

BUB1B promotes homologous recombination-mediated DNA damage repair in breast cancer cells through the PI3K/AKT signaling pathway

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Abstract. Radioresistance is a major obstacle to effective radiotherapy in breast cancer. BUB1 mitotic checkpoint serine/threonine kinase B (BUB1B) is involved in numerous biological processes associated with cancer; however, its specific role in mediating radioresistance in breast cancer remains poorly characterized. The present study first evaluated its expression profile and association with patient prognosis through bioinformatics analysis. Subsequently, BUB1B expression in various breast cancer cell lines was validated by reverse transcription-quantitative PCR. Following short hairpin RNA-mediated knockdown of BUB1B in MDA-MB-231 cells, the impact of BUB1B on the biological functions and radiosensitivity of breast cancer cells was investigated using Cell Counting Kit-8, colony formation, EdU staining, gap closure, Transwell, immunofluorescence and comet assays, flow cytometric cell cycle analysis, and *in vivo* xenograft tumor experiments. Downstream signaling pathways regulated by BUB1B were identified via RNA sequencing and western blotting. The results revealed that BUB1B expression was elevated in breast cancer tissues and cell lines, and higher BUB1B expression was associated with poorer prognosis in patients with breast cancer. Functional investigations demonstrated that BUB1B may facilitate the proliferation, invasion and migration of breast cancer cells. Furthermore, BUB1B had a significant influence on the radioresistance of breast cancer, and promoted homologous recombination-mediated DNA damage repair and cell cycle arrest. At the molecular level, BUB1B may exert its effects through regulation of the

PI3K/AKT signaling cascade. In conclusion, these findings indicated that BUB1B may be a potential therapeutic target to overcome radioresistance in breast cancer.

Introduction

On a global scale, breast cancer ranks as the second most commonly occurring malignant neoplasm, and the leading cause of cancer-associated mortality among women (1). Despite substantial advancements in molecularly targeted interventions and immunotherapeutic approaches, therapeutic outcomes and prognostic indicators for patients with breast cancer continue to demonstrate suboptimal efficacy (2,3). Within the multimodal therapeutic framework for breast carcinoma, radiation therapy serves as a crucial component, demonstrating efficacy in managing localized tumor advancement, reducing the probability of recurrence and enhancing patient survival outcomes (4-6). However, the clinical efficacy of radiotherapy is frequently compromised by inherent or progressively acquired radioresistance mechanisms (7). Among the various factors influencing radiosensitivity, dysregulation of DNA repair mechanisms and cell cycle progression have been identified as pivotal elements underlying therapeutic resistance in neoplastic cells (8). Therefore, revealing the radiation therapy resistance mechanisms in breast cancer may provide improved opportunities to overcome tumor resistance.

DNA is vulnerable to multiple types of damage; such damage encompasses harm to the nucleotides (comprising bases and sugars) that constitute the DNA framework, the formation of crosslinks, and the occurrence of single-stranded breaks and double-stranded breaks (DSBs) within the DNA molecule (9). Among them, DSBs represent the most cytotoxic form of radiation-induced damage, initiating a cascade of molecular events within the cellular DNA damage response (DDR) network. This intricate process involves DNA damage perception, signal transduction pathway activation, DNA repair machinery engagement and cell cycle regulation (7,10,11). The defense mechanism of the cell utilizes a variety of strategies to handle DSBs, with the two primary repair routes being homologous recombination (HR) and non-homologous end joining (NHEJ) (12,13). The NHEJ mechanism, predominantly active during the G₁ phase, exhibits rapid repair kinetics but

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is associated with reduced fidelity. By contrast, HR-mediated repair, which utilizes undamaged sister chromatids as templates, demonstrates superior accuracy in damage correction (14,15). While DNA damage repair occurs, cell cycle checkpoints are activated to trigger cell cycle arrest, which provides a critical repair time for cancer cells and prevents them from entering mitosis without completing repair (8,16). In normal cells, these mechanisms act synergistically to maintain genome stability; however, in tumor cells, aberrant activation of the DDR often leads to radioresistance. Consequently, one of the most crucial methods for overcoming tumor radioresistance is to target the DDR signaling pathway (7,17).

An important part of the spindle assembly checkpoint system is BUB1 mitotic checkpoint serine/threonine kinase B (BUB1B) (18), which serves a key role in maintaining chromosome stability by interacting with Bub3, Mad2 and Cdc20 to form a mitotic checkpoint complex. Emerging evidence from numerous previous studies has established a strong association between dysregulated BUB1B expression and the development of malignancies, including extrahepatic cholangiocarcinoma, lung adenocarcinoma and thyroid carcinoma, with its upregulation consistently linked to unfavorable clinical outcomes (19-21). Furthermore, BUB1B is associated with chemoradiotherapy resistance in cancer, such as bladder cancer, glioblastoma and multiple myeloma (22-24).

In the present study, the extent of BUB1B expression in breast cancer and its association with patient prognosis was assessed. Subsequently, the MDA-MB-231 cell line, which shows a high level of BUB1B expression, was selected to conduct a more in-depth exploration of its biological functions during tumor development and its response to radiation. The present study underscores the crucial role that BUB1B assumes in breast cancer and identifies a potential therapeutic target for enhancing the sensitivity of breast cancer to radiotherapy.

Materials and methods

Bioinformatics analysis. The UALCAN (The Cancer Genome Atlas module, <https://ualcan.path.uab.edu/>) and Gene Expression Omnibus (GEO, (<https://www.ncbi.nlm.nih.gov/geo/>)) databases were employed to investigate the expression state of BUB1B in breast cancer samples. The Mann-Whitney U test was used to analyze the GEO datasets GSE38959 (25) and GSE65194 (26). Subsequently, receiver operating characteristic (ROC) curve analysis was conducted to assess the clinical diagnostic capability of BUB1B. Survival analysis related to BUB1B expression was analyzed using the bc-GenExMiner website (<http://bcgenex.ico.unicancer.fr>). Gene Set Enrichment Analysis (GSEA) was performed on data from the GEO database using R language (version 4.3.1; R Foundation for Statistical Computing). Breast cancer samples were categorized into two groups based on the median expression level of BUB1B mRNA: High-expression and low-expression cohorts. Subsequently, GSEA was performed to identify distinct molecular pathways between these two groups.

Cell lines. The normal mammary epithelial cell line MCF-10A, and breast cancer cell lines MDA-MB-231, BT-549, MCF-7 and BT-474 cell lines were procured from Procell Life Science & Technology Co., Ltd. Different culture media were used for each cell line. MCF-10A cells were cultured in MCF 10A Cell

Complete Medium (cat. no. CM-0525; Procell Life Science & Technology Co., Ltd.). To culture MDA-MB-231 and MCF-7 cells, high-glucose DMEM (cat. no. C11995500BT; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; cat. no. C04001; Shanghai VivaCell Biosciences, Ltd.) was employed, whereas BT-549 and BT-474 cells were cultured in RPMI 1640 medium (cat. no. C11875500BT; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. All cells were cultured at 37°C in a 5% CO₂ incubator.

Lentivirus infection. The expression of BUB1B was first examined in normal mammary epithelial cells and different breast cancer cell lines, and, based on the experimental results, the MDA-MB-231 cell line was selected for lentiviral infection in subsequent experiments, as it had the highest expression of BUB1B. The lentiviral short hairpin RNA (shRNA) constructs, comprising non-targeting nonsense negative control (NC) sequences and specific shBUB1B1/2/3 targeting human genes, were acquired from Shanghai Hanhang Technology Co., Ltd. The shRNA sequences were as follows: NC: Top strand 5'-GATCCGTTCTCCGAACGTGTCACGTAATTC AAGAGATTACGTGACACGTTCCGAGAATTTTTTC, bottom strand 5'-AATTGAAAAAATTCTCCGAACGTGTCACGT AATCTCTTGAATTACGTGACACGTTCCGAGAACG; shBUB1B-1, top strand 5'-GATCCGAGACAACTAAAC TGCAAATTCTCGAGAATTTGCAGTTTAGTTGTCTCT TTTTGT, bottom 5'-AATTCAAAAAAGAGACAACTA AACTGCAAATTCTCGAGAATTTGCAGTTTAGTTGTC TCG-3'; shBUB1B-2, top strand 5'-GATCCGCCAGTTCTG TTTGTCAAGTAACTCGAGTTACTTGACAAACAGAAC TGGTTTTTTG-3', bottom strand 5'-AATTCAAAAAACCAG TTCTGTTTGTCAAGTAACTCGAGTTACTTGACAAAC AGAACTGGCG-3'; shBUB1B-3, top strand 5'-GATCCG CTGTATTGTTTGGCACCAATACTCGAGTATTGGTGC CAAACAATACAGTTTTTTG-3', bottom strand 5'-AATTCA AAAAAGTATTGTTTGGCACCAATACTCGAGTATT GGTGCCAAACAATACAGCG-3'. The second-generation lentiviral packaging system was used. All vectors, reagents, and cell lines utilized for lentiviral packaging were provided by Shanghai Hanhang Technology Co., Ltd. The plasmids used for transfection included: pSPAX2: 10 µg, pMD2G: 5 µg, pHBLV-U6-MCS-CMV-ZsGreen-PGK-PURO plasmid (carrying shRNA): 10 µg. These plasmids were co-transfected into the 293T packaging cells using a transfection reagent (Lipofiter™, 75 µl), and the cells were incubated at 37°C for 72 h, after which, the viral supernatant was collected by ultracentrifugation. The lentivirus was then infected into MDA-MB-231 cells using the half-volume infection method at a multiplicity of infection of 30, followed by incubation in a 37°C incubator for 24 h. After replacing the medium, the cells were cultured for a further 48 h and fluorescence was observed under a fluorescence microscope. Stable infected cell lines were selected by culturing the cells in medium containing 4 µg/ml puromycin (Beyotime Institute of Biotechnology) for 7 days.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the MCF-10A, MDA-MB-231, BT-549, MCF-7 and BT-474 cell using the Total RNA Isolation Kit (cat. no. M5105; New Cell & Molecular

Biotech). Subsequently, the concentration and purity of the extracted RNA were assessed with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). The extracted RNA was stored at -80°C . cDNA was obtained from the extracted RNA using the Script Reverse Transcription Supermix Kit (cat. no. RR047A; Takara Bio, Inc.) according to the manufacturer's instructions. Subsequently, qPCR was performed using the TB Green® Fast qPCR Mix (cat. no. RR430A; Takara Bio, Inc.). The gene expression levels were measured using the $2^{-\Delta\Delta\text{Cq}}$ method (27), with GAPDH serving as the internal reference gene. The primer sequences utilized for amplification were as follows: BUB1B, forward 5'-AAATGACCCTCTGGATGTTTGG-3' and reverse 5'-GCATAAACGCCCTAATTTAAGCC-3'; and GAPDH, forward 5'-CAGGAGGCATTGCTGATGAT-3' and reverse 5'-GAAGGCTGGGGCTCATTT-3'.

Western blot analysis. Cells were lysed with RIPA lysis solution (cat. no. P0013B; Beyotime Institute of Biotechnology) and protein supernatants were collected. Protein concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). The proteins (20 μg) were denatured by boiling and were separated by SDS-PAGE on a 4.5% stacking gel and 7.5% resolving gel. The proteins were subsequently transferred to a PVDF membrane, which was blocked with 5% skimmed milk for 1 h at room temperature. The membrane was then incubated with primary antibodies at 4°C overnight. On the next day, secondary antibody incubation was performed, using HRP-labeled secondary antibodies (cat. nos. 511203 and 511103; 1:5,000; Chengdu Zen-Bioscience Co., Ltd.) for 1 h at room temperature. After immersing the membrane in BeyoECL Plus (cat. no. P0018S; Beyotime Institute of Biotechnology) working solution for 1 min, the bands were detected using a chemiluminescence imaging system (Bio-Rad Laboratories, Inc.). Semi-quantitative analysis was performed using ImageJ software (version 1.54g; National Institutes of Health). The primary antibodies used were against BUB1B (cat. no. ab183496; 1:20,000; Abcam), β -actin (cat. no. AB0035; 1:10,000; Shanghai Abways Biotechnology Co., Ltd.), E-cadherin (cat. no. 60335-1-Ig; 1:2,000; Proteintech Group, Inc.), N-cadherin (cat. no. R380671; 1:500; Chengdu Zen-Bioscience Co., Ltd.), vimentin (cat. no. R22775; 1:500; Chengdu Zen-Bioscience Co., Ltd.), RAD51 (cat. no. R27223; 1:500; Chengdu Zen-Bioscience Co., Ltd.), PI3K (cat. no. 60225-1-Ig; 1:2,000; Proteintech Group, Inc.), AKT (cat. no. 60203-1-Ig; 1:2,000; Proteintech Group, Inc.), phosphorylated (p)-PI3K (cat. no. 341468; 1:500; Chengdu Zen-Bioscience Co., Ltd.) and p-AKT (cat. no. R381555; 1:500; Chengdu Zen-Bioscience Co., Ltd.).

Cell counting kit-8 (CCK-8) assay. A total of 5,000 cells was added to each well of a 96-well plate, and incubated in a 37°C incubator for 24, 48, 72 or 96 h. Subsequently, CCK-8 reagent (cat. no. A311-01; Vazyme Biotech Co., Ltd.) was added to each well and incubated at 37°C for 1 h. Next, a microplate spectrophotometer was employed to measure the absorbance at a wavelength of 450 nm.

Colony formation assay. To explore the development of cell colonies, cells were distributed evenly across 6-well culture plates at a density of 400 cell/well and were incubated for 10-14 days

under standard culture conditions. The medium was changed and the cell status was observed every 3 days. Colonies were defined as aggregates of ≥ 50 cells originating from a single progenitor cell and were manually counted under a light microscope. The cell colonies were fixed with 4% paraformaldehyde solution at room temperature for 15 min and stained with 5% crystal violet solution at room temperature for 15 min. After washing with PBS three times, colony quantification and photographic documentation were performed. Each experimental condition was independently replicated three times, with quantitative data normalized to the corresponding control groups.

EdU staining. Cell proliferation capacity was measured with the BeyoClick™ EdU-594 Cell Proliferation Assay Kit (cat. no. C0078S; Beyotime Institute of Biotechnology). Cells were inoculated into 6-well plates at a density of 2×10^5 cells/well and were incubated for 24 h in a 37°C incubator. Subsequently, the cells were treated with pre-warmed (37°C) 2X EdU working solution (20 μM) at equal volumes, achieving a final 1X EdU concentration. After 2 h of incubation under standard culture conditions, cellular samples were subjected to washing with PBS, followed by 4% paraformaldehyde fixation for 30 min at room temperature and 0.5% Triton X-100 permeabilization for 10 min at room temperature. Next, a Click reaction solution was performed by incubating the cells with 500 μl reaction mixture for 30 min in the dark at room temperature. Nuclear counterstaining was accomplished using 1,000 μl 1X Hoechst 33342 solution for 10 min in the dark at room temperature. Fluorescence imaging was conducted using an inverted fluorescence microscope, with quantification based on positive cell counts from five randomly selected fields per sample.

Xenograft tumor assay. A total of 10 BALB/c-nu female mice (age, 4 weeks; weight, 16-20 g) from Guangxi Medical University Laboratory Animal Center (Nanning, China) were selected for the experiment. The mice were housed in a specific pathogen-free environment with a temperature of $26-28^{\circ}\text{C}$, humidity of 40-60% and under a 12-h light/dark cycle. The mice were fed an irradiated sterilized high protein feed *ad libitum*, and the drinking water was ultrapure water, with the water bottle changed daily. The Guangxi Medical University Laboratory Animal Ethics Committee approved the present animal studies (approval no. 202410013).

BALB/c nude mice were randomly allocated into two groups ($n=5/\text{group}$): i) Control group receiving MDA-MB-231-NC cells and ii) experimental group, inoculated with MDA-MB-231-shBUB1B cells. A suspension containing 1×10^6 cells in 100 μl PBS was subcutaneously injected into the right axilla of each mouse. Tumor growth was monitored every 3 days by measuring orthogonal diameters with digital calipers, and tumor volume (TV) was calculated using the ellipsoid formula: $\text{TV} = 1/2 \times \text{length} \times \text{width}^2$. Humane endpoints were strictly enforced, and experiments were terminated when mice had a tumor volume $\geq 1,500 \text{ mm}^3$ or lost $>20\%$ of their body weight. The experiment was terminated after 5 weeks and no mice were sacrificed due to reaching the aforementioned humane endpoints. Mice were anesthetized via an intraperitoneal injection of 1.25% tribromoethanol (250 mg/kg body weight) and euthanized by cervical dislocation. All animals died from euthanasia. Death was confirmed

by continuous observation of respiratory movements for ≥ 5 min, including cessation of breathing and absence of chest rise and fall, and loss of corneal and pain reflexes.

Immunohistochemistry. For immunohistochemistry, nude mouse tissue specimens of xenograft tumor origin were incubated in 4% paraformaldehyde solution for at room temperature 72 h, embedded in paraffin and sectioned into 4- μ m slices. Tissue sections then underwent sequential processing (deparaffinization in xylene and rehydration through an alcohol gradient series), and were soaked in citrate buffer for 3 min for antigen retrieval. Bovine serum albumin (BSA; cat no. G5001; Wuhan Servicebio Technology Co., Ltd.) was used to block the sections at room temperature for 30 min. Subsequently, the sections were incubated with primary antibodies against Ki67 (cat. no. AF20068; 1:200; Hunan Aifang Biotechnology Co., Ltd.) overnight at 4°C, and then with a Polymer-HRP anti-mouse secondary antibody kit (cat. no. AFIHC002; Hunan Aifang Biotechnology Co., Ltd.) for 30 min at 37°C. DAB (cat. no. AFIHC004; Hunan Aifang Biotechnology Co., Ltd.) reagent was used for staining, whereas for counterstaining, the sections were incubated with hematoxylin at room temperature for 3 min, followed by sequential dehydration through an ethanol gradient and xylene. The staining was observed under a light microscope (E100; Nikon Corporation). Panoramic scanning was performed under a 20X objective lens using a DS-U3 imaging system (Nikon Corporation).

TUNEL staining was performed using a TUNEL kit (cat. no. AFIHC030-C; Hunan Aifang Biotechnology Co., Ltd.) to evaluate tissue apoptosis. The procedures for tissue fixation, paraffin embedding, sectioning and antigen retrieval were the same as aforementioned. Subsequently, a mixture of TDT enzyme and dUTP (mixed at a ratio of 1:50) was used to cover the tissues, followed by incubation at 37°C for 1–2 h. Nuclear staining was conducted at room temperature using 20X DAB staining solution (cat no. AFIHC004; Hunan Aifang Biotechnology Co., Ltd.). The staining was monitored under a light microscope (cat no. E100; Nikon Corporation) and the staining was terminated when brownish-yellow signals appeared. The stained sections were subjected to panoramic scanning under a 20X objective using the DS-U3 imaging system (Nikon Corporation).

Gap closure assay. Cell migration was evaluated using the ibidi Culture-Insert 2 Well (cat no. 81176; ibidi GmbH). The inserts were placed in 6-well plates, and each well was seeded with 70 μ l cell suspension containing 6×10^4 cells. After the cell confluence reached 95%, the inserts were carefully removed. The cell monolayer was then washed once with PBS and cultured in serum-free medium. The gap area was captured using a light microscope immediately after washing in PBS (designated as 0 h) and was established as the baseline. Images of the same positions were acquired at 12 and 24 h. The migratory capacity of the cells was assessed by measuring the changes in the gap area using ImageJ software (version 1.54g; National Institutes of Health, USA).

Transwell assay. Both Transwell invasion and migration assays were performed. For the invasion assay, the bottom of the 24-well Transwell inserts (pore size, 9 μ m) were coated

with Matrigel (Corning, Inc.), whereas the migration assay inserts remained uncoated. The Matrigel was thawed overnight in a refrigerator at 4°C, and the next day it was spread evenly on the bottom of the chamber and then incubated at 37°C for 1 h to solidify. A total of 200 μ l cells resuspended in serum-free DMEM (containing 2×10^4 cells/well) were seeded into the upper chamber, and 600 μ l complete medium supplemented with 10% FBS was added to the lower chamber. After 24 h of incubation at 37°C, the Transwell inserts were washed twice with PBS. The cells were then fixed with 4% paraformaldehyde for 15 min at room temperature, followed by staining with crystal violet for 15 min at room temperature. After rinsing the inserts twice with PBS, the stained cells were visualized and images were captured using a light microscope.

Immunofluorescence assay. Cells (5×10^4) were inoculated in 6-well plates and incubated at 37°C for 24 h. The cells were then treated with 8 Gy irradiation using a linear accelerator (Elekta Instrument AB) at a dose rate of 1 Gy/min before the detection of γ -H2AX by immunofluorescence assay. No irradiation was required for detection of the other indicators. Upon rinsing with PBS, cells were fixed using a 4% paraformaldehyde solution for 10 min at room temperature. After fixation, cell permeability was induced by treating the cells with a PBS solution containing 0.1% Triton X-100. Next, to prevent non-specific binding, a blocking procedure was carried out by incubating the cells with a 5% BSA solution for 30 min at room temperature. Once the blocking step was completed, the cells were incubated with the following primary antibodies overnight at 4°C: E-cadherin (cat. no. 60335-1-Ig; 1:200; Proteintech Group, Inc.), N-cadherin (cat. no. 22018-1-AP; 1:200; Proteintech Group, Inc.), vimentin (cat. no. R22775; 1:100; Chengdu Zen-Bioscience Co., Ltd.) and γ -H2AX (cat. no. ab81299; 1:250; Abcam). After rinsing with PBS, Alexa Fluor (AF)-conjugated Goat Anti-Mouse IgG H&L (AF594) (cat. no. 550042; Positive Bio) and Goat Anti-Rabbit IgG H&L (AF594) (cat. no. 550043; Positive Bio) secondary antibodies were added (dilution, 1:500) and incubated for 1 h at room temperature. Next, nuclear counterstaining was carried out using DAPI (cat. no. G1012; Wuhan Servicebio Technology Co., Ltd.) for 10 min at room temperature. Finally, fluorescence imaging of the samples was performed using a Zeiss LSM880 confocal microscope (Zeiss AG).

Neutral comet assay. DNA damage analysis was conducted 2 h after 8 Gy irradiation using a Comet Assay Kit (cat. no. KGA1302-20; Nanjing KeyGen Biotech Co., Ltd.). A total of 100 μ l 1% normal melting point agarose was added to the slide, followed by its solidification at 4°C for 10 min. Next, a cellular suspension containing 10^5 cells and 75 μ l 0.75% low melting point agarose were combined, followed by solidification under identical temperature conditions for 30 min. The slides were then immersed in lysis buffer for 2 h at 4°C, and the samples were then electrophoresed for 20 min at 22 V after being equilibrated for 30 min in a neutral electrophoresis solution. Post-electrophoresis processing included PBS immersion (pH 7.2–7.4) for 30 min at 4°C and propidium iodide staining for 10 min at room temperature. Fluorescent images were obtained with a Zeiss fluorescence microscope (Zeiss AG). Quantitative analysis was performed using Comet Assay

Software Project (28) (version 1.2.3 beta1), counting 20 cells per group, and the olive tail moments were shown.

Cell cycle analysis. A commercially available Cell Cycle Staining Kit [cat. no. CCS012; Multisciences (Lianke) Biotech, Co., Ltd.] was employed to assess the distribution of the cell cycle. Briefly, 2×10^5 cells were inoculated in a 6-well dish, placed in an incubator overnight. On the second day, the cells were treated with 8 Gy irradiation. Trypsin was used to collect cells at predetermined times post-treatment (0, 12 and 24 h), followed by washing in PBS and resuspension. Subsequently, 10 μ l permeabilization solution and 1 ml propidium iodide staining solution (containing RNase A) were incorporated into the mixture according to the Cell Cycle Staining Kit instructions. The resulting combination was then incubated at room temperature for 10 min in the dark. Data were acquired using a CytoFLEX flow cytometer (Beckman Coulter, Inc.). Upon data collection, assessment of the cell cycle phases was carried out with the aid of FlowJo analysis software (version 10.8.1; BD Biosciences).

RNA sequencing. Total RNA was extracted from NC and shBUB1B cells using the MJZol Total RNA Extraction Kit (cat no. T01-200; Shanghai Majorbio) at 8 h after 8 Gy irradiation. Subsequently, RNA quality was determined using a 5300 Bioanalyzer (Agilent Technologies, Inc.) and RNA was quantified using the ND-2000 (NanoDrop; Thermo Fisher Scientific, Inc.). The molar concentrations of libraries was determined by fluorescence quantification (Qubit™ 4.0; Thermo Fisher Scientific, Inc.). The final loading concentration was adjusted to 2 nM, and 150 bp paired-end sequencing was performed on the NovaSeq X Plus platform (PE150; Illumina, Inc.) using the NovaSeq 6000 SP reagent kit (100 cycles; cat. no. 2002746; Illumina Inc.). Bioinformatics processing and initial data analysis were performed by Shanghai Meiji Biological Co., Ltd. DESeq2 software (<http://bioconductor.org/packages/stats/bioc/DESeq2/>) was used to assess differential gene expression and significant changes were found using two criteria: $P < 0.05$ and $|\log_2 \text{fold-change}| \geq 1$. The Goatools program (<https://github.com/tanghaibao/GOatools>) was used for Gene Ontology (GO) enrichment analysis, and the Python SciPy package (<https://scipy.org/install/>) was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

Statistical analysis. All experimental procedures were independently performed in triplicate, with representative results shown. GraphPad Prism software version 10.0 (Dotmatics) was utilized to conduct statistical comparisons. Comparisons between two groups were performed using the two-tailed unpaired Student's t-test. For comparisons among multiple groups, one-way ANOVA followed by Dunnett's post hoc test or two-way ANOVA followed by Bonferroni's multiple comparisons test were applied. The quantitative outcomes are presented as the mean $\pm$ standard. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

BUB1B is highly expressed in breast cancer and is associated with poor prognosis. Analysis of the UALCAN database showed that the expression levels of BUB1B were significantly

higher in breast cancer tissues compared with in the normal tissues of healthy controls (Fig. 1A). Among them, the expression levels of BUB1B were highest in triple-negative breast cancer, followed by HER2-positive breast cancer, and it was lowest in luminal breast cancer. Analysis of GEO datasets using the Mann-Whitney U test revealed that BUB1B expression was markedly higher in breast cancer tissues vs. normal breast tissues from healthy controls in the GSE65194 dataset (Fig. 1B). Similarly, BUB1B gene expression was significantly elevated in breast cancer cells compared with in normal mammary ductal cells from healthy controls in the GSE38959 dataset (Fig. 1C). Based on the mRNA expression levels, a ROC curve was generated (Fig. 1D). The area under the ROC curve was calculated to be 0.963, which suggested that the mRNA levels of BUB1B may effectively distinguish between patients with breast cancer and healthy individuals.

The prognostic value of BUB1B was assessed via Kaplan-Meier survival analysis with log-rank testing using bc-GenExMiner. The present findings showed that higher BUB1B levels were associated with a lower probability of distant metastasis-free survival (DMFS) (Fig. 1D). Additionally, BUB1B mRNA expression was quantified in normal mammary epithelial cells (MCF-10A) and in multiple breast cancer cell lines (MDA-MB-231, BT-549, MCF-7 and BT-474) using RT-qPCR. The results demonstrated significantly elevated BUB1B mRNA levels in all breast cancer cell lines compared with in normal mammary epithelial cells (Fig. 1E). Furthermore, GSEA of GEO datasets revealed that the expression of BUB1B was associated with cell cycle, DNA replication, mismatch repair and base excision repair (Fig. 1F). These results suggested that BUB1B may be associated with biological processes, such as proliferation, cell cycle regulation and DNA damage repair in breast cancer cells.

BUB1B induces MDA-MB-231 cell proliferation in vivo and in vitro. To investigate the role of BUB1B in breast cancer, a stable knockdown of BUB1B was established in MDA-MB-231 cells (Fig. 2A and B) and BUB1B knockdown cell lines constructed using shRNA-2 were selected for cellular experiments. To assess the impact of BUB1B knockdown on cell proliferation under *in vitro* conditions, CCK-8 and colony formation assays were employed. Knocking down BUB1B significantly reduced MDA-MB-231 cell proliferation (Fig. 2C and D). EdU staining also demonstrated that DNA replication was significantly suppressed by BUB1B knockdown (Fig. 2E). To evaluate the impact of BUB1B on tumor formation in living organisms, nude mice were injected with MDA-MB-231 cells that either exhibited normal BUB1B expression or had BUB1B knockdown. The findings from the *in vivo* experiments were consistent with the aforementioned *in vitro* findings. Specifically, the results demonstrated that tumor growth was significantly reduced in the BUB1B knockdown group compared with that in the control group (Fig. 2F). Furthermore, immunohistochemical analysis revealed that BUB1B suppression resulted in decreased Ki67-positive proliferating cells and elevated TUNEL-positive apoptotic cell populations (Fig. 2G). These results suggested that knockdown of BUB1B may affect the growth of transplanted tumors *in vivo* by inhibiting cell proliferation and promoting apoptosis.

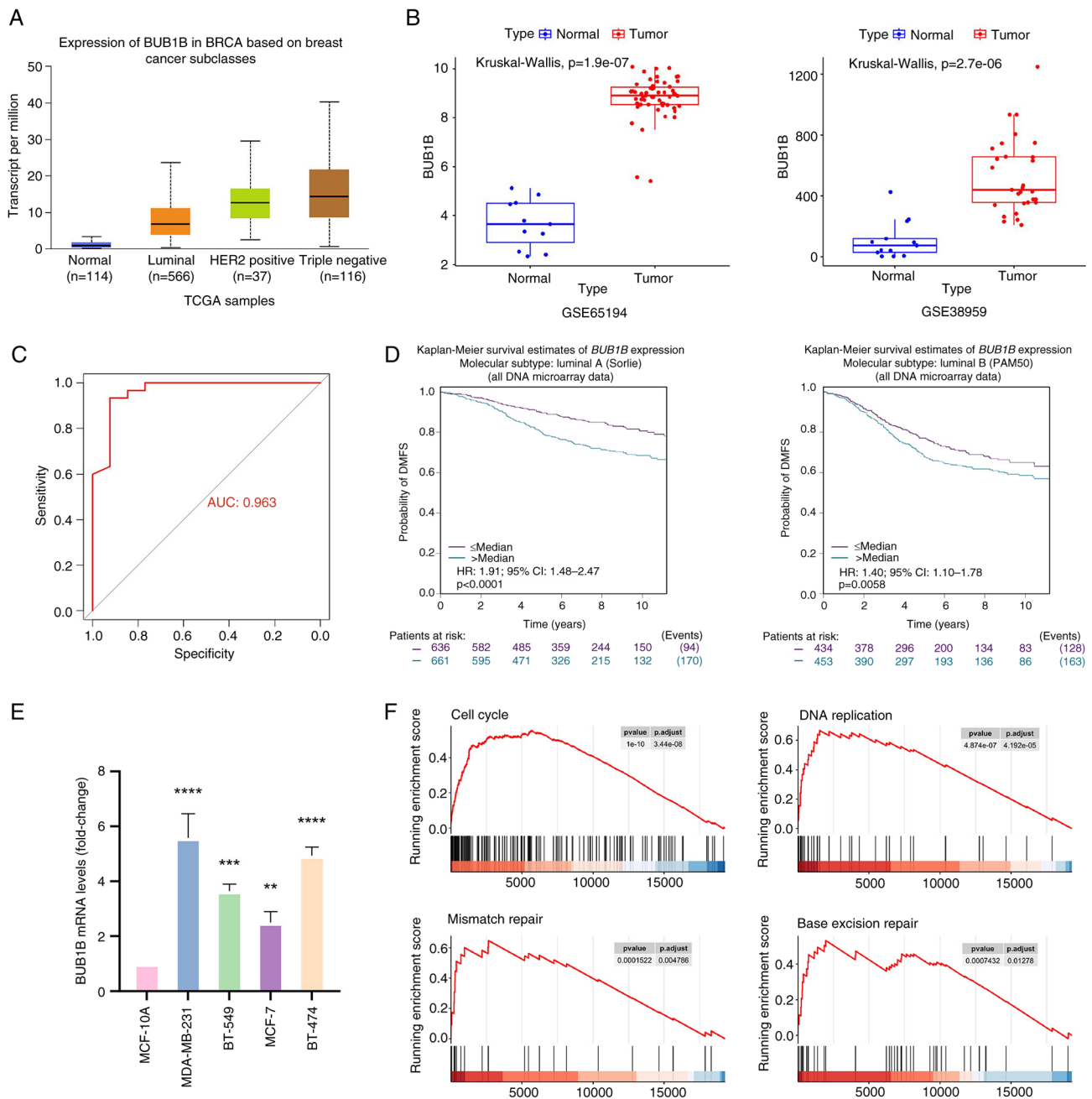


Figure 1. BUB1B is highly expressed in breast cancer and is associated with a poor prognosis. (A) BUB1B expression in healthy and breast cancer tissues in the UALCAN database. Compared with the Normal group, the P-values for Luminal, HER2-positive and triple-negative groups were all <0.001 . (B) Expression levels of BUB1B in GSE38959 and GSE65194 datasets. (C) Receiver operating characteristic curve based on BUB1B mRNA expression levels. (D) bc-GenEx-Miner website was used to evaluate the association between BUB1B and DMFS. (E) mRNA expression levels of BUB1B in normal mammary epithelial and breast cancer cells. Data are presented as the mean $\pm$ SD, $n=3$. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ vs. MCF-10A (one-way ANOVA followed by Dunnett's post hoc test). (F) Comparative GSEA was conducted using transcriptomics data from the GSE38959 dataset. Four representative plots of GSEA enrichment have been demonstrated. AUC, area under the curve; BUB1B, BUB1 mitotic checkpoint serine/threonine kinase B; DMFS, distant metastasis-free survival; GSEA, gene set enrichment analysis; TCGA, The Cancer Genome Atlas.

BUB1B promotes the migration and invasion of MDA-MB-231 cells. Gap closure and Transwell migration assays were used to assess the influence of BUB1B on the migratory and invasive capabilities of breast cancer cells. The findings indicated that downregulation of BUB1B expression suppressed the migration and invasion of MDA-MB-231 cells (Fig. 3A-D). Immunofluorescence assay was utilized to verify the influence of BUB1B on the epithelial-mesenchymal transition (EMT) of breast cancer cells. The findings showed that, when BUB1B

was knocked down in MDA-MB-231 cells, the expression levels of the epithelial marker E-cadherin were significantly increased, whereas the levels of mesenchymal markers (N-cadherin and vimentin) were decreased, compared with those in the NC group (Fig. 3E and F). This result was further verified by western blot analysis (Fig. 3G and H). These findings suggested that knocking down BUB1B may inhibit the invasion and metastatic potential of breast cancer cells through modulating the EMT process.

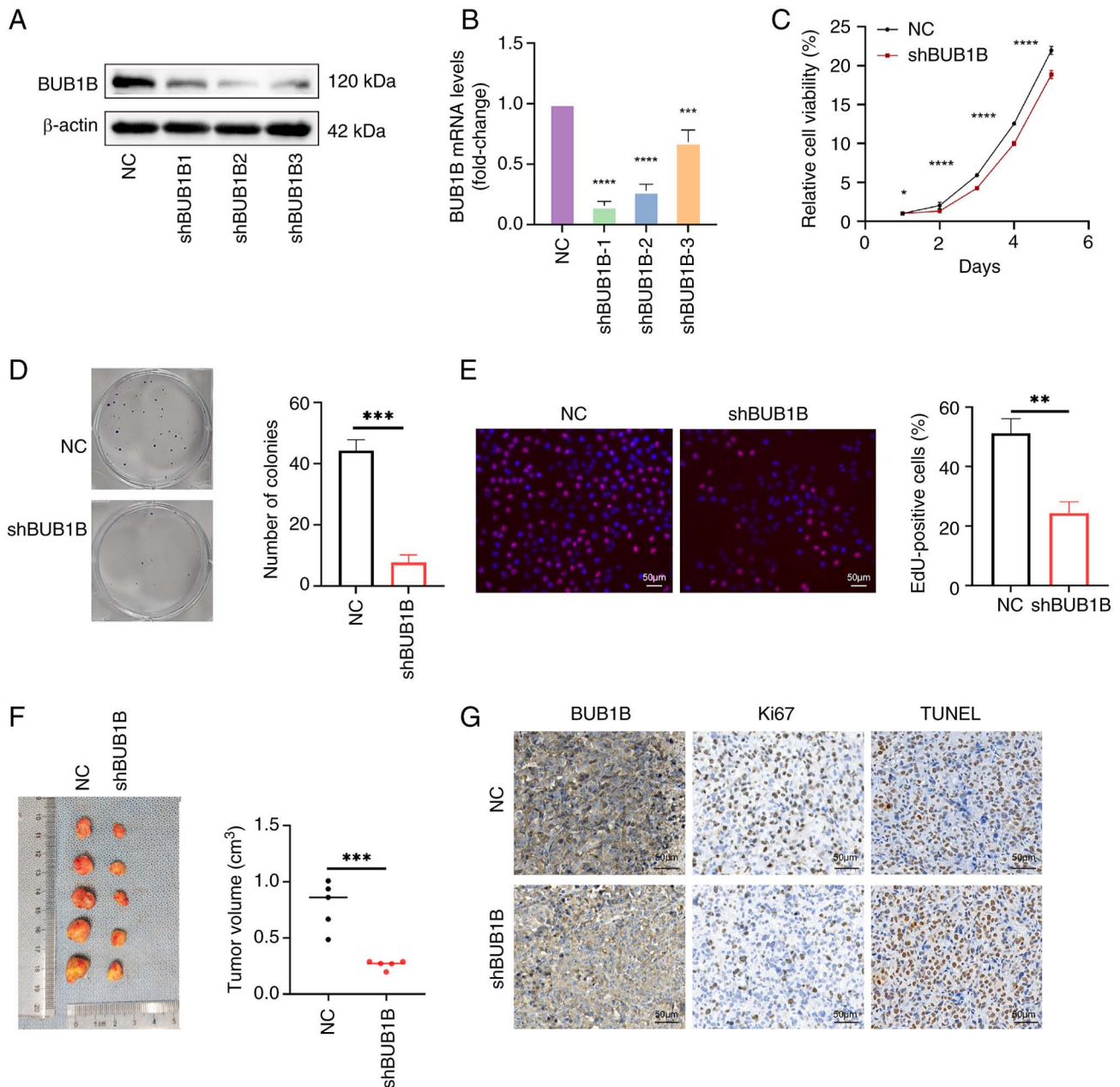


Figure 2. BUB1B promotes the proliferation of MDA-MB-231 cells *in vivo* and *in vitro*. BUB1B expression in MDA-MB-231 cells stably infected with NC or shBUB1B was detected by (A) western blotting and (B) reverse transcription-quantitative PCR. *** $P < 0.001$, **** $P < 0.0001$ vs. NC (one-way ANOVA followed by Dunnett's post hoc test). (C) Cell Counting Kit-8 assay was used to detect the proliferation of MDA-MB-231 cells. * $P < 0.05$, **** $P < 0.0001$ vs. NC (two-way ANOVA followed by Bonferroni's multiple comparisons test). (D) Colony formation assay was used to detect the colony-forming ability of MDA-MB-231 cells. *** $P < 0.001$ vs. NC (unpaired Student's t-test). (E) EdU assay of DNA replication in MDA-MB-231 cells. Scale bar, 50 μm ; magnification, x200. ** $P < 0.01$ vs. NC (unpaired Student's t-test). (B-E) Data are presented as the mean $\pm$ SD, $n=3$. (F) Xenograft tumor assay validated the effect of BUB1B on cell proliferation *in vivo*. Data are presented as the mean $\pm$ SD, $n=5$. *** $P < 0.001$ vs. NC (unpaired Student's t-test). (G) TUNEL and Ki67 were detected by immunohistochemical staining in tumor tissues. Scale bar, 50 μm ; magnification, x200. BUB1B, BUB1 mitotic checkpoint serine/threonine kinase B; NC, negative control; sh, short hairpin.

BUB1B promotes cell cycle arrest in breast cancer cells after irradiation. There is a close association between radiosensitivity and regulation of the cell cycle (29). Flow cytometry was employed to assess the impact of BUB1B on the cell cycle after exposure to ionizing radiation. The results indicated that, upon treatment with ionizing radiation, in contrast to NC cells, shBUB1B cells exhibited an increased proportion in the G₁/S phase and a concomitant decrease in the G₂/M phase (Fig. 4A and B). These results indicated that breast cancer cells mainly underwent G₂/M phase cycle arrest when exposed

to radiation. Notably, compared with that in the NC group, the percentage of BUB1B-silenced cells in G₂/M phase was considerably lower. These findings indicated that silencing BUB1B could impede the G₂/M phase arrest triggered by ionizing radiation.

BUB1B promotes DNA damage repair in a HR-biased manner. The primary mechanism of action of radiation therapy is to cause notable DNA damage (30); therefore, the present study examined how BUB1B contributes to DNA

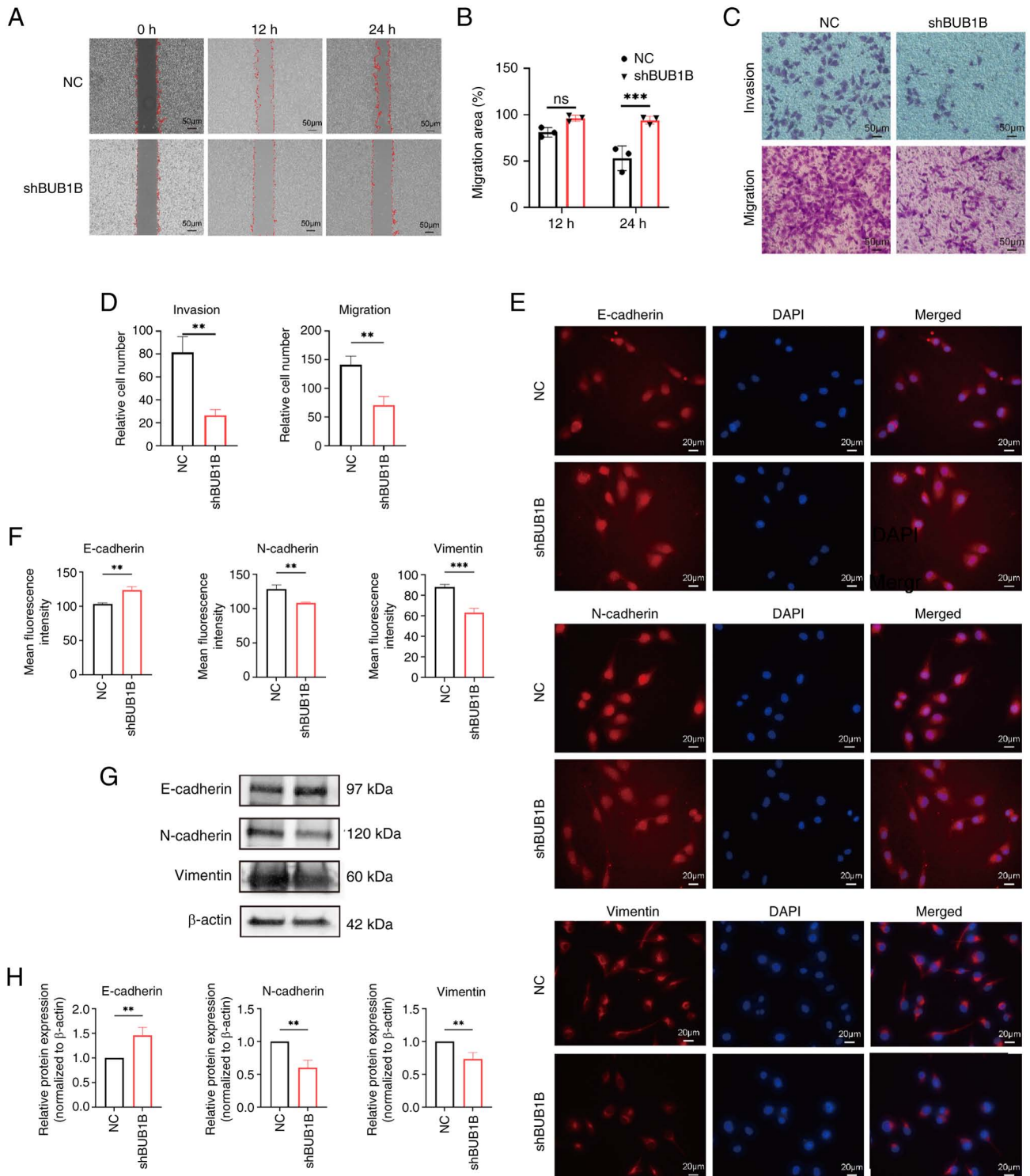


Figure 3. BUB1B promotes the migration and invasion of MDA-MB-231 cells. (A) Representative images and (B) quantitative analysis of cell migration assessed using the gap closure assay. Scale bar, 50 μ m; magnification, x200. *** P <0.001 vs. NC (two-way ANOVA followed by Bonferroni's multiple comparisons test). (C) Representative images and (D) quantitative analysis of migratory and invasive capacity of cells detected by Transwell assay. Scale bar, 50 μ m; magnification, x200. ** P <0.01 vs. NC (unpaired Student's *t*-test). (E) Representative immunofluorescence staining images of the EMT markers E-cadherin, N-cadherin and vimentin. (F) Semi-quantitative analysis of NC and shBUB1B cells after irradiation. Scale bar, 20 μ m; magnification, x400. ** P <0.01, *** P <0.001 vs. NC (unpaired Student's *t*-test). (G) Representative images and (H) semi-quantitative analysis of the EMT markers E-cadherin, N-cadherin and vimentin detected by western blot analysis. ** P <0.01 vs. NC (unpaired Student's *t*-test). All data are presented as the mean $\pm$ SD, *n*=3. BUB1B, BUB1 mitotic checkpoint serine/threonine kinase B; NC, negative control; sh, short hairpin.

repair. Immunofluorescence was used to assess the expression levels of γ -H2AX, a conventional DNA double-strand break marker, in MDA-MB-231 cells. The results revealed that cells with BUB1B knockdown had higher levels of γ -H2AX

than NC cells (Fig. 4C and D). After irradiation with 8 Gy, the comet assay was employed to detect DSBs. Compared with in the NC cell group, BUB1B-knockdown cells had a longer olive tail moment (Fig. 4E and F), which indicated that

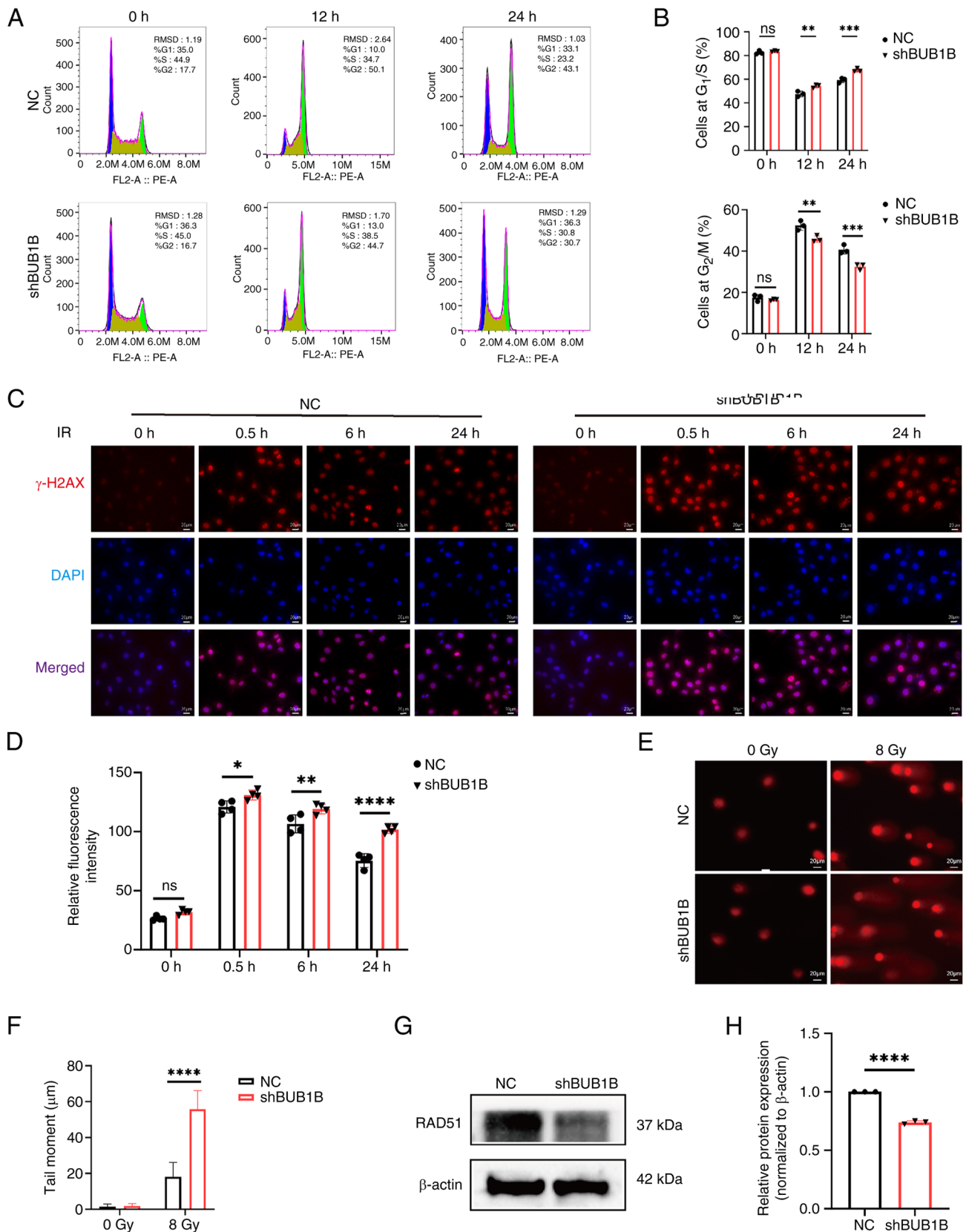


Figure 4. BUB1B serves an important role in the cell cycle arrest and DNA damage repair after irradiation. (A) Representative flow cytometry plots of cell cycle analysis and (B) quantitative analysis of NC and shBUB1B cells after irradiation. ** $P < 0.01$, *** $P < 0.001$ vs. NC (two-way ANOVA followed by Bonferroni's multiple comparisons test). (C) Representative γ -H2AX immunofluorescence images and (D) semi-quantitative analysis of NC and shBUB1B cells after irradiation. Scale bar, 20 μ m; magnification, $\times 400$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC (two-way ANOVA followed by Bonferroni's multiple comparisons test). (A-D) Data are presented as the mean $\pm$ SD, $n = 3$. (E) Representative images of the comet assay and (F) tail moment quantification analysis in MDA-MB-231 cells in the NC and shBUB1B groups after irradiation. Data are presented as the mean $\pm$ SD, $n = 20$. Scale bar, 20 μ m; magnification, $\times 400$. **** $P < 0.0001$ vs. NC (unpaired Student's t -test). (G) Representative images and (H) semi-quantitative analysis of RAD51 protein levels detected by western blot analysis. **** $P < 0.0001$ vs. NC (unpaired Student's t -test). All data are presented as the mean $\pm$ SD, $n = 3$. BUB1B, BUB1 mitotic checkpoint serine/threonine kinase B; IR, ionizing radiation; NC, negative control; sh, short hairpin.

BUB1B knockdown cells may have more pronounced DNA double-strand breaks. Together, these results suggested that BUB1B knockdown cells were more severely DNA damaged than NC cells after irradiation.

HR and NHEJ have been reported to be the two main pathways of cellular DSB repair. The present study confirmed that BUB1B knockdown can inhibit irradiation-induced G₂/M phase arrest, which primarily affects cellular HR repair. Therefore, the study further examined the effect of BUB1B knockdown on RAD51, a key protein in the HR repair pathway, through western blot analysis. The results revealed that BUB1B knockdown significantly suppressed the expression levels of RAD51 (Fig. 4G and H). These results indicated that BUB1B may promote the repair of DNA damage via HR, which could offer a novel mechanism for DNA damage repair.

BUB1B regulates the expression of the PI3K/AKT signaling pathway. To further identify the potential signaling pathways regulated by BUB1B, differentially expressed genes were screened in irradiated NC and BUB1B-knockdown cells by RNA sequencing, and GO and KEGG analyses were performed. The results showed that 297 genes were upregulated and 76 were downregulated in the BUB1B-knockdown group compared with in the NC group (Fig. 5C). Further GO functional analysis revealed that the differentially expressed genes were mainly involved in functions such as 'regulation of cellular biological processes' and 'signal transduction' (Fig. 5A). KEGG pathway enrichment analysis revealed that the activity of the 'PI3K-Akt signaling pathway' was associated with the BUB1B expression level (Fig. 5B). Several core proteins involved in the PI3K/AKT signaling pathway were detected by western blotting. The results showed that the protein levels of PI3K, AKT, p-PI3K and p-AKT were significantly reduced in MDA-MB-231 cells after downregulation of BUB1B expression (Fig. 5D and E). These data suggested that BUB1B regulates the PI3K/AKT signaling pathway.

Discussion

The present results suggested that BUB1B is upregulated in breast cancer, with higher expression levels significantly associated with poorer patient survival. Functional knockdown of BUB1B suppressed breast cancer cell proliferation, invasion, migration and tumorigenic potential. In addition, BUB1B was suggested to be a key regulator of radioresistance in breast cancer: BUB1B knockdown attenuated radiation-induced G₂/M cell cycle arrest and impaired HR-mediated DNA damage repair following irradiation. Collectively, these findings indicated that BUB1B may modulate breast cancer radiosensitivity by orchestrating cell cycle progression and DNA repair capacity, establishing it as a promising therapeutic target for improving radiation efficacy in breast cancer treatment.

Increasing evidence has indicated that BUB1B drives malignant progression in hepatocellular carcinoma, extrahepatic cholangiocarcinoma, lung adenocarcinoma and renal cell carcinoma by promoting cell proliferation and oncogenic signaling pathways, which is associated with poor patient prognosis (19,20,31,32). For example, in extrahepatic cholangiocarcinoma, aberrant BUB1B upregulation is significantly associated with a shorter overall survival and disease-free

survival. Mechanistically, BUB1B promotes tumor cell proliferation, migration and invasion via activation of the JNK/c-Jun signaling axis (19). Additionally, knockdown of BUB1B in human breast cancer cells has been shown to inhibit carcinomatous growth and induce chromosomal abnormalities (33). These findings align with the current study, which confirmed that BUB1B was upregulated in breast cancer and demonstrated a significant association between high BUB1B expression and shorter DMFS. Functional analyses further revealed that BUB1B may enhance breast cancer cell proliferation and invasiveness. Collectively, these results established BUB1B as a tumor promoter with critical prognostic significance in breast cancer. The long-term effects of BUB1B on breast cancer cells, including tumor recurrence and metastasis, require further validation in the future by constructing nude mouse metastatic tumor models.

Certain studies have proposed that BUB1B is involved in radioresistance in malignant tumors, but its biological mechanism remains controversial. For example, Komura *et al* (23) showed that, in bladder cancer, BUB1B interacts with ATM proteins to enhance cellular DNA damage repair via mutagenic NHEJ, leading to radioresistance in bladder cancer. Ma *et al* (22) demonstrated that, in glioblastoma, FOXM1 transcriptionally regulates BUB1B expression, thereby inducing tumor cell radioresistance. By contrast, treatment with a FOXM1 inhibitor could attenuate tumor radioresistance *in vitro* and *in vivo* (22). The present findings support the hypothesis that increased BUB1B expression is associated with the development of radioresistance. In the current study, GSEA indicated that BUB1B may modulate cell cycle progression and DNA damage repair. Molecular biology experiments validated that BUB1B knockdown reduced irradiation-induced G₂/M phase arrest and exacerbated DNA damage in cells. DNA damage repair is dependent on HR and NHEJ (34,35). G₂/M phase arrest provides a temporal window for HR repair, such that inhibiting G₂/M arrest predominantly impairs HR repair efficiency. As RAD51 is a major component of HR-mediated DNA repair, the present study demonstrated that BUB1B knockdown downregulated IR-induced RAD51 protein expression, thus suggesting that BUB1B knockdown may modulate breast cancer radiosensitivity by suppressing HR-mediated DNA damage repair. However, the impact of BUB1B knockdown on DNA damage repair efficiency currently lacks more direct experimental validation, such as verification using the direct repeat-green fluorescent protein reporter assay system.

The PI3K/AKT signaling pathway serves an important role in regulating tumor proliferation, invasion and apoptosis (36-38). Previous research has shown that activation of the PI3K/AKT pathway is also associated with tumor radiotherapy resistance (39). Inhibition of the PI3K/AKT pathway has been reported to increase the radiosensitivity of cancer cells in different types of tumor, including nasopharyngeal carcinoma (40), non-small cell lung cancer (41), oral squamous cell carcinoma (42) and glioblastoma (43,44). In breast cancer, No *et al* (45) showed that inhibition of the PI3K/AKT/mTOR signaling pathway can enhance the radiosensitivity of SKBR3 cells by inhibiting DNA damage repair, suggesting that the PI3K/AKT signaling pathway may be a key pathway influencing radioresistance in breast cancer. The present results of transcriptome sequencing analysis suggested that the PI3K/AKT signaling pathway may be a downstream signaling

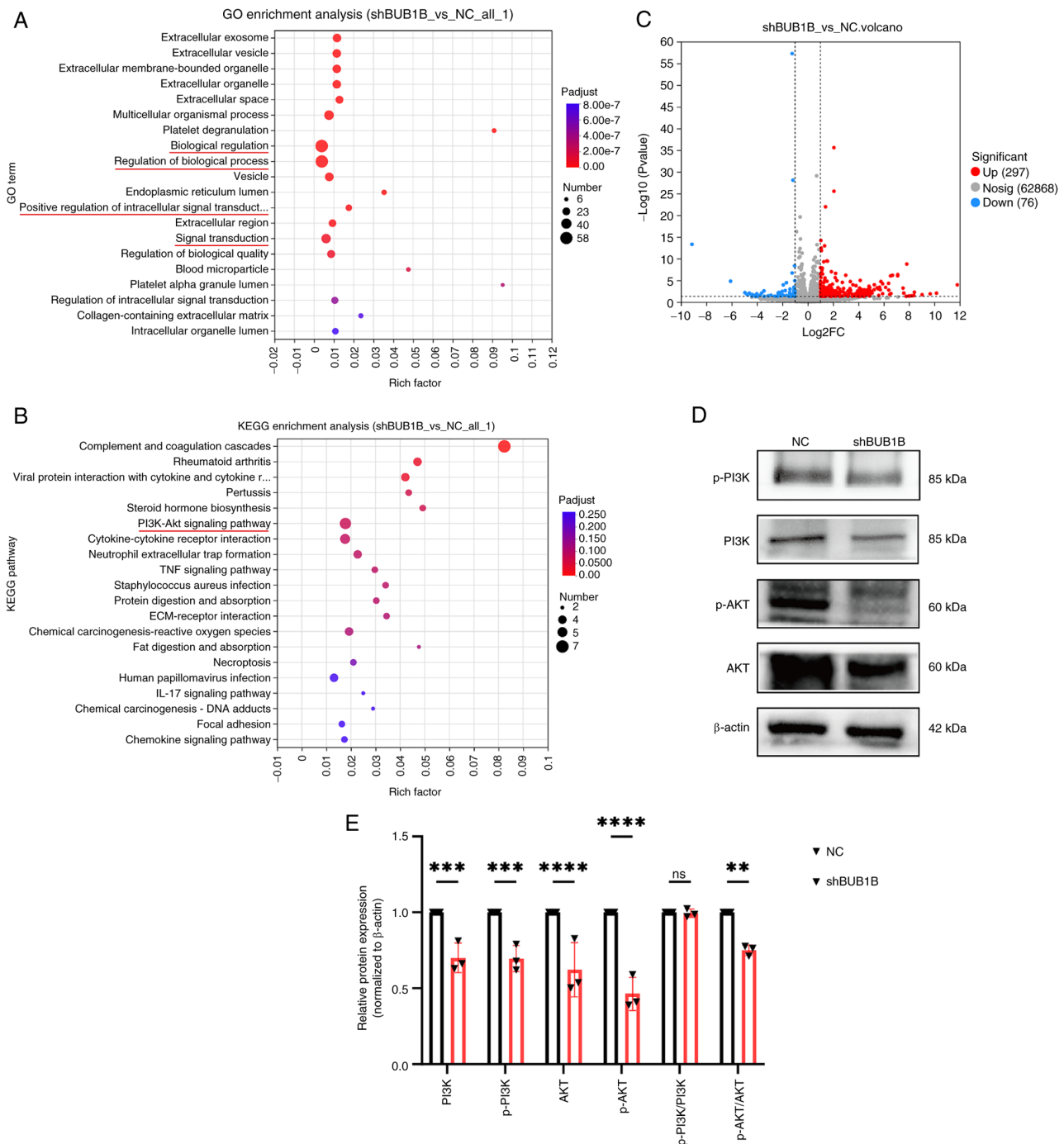


Figure 5. BUB1B regulates the PI3K/AKT signaling pathway. (A) GO and (B) KEGG enrichment analyses of differentially expressed genes in irradiated shBUB1B MDA-MB-231 cells vs. irradiated NC MDA-MB-231 cells. (C) After irradiation, a volcano plot of the genes that were differentially expressed in irradiated shBUB1B MDA-MB-231 cells vs. irradiated NC MDA-MB-231 cells was generated ($P < 0.05$, $|\log_2FC| \geq 1$). (D) Representative images and (E) semi-quantitative analysis of the protein levels in the PI3K/AKT signaling pathway determined by western blotting. Data are presented as the mean $\pm$ SD, $n=3$. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. NC (two-way ANOVA followed by Bonferroni's multiple comparisons test). BUB1B, BUB1 mitotic checkpoint serine/threonine kinase B; GO, Gene Ontology; IR, ionizing radiation; KEGG, Kyoto Encyclopedia of Genes and Genomes; NC, negative control; ns, not significant; p-, phosphorylated; sh, short hairpin.

pathway regulated by BUB1B. Further studies demonstrated that the expression levels of PI3K, p-PI3K, AKT and p-AKT were downregulated in BUB1B-knockdown breast cancer cell lines. The concurrent reduction in both total and phosphorylated levels of PI3K/AKT upon BUB1B knockdown may be attributed to the synergistic interplay of multiple mechanisms. BUB1B may enhance the expression of total PI3K/AKT proteins through interactions with transcription

regulatory factors, or alternatively, it could modulate their total protein levels by suppressing ubiquitin-mediated degradation. The decreased phosphorylated forms (p-PI3K, p-AKT) might either represent a secondary effect of reduced total protein abundance or result from the direct regulation of phosphorylation-related machinery by BUB1B. These results demonstrated that BUB1B may serve as an upstream regulator of the PI3K/AKT signaling pathway. Collectively, the findings

of the current study lead to a reasonable speculation that BUB1B may promote cell cycle arrest and DNA damage repair in breast cancer cells by activating the PI3K/AKT signaling pathway, thereby contributing to radioresistance. Therefore, the combined inhibition of BUB1B and targeted suppression of the PI3K/AKT signaling pathway could represent an effective strategy for radiosensitization in breast cancer.

PARP inhibitors exert therapeutic effects in tumors with HR repair deficiencies through synthetic lethality, such as in BRCA1/2-mutated breast and ovarian cancer (46). However, they exhibit limited efficacy in tumors with intact HR repair function. The present study demonstrated that BUB1B knockdown inhibited the HR repair pathway by downregulating RAD51, a key protein in HR repair. The current findings highlight the potential of combining BUB1B inhibition with PARP inhibitors for treating cancer insensitive to PARP inhibitors alone.

The present study has the following limitations: Firstly, in terms of clinical validation, the current study lacks validation of the expression level of BUB1B in clinical samples and its association with clinical parameters, such as patient prognosis and treatment response. Secondly, at the experimental model level, the functional validation in the present study was only performed in the MDA-MB-231 cell line, and further validation in other breast cancer cell models is needed. Furthermore, when validating the effect of BUB1B on DNA damage repair efficiency, a control group using DNA damage repair inhibitors was lacking. Additionally, since both immunofluorescence and western blotting are protein-level verification experiments, only immunofluorescence verification of γ -H2AX was performed, not western blotting. In addition, at the level of molecular mechanism, the detailed mechanism underlying the regulatory effects of BUB1B on the PI3K/AKT pathway remains to be identified and verified by further experiments. Despite the aforementioned limitations, the present study contributed to an increased understanding of the molecular mechanisms of breast cancer radiosensitivity, and provides novel molecular targets and experimental bases for the in-depth exploration of the regulatory network of tumor radiosensitivity.

In conclusion, the present study identified BUB1B as a gene associated with breast cancer radiotherapy resistance, which is involved in cellular DNA damage repair, especially HR repair, by regulating the PI3K/AKT signaling pathway. The current findings provide a novel mechanism for breast cancer radiotherapy resistance and suggest that BUB1B may be a potential target for improving the efficacy of breast cancer radiotherapy.

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Availability of data and materials

The RNA-sequencing data generated in the present study may be found in the NCBI BioProject database under accession number PRJNA1277467 or at the following URL: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1277467>. The other data generated in the present study may be requested from the corresponding author.

Authors' contributions

XL and WZ confirm the authenticity of all the raw data. XL conceptualized the study, developed the methodology, conducted the investigations and drafted the original manuscript. YW performed the investigations, and contributed to visualization and data curation. HL carried out validation and formal analysis. NX conducted the investigations and contributed to visualization. WZ conceptualized the study, provided supervision, secured funding, and revised and edited the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experimental protocols involving animal subjects were carried out in compliance with ethical guidelines and received formal approval from the Institutional Animal Care and Use Committee at Guangxi Medical University (Ethical Approval Number 202410013).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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