Deubiquitinase BRCC3 increases the stability of ZEB1 and promotes the proliferation and metastasis of triple negative breast cancer cells

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Original Article

The deubiquitinase BRCC3 increases the stability of ZEB1 and promotes the proliferation and metastasis of triple-negative breast cancer cells

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Abstract

Triple negative breast cancer (TNBC) has a high recurrence rate, metastasis rate and mortality rate. The aim of this study is to identify new targets for the treatment of TNBC. Clinical samples are used for screening deubiquitinating enzymes (DUBs). MDA-MB-231 cells and a TNBC mouse model are used for *in vitro* and *in vivo* experiments, respectively. Western blot analysis is used to detect the protein expressions of DUBs, zinc finger E-box binding homeobox 1 (ZEB1), and epithelial–mesenchymal transition (EMT)-related markers. Colony formation and transwell assays are used to detect the proliferation, migration and invasion of TNBC cells. A wound healing assay is used to detect the mobility of TNBC cells. Immunoprecipitation assay is used to detect the interaction between breast cancer susceptibility gene 1/2-containing complex subunit 3 (BRCC3) and ZEB1. ZEB1 ubiquitination levels, protein stability, and protein degradation are also examined. Pathological changes in the lung tissues are detected via HE staining.

Our results show that a significant positive correlation between the expressions of BRCC3 and ZEB1 is detected in clinical TNBC tissues. Interference with BRCC3 inhibits TNBC cell proliferation, migration, invasion and EMT. BRCC3 interacts with ZEB1 and interferes with BRCC3 to inhibit ZEB1 expression by increasing ZEB1 ubiquitination. Interference with BRCC3 inhibits TNBC cell tumorigenesis and lung metastasis *in vivo*. In all, BRCC3 can increase the stability of ZEB1, upregulate ZEB1 expression, and promote the proliferation, migration, invasion, EMT and metastasis of TNBC cells, providing a new direction for cancer therapy.

**Keywords:** BRCC3, ZEB1, epithelial-mesenchymal transition, triple negative breast cancer
Introduction

Globally, breast cancer is one of the most common malignancies and the second leading cause of cancer death in women [1]. Triple-negative breast cancer (TNBC), which accounts for approximately 15% of all breast cancers, is highly aggressive and characterized by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [2]. Compared to other pathological types of breast cancers, TNBC tends to be associated with a younger age of onset, higher histologic grade, larger tumor volume, greater tendency toward recurrence and distant metastasis, and worse prognosis [3, 4]. Due to the lack of clear molecular markers, chemotherapy is currently the only effective systematic therapy for TNBC. Early-stage TNBC may still recur even if patients are in remission after chemotherapy, and the overall survival (OS) of patients with metastatic lesions is only 13–18 months [5]. Current therapeutic regimens failed to improve the survival rate of TNBC patients, and there is an urgent need to explore new molecular targets for TNBC treatment.

It is well known that epithelial-mesenchymal transition (EMT) promotes tumor invasion and metastasis and is associated with the recurrence and metastasis of malignant tumors [6-8]. The zinc finger E-box binding homeobox (ZEB) family (ZEB1 and ZEB2) comprises important transcription factors that regulate EMT. Many previous studies have identified a role of ZEB1 in the induction of EMT and metastasis in TNBC [9-12]. Furthermore, ZEB1 expression was upregulated in TNBC tissues compared to adjacent normal tissues, and downregulation of ZEB1 inhibited cell proliferation, leading to the alleviation of cell migration and invasion [13, 14]. Therefore, ZEB1 plays a very important role in TNBC proliferation and metastasis, and inhibition of ZEB1 will become a research hotspot for TNBC treatment.

The ubiquitin-proteasome system (UPS) is a crucial pathway for intracellular protein degradation and is involved in a wide range of cellular activities, including apoptosis, the cell cycle, and tumorigenesis [15]. Ubiquitination is a reversible process regulated by deubiquitinating enzymes (DUBs), which increase protein stability by removing ubiquitin from the substrate and preventing the degradation of the target protein via
the UPS [16]. ZEB1 is regulated by DUBs, which ultimately leads to enhanced tumor invasion and metastatic capacity [17-20]. In addition, recent studies have suggested that DUBs play key roles in the occurrence and development of TNBC. Therefore, DUBs may be potential targets for TNBC therapy, and an in-depth investigation of this mechanism is highly important for the treatment of TNBC.

In the present study, we identify BRCC3, a DUB that binds to ZEB1, but the role of BRCC3 in TNBC proliferation and metastasis has not yet been reported. The aim of this study was to investigate the role of BRCC3-mediated increased ZEB1 stability in influencing the progression of TNBC.

**Materials and Methods**

**Clinical samples**

Twenty TNBC patients who underwent surgical treatment at the First Affiliated Hospital of Wenzhou Medical University (January 2021–December 2021) were randomly selected. The patients had not undergone neoadjuvant therapy before surgery, and the diagnosis of TNBC was confirmed by pathology and immunohistochemistry in our Pathology Department. Tumor tissues and adjacent nontumor tissues approximately 5 cm away from the tumor tissue were paired. The patients provided signed informed consent, and the study was ethically approved by the Medical Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

**Cell culture and transfection**

Human mammary epithelial cell line (MCF-10A) and TNBC cell lines (MDA-MB-231, MDA-MB-453, MDA-MB-468, and BT-549) were purchased from American Tissue Culture Collection (ATCC; Manassas, USA). MCF-10A cells were cultured in DMEM/F12 complete medium (Gibco, Carlsbad, USA) supplemented with 5% horse serum (Sigma, St Louis, USA) and 10 μg/mL insulin (Procell Life Science & Technology, Wuhan, Chino., Ltd.). 5 μg/mL hydrocortisone (Sangon Biotech, Shanghai, China), and 20 ng/mL epidermal growth factor (PeproTech,
Waltham, USA) at 37°C with 5% CO₂. TNBC cells were cultured in 1640 complete medium (Gibco) supplemented with 10% fetal bovine serum at 37°C with 5% CO₂.

MDA-MB-231 and BT-549 cells in the logarithmic growth phase were inoculated into 6-well plates at 5×10⁵ cells/well and then cultured at 37°C with 5% CO₂. MDA-MB-231 cells were divided into shRNA, shRNA-BRCC3, shRNA+vector, shRNA-BRCC3+vector and shRNA-BRCC3+ZEB1 groups. The BT-549 cells were divided into pcDNA and pcDNA-BRCC3 groups. When the cell confluence reached 70%, transient transfection of the cells in the experimental groups was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). After 5 h of cell transfection, the culture medium was replaced with culture medium, and the cells were cultured for an additional 48 h. Each group of cells was collected for subsequent experiments. The sequences of sense strand (5′→3′) for shRNA and shRNA-BRCC3 were as follows: shRNA: UCACUGCGCUCGAUGCAGUTT; and shRNA-BRCC3: GUACUGGGUUUGUUACAGAUU.

**Western blot analysis**

Approximately 50 mg of tissue fragments were removed and rapidly ground by adding a small amount of liquid nitrogen. Tissues or cells were collected, and RIPA lysis buffer was added to the cells on ice. Cell lysates were collected, centrifuged and subsequently measured using a BCA kit (Sigma). Fifty micrograms of protein sample was added to sodium dodecyl sulfate-polyacrylamide gels for electrophoresis, transferred to PVDF membranes (Millipore, Billerica, USA), blocked with 5% skim milk for 1 h and incubated overnight with primary antibodies as follows: anti-ZEB1 (ab203829; 1:500; Abcam, Cambridge, UK); anti-STAMBP (sc-398480; 1:1000; Santa Cruz, Santa Cruz, USA); anti-BAP1 (ab245391; 1:2000; Abcam); anti-UCHL1 (sc-271639; 1:1000; Santa Cruz); anti-USP9X (ab19879; 1:1000; Abcam); anti-BRCC3 (ab115172; 1:2000; Abcam); anti-E-cadherin (sc-8426; 1:1000; Santa Cruz); anti-vimentin (sc-6260; 1:1000; Santa Cruz); and anti-β-catenin (ab184919; 1:500; Abcam). The membrane was washed three times with TBST and incubated with an HRP- conjugated secondary antibody (1:3000; Cell Signaling Technology,
Beverly, USA) for 30 min. The experiments were repeated three times. ChemiDoc Image Lab software (Bio-Rad, Hercules, USA) was used for analysis.

**Immunofluorescence detection**

MDA-MB-231 and BT-549 cells in the logarithmic growth phase were collected, washed three times with PBS and fixed with 4% paraformaldehyde (Beyotime, Shanghai, China) for 30 min. After washing with PBS, MDA-MB-231 and BT-549 cells were added with 0.5% Triton X-100+1% normal goat serum (Yeasen Biotechnology, Shanghai, China), and the cells were lysed and fixed on ice for 10 min. Next, MDA-MB-231 and BT-549 cells were blocked with 1% normal goat serum+PBS, incubated with primary antibodies as follows: anti-BRCC3 (ab62075; 20 µg/mL; Abcam); anti-ZEB1 (ab203829; 1:250; Abcam); anti-E-cadherin (sc-8426, 1:1500; Santa Cruz); and anti-vimentin (sc-6260; 1:500; Santa Cruz), incubated at 4°C for 2 h, and washed three times with 1% normal goat serum. The following steps were performed in the dark. MDA-MB-231 and BT-549 cells were incubated with secondary antibody for 1 h and washed 3 times with PBS. The nuclei were stained with DAPI (Sigma), and the cells were washed 3 times with PBS. The colocalization of BRCC3 and ZEB1 was observed under a microscope.

**Real-time reverse transcription quantitative polymerase chain reaction (RT-qPCR)**

The collected cells were subjected to RNA extraction using Trizol reagent (Invitrogen, Carlsbad, USA). Reverse transcription was performed using the PrimeScript RT kit (TaKaRa, Dalian, China). RT-qPCR was performed on an ABI 7500 PCR system (Foster City, USA) using the SYBR® Premix Ex TaqTM II kit (TaKaRa). The relative mRNA level of breast cancer susceptibility gene 1/2 (BRCA1/BRCAl2)-containing complex subunit 3 (BRCC3) was detected by the 2^−ΔΔCt method. β-Actin was used as an internal reference. The primers used for the experiments were synthesized by Sangon Biotech (Shanghai, China) and as shown in Table 1. All the experiments were repeated three times.
Colony formation assay
A total of $2.5 \times 10^6$ cells/well were inoculated in a 6-well plate, and adherent cells were treated according to the experimental methods. After digestion and resuspension, the cells were inoculated at $1 \times 10^3$ cells/well in a new 6-well plate, and the culture was terminated when cell colonies appeared. The cells in each well were fixed with 4% paraformaldehyde fixative (1.5 mL) for 30 min, stained with 0.1% crystal violet (1.5 mL) for 30 min, washed, air-dried, photographed, and counted for colonies of more than 50 cells. Three replicate wells of each group were set up.

Transwell assay
The cells were starved with serum-free medium for 12 h, after which the cell concentration was adjusted to $0.5 \times 10^6$ cells/mL. The cell suspension (200 μL) was placed in the upper chamber and evenly coated. Serum-containing medium (600 μL) was added to the lower chamber, which was subsequently incubated at 37°C for 24 h. During this period, avoiding air bubbles was avoided. The chambers were gently removed, and the residual cells that had not penetrated the membrane were removed to avoid affecting the calculation results. The cells were fixed with 4% paraformaldehyde for 20 min and then stained with 1% crystal violet for 15 min. The cells were observed and then counted under a microscope. The procedure for the cell invasion experiment was the same as above, but the transwell chambers were mixed with matrix gel mixed with serum-free medium at a 1:8 ratio.

Wound healing assay
After 48 h of transfection, the cells were collected by trypsin digestion and inoculated in 6-well plates at $1 \times 10^6$ cells/well. After adhesion, the cells were scratched with a 200 μL pipette tip and washed with precooling phosphate buffer 3 times. Then, serum-free medium was added for routine culture. At 0 and 24 h, images were recorded using an inverted microscope. ImageJ software was used to calculate the rate of scratch healing as follows: rate of scratch healing = (the scratch width at 0 h – the
scratch width at 24 h)/the scratch width at 0 h×100%. Three replicate wells were set up for each group.

**Coimmunoprecipitation (IP)**

MDA-MB-231 cells were transfected with the Flag-ZEB1 or Myc-BRCC3 plasmid for 48 h and then treated with MG132 (20 μM; Invitrogen). The precipitate was repeatedly washed 4-5 times by adding 0.5 mL of precooled NP40 (Beyotime) for 4 h. The cells were washed 3 times with phosphate-buffered saline, 500 μL of NP40 lysate containing protease inhibitor was added, and the mixture was resuspended on ice for 20 min. After ultrasonic lysis and centrifugation at 4°C and 3000 g for 10 min, 50 μL of cell supernatant was used as the input. Thirty microlitres of the corresponding antibody (anti-Flag or anti-Myc) was added, and the supernatant was incubated overnight at 4°C. Protein A+G agarose (Santa Cruz) was added, and the mixture was subsequently incubated at 4°C for 4 h. After centrifugation at 4°C and 3000 g for 3 min, the precipitate was repeatedly washed 4–5 times by adding 0.5 mL of precooled NP40. Finally, loading buffer was added, and the proteins were denatured for western blot analysis.

To examine the interaction between endogenous BRCC3 and ZEB1 in MDA-MB-231 cells, 20 μL of Protein A/G Agarose was combined with 5 μL of a ZEB1 antibody (ab276129; Abcam) or a BRCC3 antibody (ab115172; Abcam) for 2 h at room temperature, after which the supernatant was added and incubated overnight at 4°C. IgG was used as a negative control. The next day, the supernatant was discarded, an appropriate amount of loading buffer was added, and 25 μg of protein was loaded for western blot analysis.

**Ubiquitination assay**

MDA-MB-231 cells were transfected with shRNA-BRCC3 or the negative control and were grouped into HA-Ub, Flag-ZEB1+HA-Ub, Flag-ZEB1+shRNA+HA-Ub, or Flag-ZEB1+shRNA-BRCC3+HA-Ub groups. BT-549 cells were transfected with wild-type (WT) BRCC3 or mutant (Mut) BRCC3 and grouped into the following
groups: Flag-ZEB1+HA-Ub, Flag-ZEB1+WT-BRCC3+HA-Ub, and Flag-ZEB1+Mut-BRCC3+HA-Ub. After MG132 (20 μM) was added, the cells were collected after 8 h and centrifuged at 4°C and 1600 g for 5 min. The supernatant was discarded, and the mixture was placed on ice. The cells were resuspended by adding 400 μL of HEPES buffer (Thermo Fisher, Waltham, USA), lysed by ultrasonication, and centrifuged at 4°C and 3000 g for 10 min. 40 μL of supernatant was mixed with 2× loading buffer, and the expression of intracellular proteins was detected by a water bath at 100°C for 5 min. The remaining 360 μL of supernatant was added to 2 μL of anti-Flag and incubated at 4°C for 4–6 h. Then, 40–60 μL of protein A/G agarose was added and incubated at 4°C for 8–10 h. After centrifugation at 4°C and 1600 g for 3 min, 960 μL of the supernatant was discarded, and the mixture was mixed with 2×loading buffer. After 10 min in a water bath at 100°C, western blot analysis was performed.

**Cycloheximide chase assay**

Cycloheximide (CHX; 10 μg/mL; Gibco) was added to MDA-MB-231 cells in the shRNA-BRCC3 or shRNA groups. Protein samples were collected at different time points (0, 1, 2, 3 and 4 h) after which Western blotting was performed to detect ZEB1 protein expression.

**MG132 treatment**

MDA-MB-231 cells in the shRNA-BRCC3 or shRNA groups were treated with or without 10 μM MG132 for 4 h. Protein samples were collected, and Western blot analysis was subsequently performed to detect ZEB1 protein expression.

**Animal experiment**

MDA-MB-231 cells expressing LV-shRNA, LV-shRNA-BRCC3 or LV-shRNA-BRCC3+LV-ZEB1 were prepared as a 3×10^6 cell suspension/mL. Two hundred microliters of the cell suspension was inoculated into the axilla of the right upper limb of BALB/c nude mice (4–6 weeks old, female; Beijing Vital River
Laboratory Animal Technology Co., Ltd., Beijing, China). Mice were divided into the control, LV-shRNA, LV-shRNA-BRCC3, and LV-shRNA-BRCC3+LV-ZEB1 groups (6 mice/group). Tumor diameters were measured regularly on a weekly basis using Vernier callipers, and tumor volumes were calculated. Mice were euthanized six weeks later, after which tumor tissues and lung tissues were excised. Animal experiments were approved by the Animal Experiments Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

**HE staining**

The lung tissues were cleaned with normal saline and fixed overnight with 4% paraformaldehyde. On the second day, paraffin embedding and sectioning were performed, and lung tissue sections were stained according to the instructions of the HE staining kit (Beyotime). After staining, the lung tissue sections were sealed, observed under a microscope, photographed and recorded.

**Immunohistochemistry assay**

Specimens were serially sectioned (4 μm), deparaffinized, hydrated, and treated with 3% H$_2$O$_2$ to block endogenous peroxidase activity. After antigen retrieval in sodium citrate buffer (pH 9.0), diluted primary antibodies as follows: anti-BRCC3 (ab115172; 1:500; Abcam); anti-ZEB1 (ab203829; 1:100; Abcam); anti-E-cadherin (sc-8426; 1:500; Santa Cruz); and anti-vimentin (sc-6260; 1:500; Santa Cruz) were added, and the cells were incubated at 4°C overnight. The mixture was incubated for 20 min by the addition of a biotin-labelled secondary antibody. DAB was added for 2 min, after which the sections were counterstained with hematoxylin for 1 min. The sections were examined under a microscope after sealing [21]. PBS was used instead of the primary antibody as a negative control.

**Statistical analysis**

SPSS 17.0 software was used for statistical analysis. Measurement data are expressed as the mean ± standard deviation. Comparisons between two groups were performed.
by t test. Comparisons between multiple groups were analyzed by one-way ANOVA with Tukey's or Bonferroni's multiple comparison and two-way ANOVA with Bonferroni's multiple comparison. A $P$ value <0.05 indicated that the difference was statistically significant.

Results

Screening of ZEB1-associated DUBs in TNBC

We used the online software Ubibrowser 2.0 to predict and screen five DUBs with the highest binding potential (i.e., high prediction score) to ZEB1, including signal transducing adaptor molecule binding protein (STAMBP), BRCA1 association protein 1 (BAP1), ubiquitin carboxy-terminal hydrolase-1 (UCHL1), ubiquitin-specific peptidase 9X-linked (USP9X), and BRCC3 (Figure 1A). The protein expression levels of ZEB1, STAMBP, BAP1, UCHL1, USP9X, and BRCC3 were obviously greater in the 20 TNBC tissue samples than in the adjacent nontumor tissue samples (Figure 1B and Supplementary Figure S1). Next, Pearson correlation analysis was performed between the protein expression of five DUBs and ZEB1 protein expression via western blot analysis of the 20 TNBC tissues collected in this study. In addition, there were no significant correlations between the expression of STAMBP, BAP1, UCHL1, USP9X or ZEB1 (Figure 1C–F). Figure 1G shows that there was a positive correlation between BRCC3 and ZEB1 protein expression. Compared with those in MCF-10A cells, the mRNA and protein expression levels of BRCC3 in TNBC cells were significantly greater, and the BRCC3 expression level was greatest in MDA-MB-231 cells (Figure 1H–J). Furthermore, Figure 1I,K show that the change in ZEB1 protein expression was consistent with that in BRCC3, and ZEB1 expression was greatest in MDA-MB-231 cells.

Interference with BRCC3 inhibits TNBC cell proliferation, migration, invasion and EMT

Based on the above findings, we next explored the influence of BRCC3 on the
development of TNBC. In MDA-MB-231 and BT-549 cells, ZEB1 and BRCC3 were colocalted in the nucleus (Supplementary Figure S2A). MDA-MB-231 cells were divided into shRNA and shRNA-BRCC3 groups. Figure 2A shows that there was less colony formation in the shRNA-BRCC3 group than in the shRNA group. Transwell assays were used to detect cell migration and invasion in each group (Figure 2B). The number of migrated cells and the scratch mobility of cells in the shRNA-BRCC3 group were dramatically lower than those in the shRNA group (Figure 2C,E), indicating that interference with BRCC3 had a significant inhibitory effect on the migration ability of TNBC cells. Figure 2D shows that the number of invading cells in the shRNA-BRCC3 group was significantly less than that in the shRNA group, indicating that BRCC3 knockout inhibited TNBC cell invasion. Moreover, interfering with BRCC3 increased the protein expression of the epithelial cell marker E-cadherin during EMT but downregulated the protein expression of ZEB1, BRCC3 and the mesenchymal marker vimentin in MDA-MB-231 cells (Figure 2F).

Next, the BT-549 cells were grouped into a pcDNA group and a pcDNA-BRCC3 group. Figure 2G shows that overexpression of BRCC3 promoted colony formation in the pcDNA-BRCC3 group, indicating that BRCC3 could regulate the clonogenic ability of TNBC cells. Transwell assays showed that overexpression of BRCC3 promoted the migration and invasion of BT-549 cells (Figure 2H–J). In addition, wound healing assays showed that overexpression of BRCC3 promoted cell migration (Figure 2K). Compared with those in the pcDNA group, the protein expression levels of ZEB1, BRCC3 and vimentin were upregulated, and the protein expression level of E-cadherin was downregulated in the pcDNA-BRCC3 group (Figure 2L). The above experiments showed that interference with BRCC3 inhibited TNBC cell proliferation, migration, invasion and EMT.

**Interference with BRCC3 promotes ZEB1 protein degradation and inhibits ZEB1 expression by increasing its ubiquitination level**

Since we confirmed that ZEB1 expression was regulated by BRCC3, we further verified the interaction between BRCC3 and ZEB1 using a co-IP assay. After
MDA-MB-231 cells were transfected with Flag-ZEB1 and treated with MG132, BRCC3 was pulled down by co-IP with an anti-Flag antibody (Figure 3A). After MDA-MB-231 cells were transfected with Myc-BRCC3 and treated with MG132, ZEB1 was pulled down by co-IP with an anti-Myc antibody (Figure 3B), which indicated the interaction between BRCC3 and ZEB1. We also performed a co-IP assay in MDA-MB-231 cells and found that endogenous BRCC3 was immunoprecipitated by an anti-ZEB1 antibody (Supplementary Figure S2B) and that endogenous ZEB1 was immunoprecipitated by an anti-BRCC3 antibody (Supplementary Figure S2C). After interfering with BRCC3, the ubiquitination and protein degradation of ZEB1 in MDA-MB-231 cells were significantly increased (Figure 3C and D). Furthermore, WT-BRCC3 significantly inhibited ZEB1 ubiquitination, whereas Mut-BRCC3 had no significant effect on ZEB1 ubiquitination in BT-549 cells (Supplementary Figure S2D). We also found that interfering with BRCC3-mediated ZEB1 degradation was completely inhibited by the proteasome inhibitor MG132 (Figure 3E).

**Interference with BRCC3 inhibits TNBC cell proliferation, migration, invasion and EMT by downregulating ZEB1**

To confirm that the functions of BRCC3 in this study depend on its ability to regulate ZEB1 expression, we cotransfected cells with the BRCC3 interference plasmid and the ZEB1 overexpression plasmid for further study. Interfering with BRCC3 inhibited colony formation, and overexpressing ZEB1 in the shRNA-BRCC3+ZEB1 group reversed this effect (Figure 4A). Transwell assays revealed that overexpression of ZEB1 reversed the reduction in migration and invasion caused by interference with BRCC3 (Figure 4B–D). In addition, upregulation of ZEB1 reversed the effect of BRCC3 downregulation and improved MDA-MB-231 cell mobility (Figure 4E). Western blot assays showed that interference with BRCC3 upregulated E-cadherin protein expression and downregulated BRCC3, ZEB1 and vimentin protein expression, and overexpression of ZEB1 reversed these effects (Figure 4F). In addition, positive expression of E-cadherin and vimentin was detected by
immunofluorescence in MDA-MB-231 cells. **Supplementary Figure S3** shows that overexpression of ZEB1 reversed the effect of shRNA-BRCC3, downregulated the expression of E-cadherin and upregulated the expression of vimentin. The above results demonstrated that interfering with BRCC3 inhibited TNBC cell proliferation, migration, invasion and EMT by downregulating ZEB1.

**Interference with BRCC3 inhibits tumorigenesis and lung metastasis in TNBC in vivo**

The changes in the transplanted tumor volume in the mice are shown in **Figure 5A**. After the inoculation of MDA-MB-231 cells in the control group, the subcutaneous tumors grew rapidly, and interfering with BRCC3 expression significantly inhibited tumor growth after 3 weeks (**Figure 5B**). HE staining revealed deep staining of lung metastases, clear boundaries with surrounding tissues, closely arranged tumor cells and abundant capillaries in the control and LV-shRNA groups, and LV-shRNA-BRCC3 reversed these effects (**Figure 5C**). Next, the expression levels of BRCC3, ZEB1, E-cadherin and vimentin in tumor tissues were detected via immunohistochemistry. We found that RCC3 knockdown in vivo upregulated E-cadherin expression and downregulated BRCC3, ZEB1 and vimentin expression (**Figure 5D–G**). These results suggest that interference with BRCC3 inhibits EMT, TNBC cell tumorigenesis and distant metastasis.

**Interfering with BRCC3 inhibits the tumorigenesis and lung metastasis of TNBC cells in vivo by downregulating ZEB1**

To further confirm the molecular mechanism above in vivo, we divided the mice into LV-shRNA, LV-shRNA-BRCC3, and LV-shRNA-BRCC3+LV-ZEB1 groups. Tumor tissue lysates from LV-shRNA and LV-shRNA-BRCC3 mice were subjected to IP with an anti-ZEB1 antibody, followed by Western blotting with an anti-ubiquitin antibody. Mice in the LV-shRNA-BRCC3 group exhibited increased ZEB1 ubiquitination (**Figure 6A**). According to the tumor tissue images and statistical analysis of the tumor volume, LV-ZEB1 reversed the effect of LV-shRNA-BRCC3
and promoted tumor growth (Figure 6B). The results of HE staining demonstrated that LV-ZEB1 promoted lung metastasis (Figure 6C). The immunohistochemistry results showed that BRCC3 interference upregulated E-cadherin expression and downregulated ZEB1 and vimentin expression, and ZEB1 abolished these effects (Figure 6D–F).

Discussion

The main factors leading to poor prognosis and death in TNBC patients are tumor recurrence and metastasis. Therefore, further research on the mechanism of TNBC occurrence and development is crucial for its early diagnosis and treatment. We examined the expressions of five DUBs and ZEB1 in TNBC patients and performed a correlation analysis. Only BRCC3 expression was positively correlated with ZEB1 expression, whereas STAMBP, BAP1, UCHL1, and USP9X were not significantly correlated with ZEB1 expression. BRCC3 has been reported to be a procarcinogenic factor in other cancer diseases. For example, in nasopharyngeal carcinoma, BRCC3 knockdown increased cell survival, attenuated DNA damage repair, and led to G2/M cell cycle arrest in radioresistant nasopharyngeal carcinoma cells [22]. In cervical cancer, interference with BRCC3 inhibited cervical cancer cell viability, invasion and migration ability [23]. However, the role of BRCC3 in TNBC metastasis has not been reported. Considering our findings, we hypothesized that BRCC3 increases ZEB1 stability and upregulates ZEB1 expression through deubiquitination, thereby promoting TNBC cell proliferation, migration, invasion, EMT and metastasis.

It is well known that EMT is important for the invasion and metastasis of cancers, including ovarian, breast, colon, lung and liver cancers [24]. In TNBC, prominent metastatic and invasive abilities are both significant features, and EMT is closely associated with the invasion and migration of many kinds of tumor cells. Therefore, effective inhibition of EMT is highly important for the treatment of TNBC. The expressions of EMT molecular markers are tightly regulated by different transcription factors, which are known as EMT-inducing transcription factors (EMT-TFs), including Slug, Snail, Twist1, and ZEB1/2 [25]. EMT-TFs are extremely unstable
proteins that are tightly controlled at the protein level by the UPS. Under pathological conditions, dysfunctional DUBs negatively affect the UPS, thereby enhancing protein stability and aggregation [26]. It was found that USP51 and constitutively photomorphogenic 9 signalsome subunit 5 promote EMT by stabilizing the expression of ZEB1, leading to increased tumor invasion and metastasis [27].

In this study, five deubiquitinating enzymes with the highest binding potential (highest score) to ZEB1, namely, STAMBP, BAP1, UCHL1, USP9X and BRCC3, were screened via the deubiquitination prediction software Uhibrowser 2.0. Knockdown of STAMBP could inhibit the proliferation, migration and invasion of multiple TNBC cell lines [28]. Studies have reported that BAP1 upregulates β-catenin, which further promotes TNBC tumorigenesis [29]. UCHL1 plays a role in the malignant progression of TNBC by maintaining cell stemness and promoting invasion [30]. Moreover, USP9X inhibitors inhibited TNBC cell migration, invasion and metastasis and increased cell sensitivity to cisplatin and paclitaxel [31]. Moreover, mRNA expression profiling of BRCC3 has been reported in human breast cancer cells, and exogenous BRCC3 expression is associated with delayed death and increased breast cancer cell proliferation [32]. In vitro experiments showed that interference with BRCC3 increased the level of ZEB1 ubiquitination and promoted ZEB1 protein degradation, thereby inhibiting ZEB1 expression.

E-cadherin and vimentin are more mature cell markers studied during the EMT process. E-cadherin is a transmembrane glycoprotein with adhesion properties that plays a role in stabilizing epithelial cell morphology and maintaining tissue structural integrity. Vimentin is generally expressed in normal mesenchymal cells and mesenchymal tumor cells and can weaken epithelial cell adhesion and promote tumor cell invasion and migration [8]. In the present study, interfering with BRCC3 inhibited the migration and invasion of TNBC cells, upregulated E-cadherin, and downregulated ZEB1 and vimentin, and these effects were reversed by the ZEB1 overexpression plasmid. Further in vivo experiments confirmed that interfering with BRCC3 inhibited the EMT, tumorigenic ability and lung metastasis of TNBC cells.

In conclusion, BRCC3 promotes TNBC cell proliferation and metastasis by
increasing the stability of ZEB1 under pathological conditions. In the future, molecular methods for BRCC3 interference could lead to new ideas and provide a clinical and experimental basis for TNBC treatment. However, the clinical sample size was small, and most of the experiments were performed at the protein level, leading to a lack of depth in the study. In the future, more accurate large-scale clinical studies and in-depth experimental studies can be conducted.

**Supplementary Data**
Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

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

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13. Luo N, Zhang K, Li X, Hu Y. ZEB1 induced-upregulation of long noncoding RNA ZEB1-AS1 facilitates the progression of triple negative breast cancer by binding with ELAVL1 to maintain the stability of ZEB1 mRNA. *Journal of cellular biochemistry* 2020, 121(10):4176-4187.


Figure legends

**Figure 1. Screening of ZEB1-associated DUBs in TNBC**  
(A) The online software Ubibrowser was used to screen the DUBs with the highest probability of binding to ZEB1. (B) Western blot analysis was used to detect the expressions of ZEB1, STAMBP, BAP1, UCHL1, USP9X, and BRCC3 in TNBC tissues (n=20) and adjacent nontumor tissues (n=20). (C–G) Pearson correlation analysis of the protein expression of five DUBs and ZEB1 in 20 TNBC tissues collected in this study was performed. (H) RT-qPCR was used to detect the mRNA levels of BRCC3 in MCF-10A, MDA-MB-231, MDA-MB-453, MDA-MB-468 and BT-549 cells. (I–K) Western blotting was used to detect the protein expressions of BRCC3 and ZEB1 in MCF-10A, MDA-MB-231, MDA-MB-453, MDA-MB-468 and BT-549 cells. *P<0.05, **P<0.01, and ***P<0.001 vs MCF-10A cells.

**Figure 2. Interference with BRCC3 inhibits TNBC cell proliferation, migration, invasion and EMT**  
MDA-MB-231 cells were divided into shRNA and shRNA-BRCC3 groups. (A) Cell proliferation was detected by a colony formation assay. The colony number was analyzed. ***P<0.001 vs shRNA. (B–D) Transwell assay was used to detect cell migration and invasion. **P<0.01 vs shRNA. Scale bar=200 μm. (E) A wound healing assay was used to detect scratch mobility. **P<0.01 vs shRNA. Scale bar=200 μm. (F) Western blot analysis was used to detect the expressions of ZEB1, BRCC3, E-cadherin and vimentin. The BT-549 cells were grouped into a pcDNA group and a pcDNA-BRCC3 group. (G) Cell proliferation was detected by a colony formation assay. The colony number was analyzed. **P<0.01 vs pcDNA. (H–J) Transwell assay was used to detect cell migration and invasion. **P<0.01 vs pcDNA. Scale bar=200 μm. (K) A wound healing assay was used to detect scratch mobility. **P<0.01 vs pcDNA. Scale bar=200 μm. (L) Western blot analysis was used to detect the expressions of ZEB1, BRCC3, E-cadherin and vimentin.
Figure 3. Interference with BRCC3 promotes ZEB1 protein degradation and inhibits ZEB1 expression by increasing its ubiquitination level (A,B) The interaction between BRCC3 and ZEB1 was verified by coimmunoprecipitation (co-IP). (C) A ubiquitination assay was conducted to confirm the ubiquitination effect of BRCC3 on ZEB1 after transfection of shRNA-BRCC3. (D) The stability of the ZEB1 protein was determined by CHX chase assay after transfection of shRNA-BRCC3. *P<0.05, ***P<0.001 vs shRNA. (E) The degradation of the ZEB1 protein after transfection of shRNA-BRCC3 was detected by MG132 treatment. **P<0.01, ***P<0.001 vs shRNA.

Figure 4. Interference with BRCC3 inhibits TNBC cell proliferation, migration, invasion and EMT by downregulating ZEB1 MDA-MB-231 cells were divided into shRNA+vector, shRNA-BRCC3+vector, and shRNA-BRCC3+ZEB1 groups. (A) Cell proliferation was detected by a colony formation assay. The colony number was analyzed. ***P<0.001 vs shRNA+vector, ###P<0.001 vs shRNA-BRCC3+vector. (B–D) Transwell assay was used to detect cell migration and invasion. ***P<0.001 vs shRNA+vector, ###P<0.001 vs shRNA-BRCC3+vector. Scale bar=200 μm. (E) A wound healing assay was used to detect scratch mobility. ***P<0.001 vs shRNA+vector, ##P<0.01 vs shRNA-BRCC3+vector. Scale bar=200 μm. (F) Western blot analysis was used to detect the expressions of BRCC3, ZEB1, E-cadherin and vimentin.

Figure 5. Interference with BRCC3 inhibits the tumorigenesis and lung metastasis of TNBC in vivo Mice were divided into the control, LV-shRNA, and LV-shRNA-BRCC3 groups (n=6/group). (A) The tumor tissues of each group were recorded. (B) Tumor volume was detected after 6 weeks. **P<0.01 vs LV-shRNA. (C) Lung metastasis was detected by HE staining. Scale bar=100 μm. (D–G) The expression levels of BRCC3, ZEB1, E-cadherin and vimentin in tumor tissues were detected via immunohistochemistry. Scale bar=50 μm.
Figure 6. Interfering with BRCC3 inhibits the tumorigenesis and lung metastasis of TNBC cells in vivo by downregulating ZEB1

Mice were divided into LV-shRNA, LV-shRNA-BRCC3 and LV-shRNA-BRCC3+LV-ZEB1 groups (n=6/group). (A) Ubiquitination assay was performed on tumor tissues from the LV-shRNA and LV-shRNA-BRCC3 groups. (B) The tumor tissues of each group were recorded. Tumor volume was detected after 6 weeks. **P<0.01 vs LV-shRNA; #P<0.05 vs LV-shRNA-BRCC3. (C) Lung metastasis was detected by HE staining. Scale bar=100 μm. (D–F) The expression levels of ZEB1, E-cadherin and vimentin in tumor tissues were detected via immunohistochemistry. Scale bar=50 μm.
<table>
<thead>
<tr>
<th>Gene</th>
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<tr>
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<td><strong>Forward:</strong> AGGAAGTAATGGGGCTGTGC</td>
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<td><strong>Reverse:</strong> AGTACTTGCGCTCAGGAGGA</td>
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Supplementary Figure S1. Quantitative analysis of ZEB1, STAMBP, BAP1, UCHL1, USP9X, and BRCC3 protein expressions in TNBC tissues (n=20) and adjacent non-tumor tissues (n=20) ***P<0.001 vs adjacent.
Supplementary Figure S2. The co-localization of ZEB1 and BRCC3 and interaction between them  (A) Immunofluorescence assay was used to detect the co-localization of ZEB1 and BRCC3 in MDA-MB-231 and BT-549 cells. (B,C) The interaction between BRCC3 and ZEB1 was verified by Co-IP. (D) Ubiquitination assay was performed after transfection of wild-type BRCC3 or mutant BRCC3 in BT-549 cells.
Supplementary Figure S3. Positive expressions of E-cadherin and vimentin in MDA-MB-231 cells was detected by immunofluorescence assay
cells were divided into shRNA+vector, shRNA-BRCC3+vector, and shRNA-BRCC3+ZEB1 groups. (A) Immunofluorescence staining of E-cadherin protein in each group. (B) Immunofluorescence staining of vimentin protein in each group. Scale bar=50 μm.
297x219mm (300 x 300 DPI)
361x306mm (300 x 300 DPI)
208x313mm (300 x 300 DPI)
213x331mm (300 x 300 DPI)
To explore the molecular mechanism of the occurrence and development of triple negative breast cancer (TNBC) is helpful to search for feasible therapeutic means, and has certain clinical significance. Here, our results show that BRCC3 promotes TNBC cell proliferation and metastasis by increasing the stability of ZEB1 under pathological conditions.

1. Only deubiquitinating enzyme BRCC3 can interact with ZEB1 in the TNBC cells.
2. Interfering with BRCC3 inhibits ZEB1 expression by increasing the level of ZEB1 ubiquitination in the TNBC cells.
3. BRCC3 interference inhibits the proliferation, migration, invasion and EMT of TNBC cells by down-regulating ZEB1.