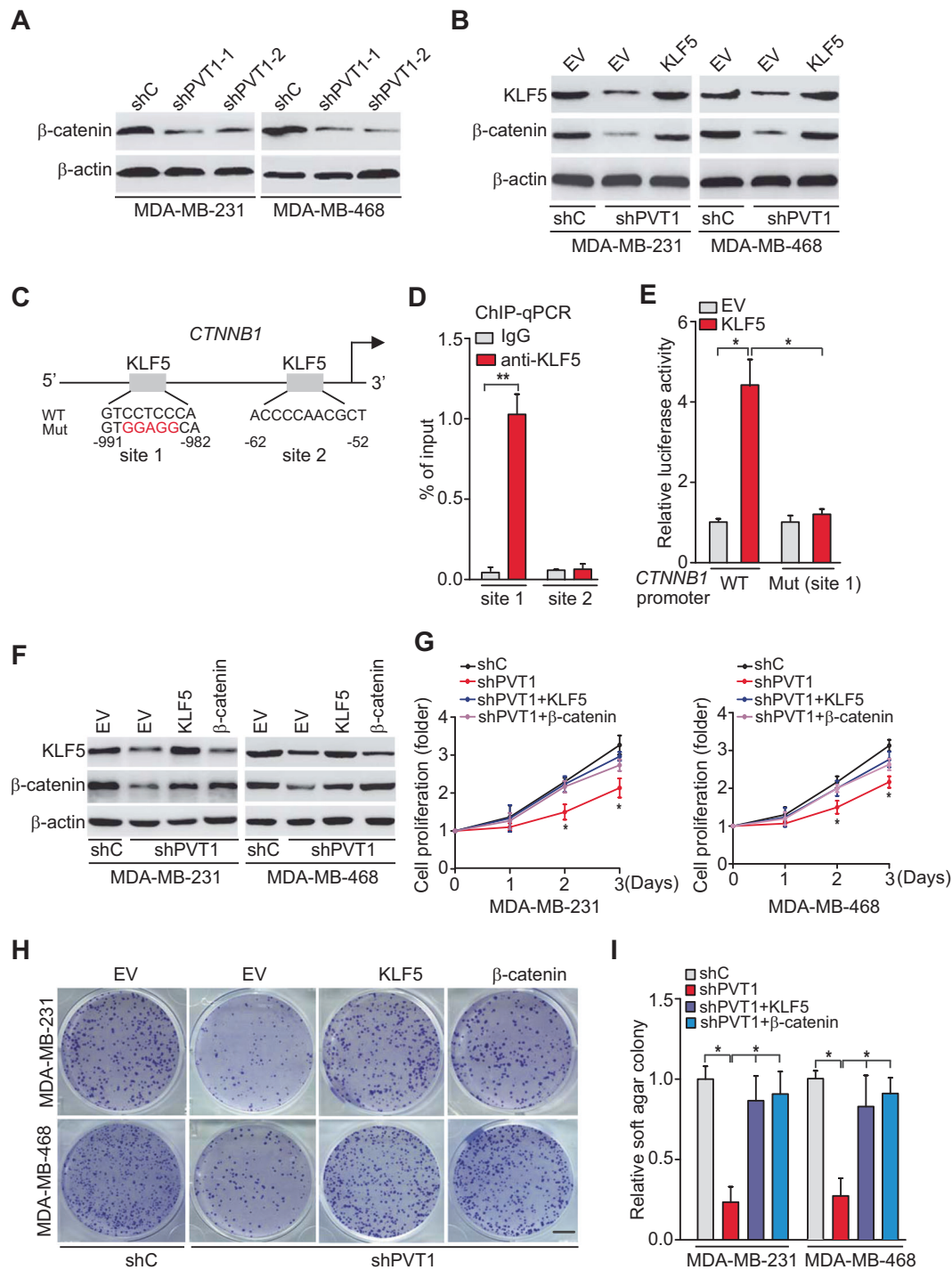
**Fig. 5** *PVT1* regulates *CTNNB1* transcription through KLF5. **a** Knockdown of *PVT1* inhibits β-catenin expression in MDA-MB-231 and MDA-MB-468 cells. **b** Overexpression of KLF5 rescues *PVT1* knockdown-inhibited β-catenin expression. **c** Schematic diagram of putative KLF5-binding sites in *CTNNB1* promoter. **d** ChIP-qPCR assay of KLF5 binding with *CTNNB1* promoter. **e** Luciferase assay of KLF5 regulation of the *CTNNB1* promoter activity. **f** Overexpression of KLF5 and β-catenin in *PVT1* knockdown cells. **g, h** Overexpression of KLF5 or β-catenin restores *PVT1* knockdown-inhibited cell proliferation (**g**) and colony formation (**h**). **i** Quantification of colony formation in (**h**). Scale bar in (**h**), 4 mm. *EV* empty vector. Error bars ± SD. *$P < 0.05$, **$P < 0.01$. Data are representative from two independent experiments

Finally, to further validate that *PVT1* binds with KLF5, we carried out RNA pull-down analysis using the Flag-MS2bp-MS2bs system, in which the FLAG-tagged MS2-binding protein (MS2BP) specifically binds RNAs that contain MS2-binding sequences (Fig. 3e). As shown in Fig. 3f, using FLAG-MS2bp-MS2bs system to pull down

**Fig. 6** *PVT1*-regulated KLF5 stability depends on BAP1. **a** Effects of *PVT1* overexpression on KLF5-BAP1 binding and β-catenin expression in MDA-MB-231 and MDA-MB-468 cells. **b** Effects of *PVT1* knockdown on KLF5-BAP1 binding and β-catenin expression. **c** BAP1 overexpression rescued *PVT1* shRNA-inhibited KLF5 stability. **d** Quantification of KLF5 protein levels in (**c**). **e** Effects of overexpression of KLF5 on KLF5-BAP1 binding and β-catenin expression in MDA-MB-231/shC and MDA-MB-231/shPVT1 cells. Error bars ± SD. *$P < 0.05$
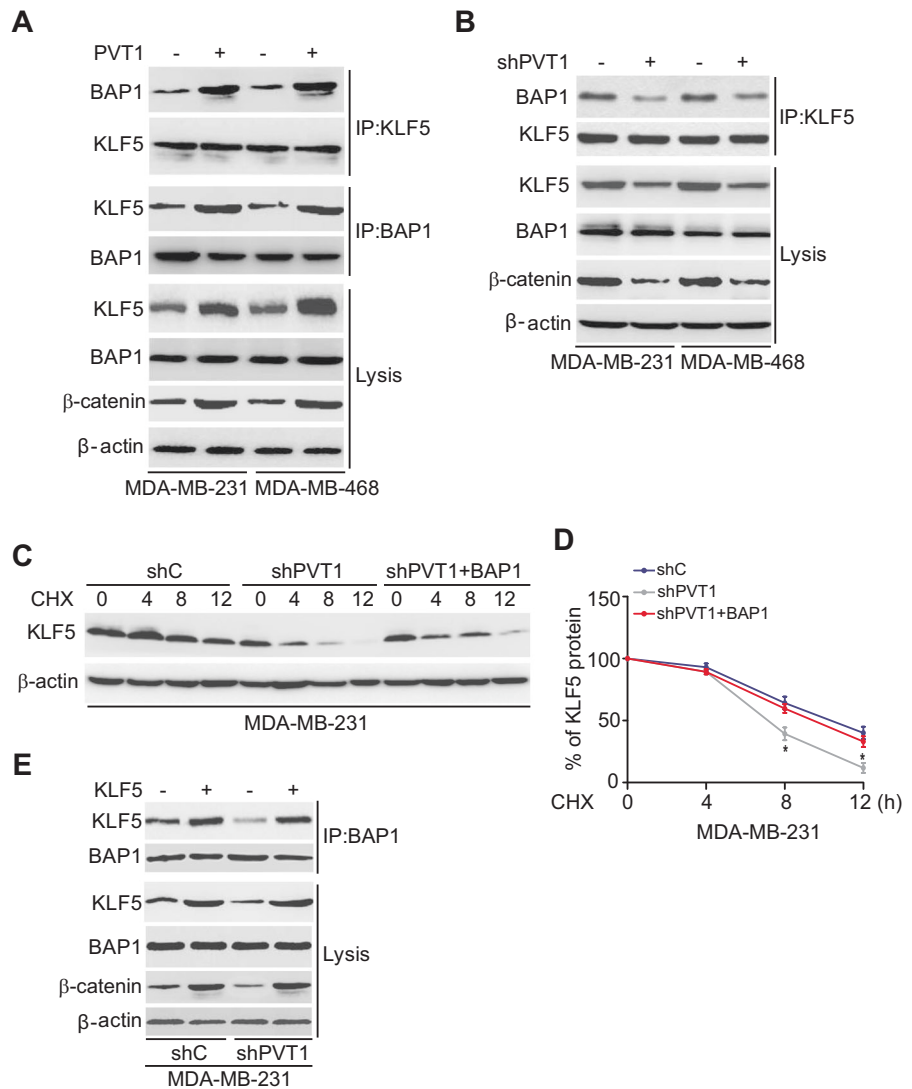


proteins associated with *PVT1*, we detected that KLF5 bound with *PVT1* wild type but not the mutant in MDA-MD-231 cells. Taken together, these data suggest that KLF5 binds with *PVT1* in TNBC cells.

## *PVT1* regulates KLF5 protein stability

To demonstrate the role of KLF5 in *PVT1*-regulated TNBC tumor growth, we first assessed KLF5 protein and mRNA expression in *PVT1* knockdown TNBC cells. As shown in Fig. 4a, compared with the controls, knockdown of *PVT1* markedly decreased KLF5 protein expression in MDA-MB-231 and MDA-MB-468 cells. However, *PVT1* depletion had no effect on *KLF5* mRNA expression (Fig. 4b). These data suggest that *PVT1* may mediate KLF5 protein stability.
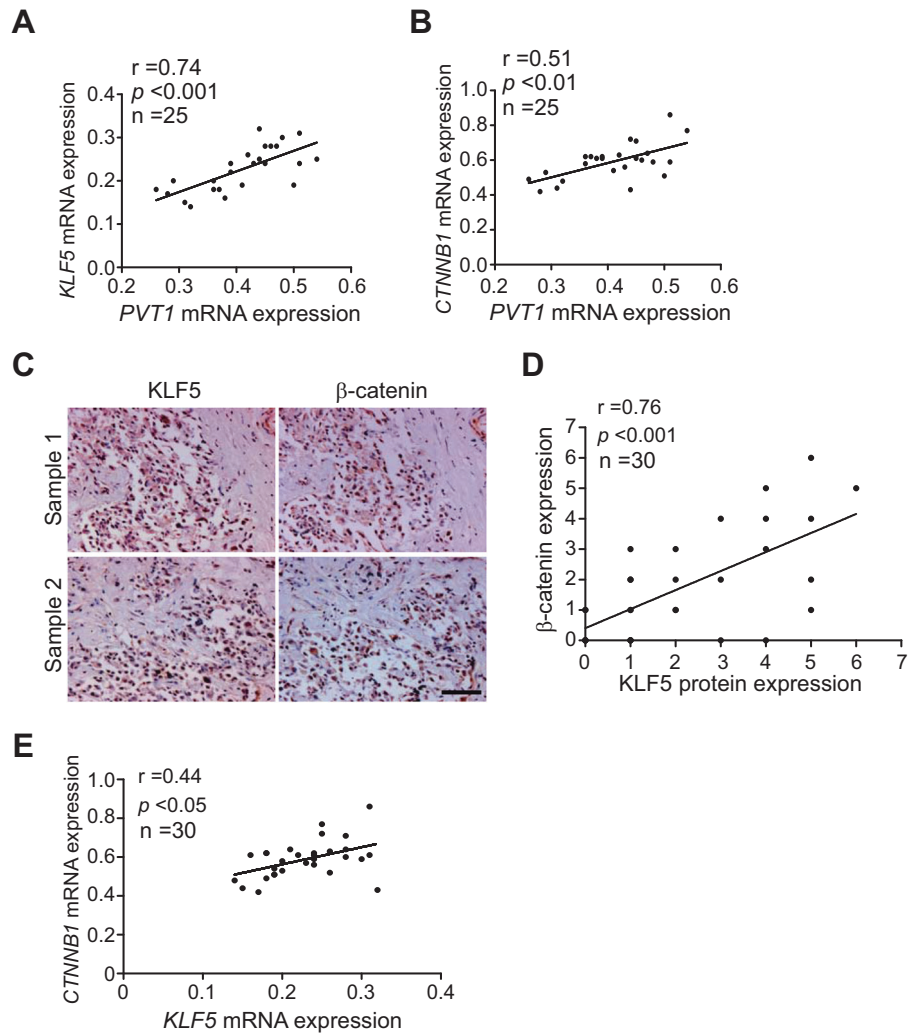
To demonstrate whether *PVT1* regulates KLF5 protein stability, MDA-MB-231 and MDA-MB-468 cells were treated with cycloheximide (CHX), an inhibitor of de novo

protein synthesis, at indicated time points (Fig. 4c, d). Compared with the untreated controls, KLF5 protein levels were significantly decreased by CHX treatment at 12 h in both cell lines (Fig. 4c, d). Knockdown of *PVT1* markedly increased KLF5 degradation compared with the control (Fig. 4c, d). Furthermore, re-expression of shRNA-resistant *PVT1* WT restored *PVT1* depletion-inhibited KLF5 stability, whereas re-expression of shRNA-resistant mutant of the KLF5 binding had no effects (Fig. 4e). These results show that *PVT1* regulates KLF5 stability in TNBC cells.

## *PVT1* regulates *CTNNB1* transcription through KLF5

Since β-catenin is an important downstream effectors of KLF5 in cancer [23, 24], we assess whether *PVT1* mediates β-catenin expression in TNBC cells. As shown in Fig. 5a, depletion of *PVT1* inhibited expression of β-catenin in

**Fig. 7** Co-expression of *PVT1*, KLF5, and β-catenin in clinical TNBC tumor samples. **a,b** Correlation of expression of *PVT1* with *KLF5* (**a**) and *CTNNB1* (**b**) in 25 snap-frozen clinical TNBC tumor specimens. **c** Representative images of immunohistochemical staining of KLF5 and β-catenin in a separate cohort of a total 30 paraffin-embedded clinical TNBC specimens. Scale bars: 50 μm. **d** Correlation analysis of KLF5 protein and β-catenin protein in (**c**). **e** Correlation analysis of between *KLF5* mRNA and *CTNNB1* mRNA from the samples in (**c**). Data are representative from two independent experiments



MDA-MB-231 and MDA-MB-468 cells. However, overexpression of KLF5 markedly restored *PVT1* knockdown-inhibited β-catenin expression (Fig. 5b). These data suggest that *PVT1* mediates KLF5 stability to regulate β-catenin expression.

To demonstrate whether KLF5 regulates β-catenin expression, we performed in silico analysis of the putative transcription factors binding to the promoter of *CTNNB1* (encoding β-catenin protein; http://jaspar.genereg.net/). Two putative KLF5-binding sites were found in the promoter of *CTNNB1* at −991 to −982 and −62 to −52 sites (Fig. 5c). To reveal whether KLF5 binds with the promoter of *CTNNB1* at these sites, we performed chromatin immunoprecipitation (ChIP)-qPCR assays in MDA-MB-231 using primers that flank a 221-bp region (containing KLF5-binding site 1) or a 252-bp region (containing KLF5-binding site 2) of the *CTNNB1* promoter. As shown in Fig. 5d, endogenous KLF5 protein bound with site 1 region but not site 2 region. To further validate this finding that KLF5 binds with the promoter of *CTNNB1*, we performed

promoter luciferase assays. KLF5 overexpression significantly promoted *CTNNB1* promoter activity compared with the empty vector (EV), whereas mutation of site 1 significantly decreased *CTNNB1* promoter activity activated by KLF5 overexpression (Fig. 5e). These data suggest that *PVT1*-regulated KLF5 functions as a transcription factor to mediate β-catenin expression.

To determine the functions of the PVT1/KLF5/β-catenin signal pathway in TNBC, we overexpressed *KLF5* and *CTNNB1* in *PVT1*-knockdown MDA-MB-231 and MDA-MB-468 cells (Fig. 5f). Overexpression of *KLF5* restored *PVT1* knockdown-inhibited β-catenin expression (Fig. 5f), cell proliferation (Fig. 5g), and colony formation (Fig. 5h, i). Overexpression of β-catenin rescued *PVT1* knockdown-inhibited cell proliferation (Fig. 5g) and colony formation (Fig. 5h, i), whereas it did not affect *PVT1* depletion-impaired KLF5 protein expression (Fig. 5f). These data further suggest that *PVT1* regulates tumor growth through mediating KLF5 stability and promoting β-catenin expression.

## PVT1-regulated KLF5 stability depends on BAP1

Since the deubiquitinase (DUB) BAP1 had been reported to mediate KLF5 stability in breast cancer cells [25], we assessed whether PVT1-regulated KLF5 stability depends on BAP1. As shown in Fig. 6a, overexpression of PVT1 increased KLF5 binding with BAP1 and β-catenin expression in both TNBC cells. In contrast, knockdown of PVT1 inhibited KLF5-BAP1 binding and β-catenin expression (Fig. 6b). Moreover, BAP1 overexpression attenuated PVT1 shRNA-enhanced KLF5 degradation (Fig. 6c, d). Consistent with previous report [25], overexpression of KLF5 increased its binding with BAP1 and β-catenin expression in MDA-MB-231 control cells (Fig. 6e). Overexpression of KLF5 markedly rescued BAP1 association and β-catenin expression inhibited by PVT1 depletion (Fig. 6e). These data support that PVT1 depends on BAP1 to mediate KLF5 stability in TNBC cells.

## Co-expression of PVT1, KLF5, and β-catenin in clinical TNBC samples

To assess the clinical relevance of our findings in this study, we determined expression levels of PVT1, KLF5, and β-catenin in clinical TNBC samples. We collected 25 clinical snap-frozen TNBC samples, and performed qRT-PCR analyses. As shown in Fig. 7a, b, based on quantification of the mRNA expression, PVT1 was revealed to significantly correlate with KLF5 and CTNNB1 using Spearman's rank correlation analysis. We also performed immunohistochemical (IHC) staining for the expression of KLF5 and β-catenin in a separate cohort of 30 paraffin-embedded clinical TNBC specimens, and found that the protein or mRNA level of KLF5 markedly co-expressed with that of β-catenin protein or CTNNB1 mRNA (Fig. 7c, e). Taken together, these data support that PVT1/KLF5/β-catenin signaling plays a critical role in tumor progression in human TNBC.

## Discussion

In this study, we demonstrate that lncRNA PVT1 is critical for TNBC cell proliferation and tumor growth through regulating KLF5/β-catenin signaling. PVT1 is amplified and overexpressed in clinical TNBC samples compared with normal breast or paired peritumoral tissues, and patients with high PVT1-expressing TNBC have a worse prognosis. PVT1 binds and stabilizes KLF5 via BAP1, which promotes β-catenin expression, resulting in enhanced tumorigenesis.

Our data demonstrate that lncRNA PVT1 regulates TNBC. Increasing evidence indicates that abnormal expression of lncRNAs is critical for the development of the malignant phenotype of breast cancer [5, 6, 21]. PVT1 encodes an lncRNA, which was found to be frequently co-amplified with MYC in several cancers [7–9], including breast cancer [8]. Recent PVT1 was shown to be amplified in TNBC with Rb/p53 deficiency [14]. Here we report that PVT1 is amplified and upregulated in clinical TNBC, and a higher level of PVT1 is inversely correlated with prognosis of TNBC patients. Depletion of PVT1 inhibited TNBC cell proliferation, colony formation, and tumor growth. These results support that PVT1 is critical for TNBC.

We also demonstrate that lncRNA PVT1 mediates TNBC through KLF5. KLF5 has been revealed to have a critical role in breast cancer cell proliferation and metastasis [25–27]. PVT1 plays as a molecular sponge to regulate miR-203 [28] and miR-195 [29]. PVT1 was also demonstrated to regulate breast and hepatocellular carcinoma through binding and stabilizing Myc [12] and Nop2 [13], respectively. In this study, we reveal that PVT1 binds with KLF5 and regulates its stability via BAP1. We identified that PVT1 binds with KLF5 at two sites. Knockdown of PVT1 inhibited the binding of BAP1 and KLF5, and enhanced KLF5 degradation. In contrast, PVT1 overexpression enhanced their binding and inhibited KLF5 degradation. Overexpression of KLF5 restored PVT1 depletion-impaired BAP1-KLF5 binding, cell proliferation, and colony formation. Moreover, PVT1 and KLF5 are co-expressed in clinical TNBC samples. These findings show that PVT1 regulates KLF5 stability to mediate TNBC.

Our data also reveal that lncRNA PVT1 regulates TNBC through KLF5-upregulating β-catenin. Cytosolic/nuclear expression of β-catenin or overexpression of its target cyclin D1 was demonstrated to be related with a poor prognosis in patients with breast cancer [30]. Cytosolic/nuclear localization of β-catenin was more often observed in basal-like breast cancers [31]. KLF5 was reported to regulate β-catenin-TCF4 (T-cell factor 4) interaction in response to lysophosphatidic acid (LPA) in colon cancer [24]. Knockdown of KLF5 inhibited β-catenin activation stimulated by LPA [24]. Deletion of KLF5 also completely suppressed β-catenin oncogenic mutant-induced intestinal carcinomas [32]. However, the relationship of KLF5 and β-catenin in breast cancer remains little studied. Here, we reveal that KLF5 binds the promoter of CTNNB1, and regulates its transcription. We identified that KLF5 binds with the CTNNB1 promoter at the site −991 to −982. PVT1 knockdown inhibited KLF5 protein stability and β-catenin expression. Consistent with this, overexpression of KLF5 restored PVT1 knockdown-inhibited β-catenin expression, cell proliferation, colony formation, and the promoter activity of CTNNB1 wild-type but not the mutation of KLF5 binding. Moreover, PVT1 and CTNNB1, and KLF5 protein and β-catenin protein are co-expressed in human clinical TNBC specimens. These lines of evidence suggest that

*PVT1* promotes TNBC by binding and stabilizing KLF5, which results in β-catenin upregulation.

Taken together, our results demonstrate that lncRNA *PVT1* acts as a critical regulator of TNBC through mediating KLF5/β-catenin signaling. The findings of this study have significant implications regarding our understanding of TNBC. Our study also indicates *PVT1* as a new effective target for improving treatment of TNBC.

## Materials and Methods

### Cell lines

BT549, MCF7, T47D, MDA-MB-231, MDA-MB-453, MDA-MB-468, MCF10A, and ZR-7530 cells were from ATCC (Manassas, VA, USA), and were cultured in 10% fetal bovine serum/Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA). MCF10A cells were cultured in DMEM/F12 (Invitrogen) supplemented with 20 ng/ml epidermal growth factor, 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 100 ng/ml cholera toxin, and 100 μg/ml penicillin–streptomycin. All cell lines in this study were authenticated using STR DNA fingerprinting by Shanghai Biowing Applied Biotechnology Co., Ltd (Shanghai, China) in January 2017, and mycoplasma infection was detected using PCR Detection kit (Sigma-Aldrich).

### Plasmids

*KLF5*, *BAP1*, *CTNNB1*, *LncRNA PVT1-203*, and *PVT1-206* variant cDNAs were amplified by PCR from normal breast tissues, sequenced, and then subcloned into a pcDNA3.1 vector (Invitrogen). *LncRNA PVT1-203* and *PVT1-206* variant cDNAs were also subcloned into a lentivirus pLVX-Puro vector (Clontech). *CTNNB1* promoter was subcloned into a pGL3 vector (Promega). *PVT1* shRNAs were designed to target PVT1-203 (shPVT1 target sequence: 5′-GCCATCATGATGGTACTTTAA-3′; shPVT1 target sequence: 5′-GCCAGGACACTGA-GATTTGGA-3′) following the method described in http://rnaidesigner.invitrogen.com/rnaiexpress (Supplementary Figure S2). *PVT1* point mutations were constructed using a QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to a protocol provided by the manufacturer.

### RNA extraction and quantitative RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen). First-strand cDNA was generated using the PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Real-time PCR was performed in the StepOne Real-Time PCR System (Applied Biosystems, Foster City, USA). *ACTB* was used as a control. Primers were listed in Supplementary Table S6.

### Cell proliferation and colony formation in soft agar

Cell proliferation assay was performed using a WST-1 Assay Kit (Roche). Briefly, cells were seeded in triplicate in a 96-well plate, and incubated at 37 °C. Cell proliferation was measured using a WST-1 assay kit. For colony formation assay in soft agar, cells were seeded in a 0.4% top agar layer with a bottom 0.8% layer in triplicate in a 12-well plate. Colonies were fixed and stained with 1% crystal violet solution after 2–3 weeks. The visible colony numbers scored and data were analyzed.

### RIP and RNA pull-down assays

RIP analysis was performed using an EZ-Magna RIP Kit (Millipore) according to the manufacturer's protocol. Briefly, 5 μg of anti-KLF5 antibody (ab24331, Abcam) was used to pull down RNAs. Then, the RNAs were extracted and assessed by qPCR. Primers are listed in Supplementary Table S6. RNA pull-down assay was performed using the Flag-MS2bp-MS2bs system.

### ChIP-qPCR assay

A Chromatin Immunoprecipitation Kit (Millipore-Upstate) was used to immunoprecipitate DNAs according to the manufacturer's instructions, and then purified DNAs were measured using qPCR. Primers are listed in Supplementary Table S6.

### Luciferase promoter assay

For the luciferase promoter assay, pGL3-*CTNNB1* promoter wild-type or mutant of the KLF5 binding was co-transfected with or without KLF5 into MDA-MB-231 cells with a *PVT1* or control shRNA using the Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific). pRL-TK Renilla plasmid (Promega) was used as a control. A Dual-Luciferase Reporter Assay Kit (Promega) was used to measure Luciferase and Renilla signals.

### Western blot assay

Western blot was carried out against KLF5 (ab24331, 1:1000, Abcam), β-catenin (D10A8, 1:1000, Cell Signaling Technology), β-actin (ab8229, 1:1000, Abcam), BAP1 (ab199396, 1:500, Abcam), and Flag (MS2, 1:1000, Sigma-Aldrich) antibodies, as we previously described [33].

## shRNA knockdown and transfection

Lentivirus production was carried out in HEK293T cells as previously described [33]. Cells were infected, and then exogenous protein expression was validated.

## Tumorigenesis studies

Six-week-old female athymic (Ncr nu/nu) mice from SLAC (Shanghai, China) were randomly divided into five per group, and cells ($2 \times 10^6$) were injected into mouse mammary fat pads. All animal experiments were approved by Shanghai Jiao Tong University Institutional Animal Care and Use Committee. Bioluminescence imaging was performed using the IVIS Lumina imaging station (Caliper Life Sciences). Two investigators independently performed animal group allocation, surgery, and assessing the outcome.

## IHC staining

IHC assay was performed on paraffin sections of 30 clinical breast cancers as previously described [33] using a primary antibody against KLF5 (1:50, Abcam) and β-catenin (1:100, Cell Signaling Technology). All the work related to human tissues was approved at Shanghai Jiao Tong University, according to the Declaration of Helsinki, and the investigators obtained informed written consent from the subjects. IHC staining was scored as 0–7 according to the percentage of positive cells and staining intensity. The slides were examined and scored by two separate individuals.

## Statistical analysis

All statistical analyses were carried out using a GraphPad Prism version 5.0 for Windows (GraphPad Software Inc., San Diego, CA, USA). The significance of the data between experimental groups was determined by one-way analysis of variance with Newman–Keuls post test and unpaired two-tailed Student's $t$-test. Correlations between study variables were calculated by Spearman's rank correlation coefficients. A $P$ value $< 0.05$ was considered statistically significant.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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