DAXX, as a Tumor Suppressor, Impacts DNA Damage Repair and Sensitizes BRCA-Proficient TNBC Cells to PARP Inhibitors[1]

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Abstract

Treatment options are limited for patients with triple negative breast cancer (TNBC). Understanding genes that participate in cancer progression and DNA damage response (DDR) may improve therapeutic strategies for TNBC. DAXX, a death domain-associated protein, has been reported to be critically involved in cancer progression and drug sensitivity in multiple cancer types. However, its role in breast cancer, especially for TNBC, remains unclear. Here, we demonstrated a tumor suppressor function of DAXX in TNBC proliferation, colony formation, and migration. In Mouse Xenograft Models, DAXX remarkably inhibited tumorigenicity of TNBC cells. Mechanistically, DAXX could directly bind to the promoter region of *RAD51* and impede DNA damage repair, which impacted the protection mechanism of tumor cells that much depended on remaining DDR pathways for cell growth. Furthermore, DAXX-mediated inefficient DNA damage repair could sensitize BRCA-proficient TNBC cells to PARP inhibitors. Additionally, we identified that dual RAD51 and PARP inhibition with RI-1 and ABT888 significantly reduced TNBC growth both *in vitro* and *in vivo*, which provided the first evidence of combining RAD51 and PARP inhibition in BRCA-proficient TNBC. In conclusion, our data support DAXX as a modulator of DNA damage repair and suppressor of TNBC progression to sensitize tumors to the PARP inhibitor by repressing RAD51 functions. These provide an effective strategy for a better application of PARP inhibition in the treatment of TNBC.

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Background

Breast cancer remains a major public health concern affecting women worldwide. Triple-negative breast cancer (TNBC) is defined as estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative tumor. This subtype represents 15% of all breast cancer and is associated with aggressive tumor pathology, and poor clinical prognosis [1]. Although vast improvements have been achieved in treatment, TNBC remains a breast cancer type with limited options for treatment and a median survival of 19 months [2]. Chemotherapy remains the mainstay for treating early and advanced TNBC. Therefore, a better application of therapeutic options for TNBC is urgently needed.

DAXX, a death domain-associated protein, is a multifunctional protein that has been implicated in pro-apoptosis, anti-apoptosis, and transcriptional regulation processes. When it is localized in the

nucleus, DAXX has been shown to directly interact and suppress many transcription factors, including androgen receptor, p53, E2F1, and Pax gene family members, and it is involved in multiple biological functions [3–6]. Also, DAXX can recruit histone deacetylase (HDAC) and transcriptional regulator (ATRX) to the promoter region and subsequently alter gene transcription [7,8]. Besides, an accumulating evidence has demonstrated that dysregulation of Daxx and its associated proteins is critically involved in cancer development and progression in cancer cells [9–12]. However, the role of DAXX in cancer remains controversial and conflicting on whether DAXX functions as a tumor suppressor or an oncogene. For example, DAXX is highly expressed in human ovarian cancer and promotes cell proliferation and chemoresistance in ovarian cancer cells [12]. In prostate cancer, DAXX was reported to have potent growth-enhancing effects on primary prostatic malignancy through repressing expression of essential autophagy modulators DAPK3 and ULK1 [13]. Although DAXX behaves as an oncogene in several cancers, recent data suggest that it functions as a tumor suppressor by repressing several oncogenes in other types of cancer, including pancreatic neuroendocrine tumors (PanNETs), colon cancer, and lung cancer [9,11,14]. In PanNETs, DAXX loss is associated with malignant progression. DAXX plays as a tumor suppressor, and DAXX/H3.3 complex suppresses target genes by promoting H3K9me3 [9]. Furthermore, DAXX

could act as repressor in controlling cell invasion in HIF-1α/HDAC1/Slug-mediated lung cancer. DAXX could bind to the DNA-binding domain of Slug, impeding HDAC1 recruitment and inhibiting Slug E-box binding [11]. DAXX, as a multifunctional protein, plays a different role in various cancer types. Its role in oncogenesis may be tissue and cell-specific. However, the role of DAXX in breast cancer, especially in TNBC, has thus far not been elucidated.

The DNA damage response (DDR) is a collective term for the plethora of different intra- and inter-cellular signaling events and enzyme activities that result from the induction and detection of DNA damage [15]. Although DDR is invoked to maintain genomic stability in normal cells, the aspect of cancer DDR is different from that of normal cells. TNBC, as the most aggressive type of breast cancer, displays an increased genomic instability with inefficient DDR processes. TNBC greatly depends on remaining DDR pathways for cancer cell growth [16]. Recently, accumulating evidence suggests that DAXX has been critically involved in DNA damage response [3,12,17–20]. An interaction between DAXX and tumor suppressor p53 has been observed in overexpression experiments. DAXX enhances the intrinsic E3 activity of Mdm2 towards p53. In response to DNA damage, DAXX dissociates from MdM2 and contributes to p53 activation [17]. DAXX also cooperates with ATR to affect DNA repair synthesis [21]. Based on the critical role of DAXX in DNA damage response, we assessed the role of DAXX in the regulation of DNA damage response in TNBC by investigating its possible involvement to PARP inhibition.

Therefore, in the present study, we used TNBC cell lines to investigate the involvement of DAXX in TNBC cell proliferation, migration, and DNA damage-repair response. We found that DAXX might function as a tumor suppressor to inhibit TNBC proliferation and invasion. Mechanistically, DAXX overexpression could impair the DNA damage-repair process by repressing RAD51, which may be as a barrier for uncontrolled cancer cell growth, thus inducing PARP inhibitor sensitivity in TNBC. These results suggest that DAXX

functions as a critical tumor suppressor during TNBC progression. These support its utility for increasing cellular sensitivity to PARP inhibition.

Methods

Cell Culture and Cell Viability Assay

The MDA-MB-231, MDA-MB-157 cell lines were purchased in 2017–2018 from the Chinese Academy of Science Committees Type Culture Collection Cell Bank (Shanghai, China). The authenticity of these cell lines was done by Chinese Academy of Science Committee Type Culture Collection Cell Bank before purchase by STR DNA typing methodology. MDA-MB-231, MDA-MB-157 cells were grown in RMPI 1640 supplemented with 10% fetal bovine serum (FBS). All culture medium contained 100 units/ml of penicillin and 100 units/ml of streptomycin, and all cell lines were cultured at 37°C and in a 5% CO^2 atmosphere. For cell viability assay, cells were seeded in 96-well plates at 4000 to 6000 cells per well. We treated the cells with different doses of agents and after 72-hour exposure cell survival was measured with the Cell Counting Kit-8 (CCK8) assay from MedChem Express.

Plasmid and Cell Transfection

We transfected the plasmids into cells by using Turbofect Transfection Reagent (ThermoFisher, USA) according to the instructions. The plasmids (pLenti-DAXX, pLenti-siDAXX) and matched controls (plenti-Blank) were purchased from ABM technology (ABM, Canada). We bought the plasmid (pCMV-RAD51) and matched controls from TranSheepBio technology (TranSheepBio; China). Transfected cells were maintained at 37°C/5% CO_2 for 48 h. Cells were then cultured in puromycin (1-5ug/ml) to allow for selection.

Antibodies and Agents

We purchased antibodies against DAXX (25C12), E-cadherin (24E10), N-cadherin (D4R1H), Vimentin (D21H3), Cleaved caspase 3 (D3E9),γH2AX (20E3), β-actin (13E5), and GAPDH (14C10) from Cell Signaling Technologies (Cell signaling; USA). We obtained DAXX antibody (H-7) from Santa Cruz Biotechnology (Santa Cruz; USA) and Anti-RAD51 (ab88572) from Abcam (Abcam; UK). The HRP-conjugated Goat Anti-Rabbit IgG and CL594-conjugated Goat Anti-Rabbit IgG antibody were purchased from Proteintech (Proteintech; USA). ABT888 and RI-1 were obtained from MedChem Express.

Colony Formation Assays

By performing the colony formation assay, cells were seeded at 500 cells per cell in 6-well plates. The following day, cells were treated with different agents at different doses for 24 hours. Cells were further washed with PBS then cultured in fresh medium for 10–14 days. We then stained the cells with 0.5% crystal violet for 30 min. For the colony formation assay that we conducted in 96-well plates, cells were seeded in 96-well plates at a concentration of 3×10^4 cells/ml in 100uL of medium per well and treated with DMSO, ABT888, RI-1, or a combination of ABT888 and RI-1 for another five days. After fixation, we stained the colonies with 0.5% crystal violet for 30 min.

Protein Isolation and Western Blot

Cells after treatment were washed two times with PBS and lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails.

We determined the protein concentrations with the BCA kit (All kits from KeyGEN Biotech, Nanjing, China). Cell extracts containing 30ug of protein were resolved by SDS-PAGE and transferred to PVDF membranes (Millipore Corp., Bedford, MA, USA). Western blot was performed using the primary antibodies, followed by incubation with secondary antibodies conjugated with HRP. Bound antibodies were visualized using an enhanced chemiluminescence detection kit (NEM Biotech, Suzhou, China).

Chromatin Immunoprecipitation (ChIP) q-PCR Assay

The ChIP assay was performed using Magnetic ChIP kit (Thermo Scientific, USA). Briefly, we fixed 70% confluent MDA-MB-231 or MDA-MB-157 cells in 1% formaldehyde for 15 min. Centrifugation for 5 min, and sonicate the lysate to shear the cross-linked DNA to an average length of 100-1000 bp. Remove the insoluble material by centrifugation. The immuno-cleared chromatin was immunoprecipitated with anti-DAXX (Santa Cruz; USA) or control IgG antibody overnight at 4°C. Reverse the cross-links by proteinase K in ChIP Elution buffer for 1.5 h at 65°C. 1 μL of each of the purified DNA was used to determine the concentration. We used each sample as a template for PCR with specific primers. PCR analysis was performed in a 20 μL volume with amplification conditions: 95°C for 5 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; 72°C for 10 min. PCR products were separated on 2% agarose gels, stained with ethidium bromide and photo each.

The primers sequences of genes covering the DAXX binding sites are:

BABAM2 forward primer: 5′-ATGATGCAGAAGGCTTTA CAAAACT-3′;
BABAM2 reverse primer: 5′-CCTCCTGGAAT TTGCTCTTTTGG-3′;
ATM forward primer: 5′-CATCTAATGGTGCTATTTACG GAGC-3′;
ATM reverse primer: 5′-GAACAATTCTAACCACTGT TGCTGA-3′;
BRCA1 forward primer: 5′-TCTGGGTAAAGTTCATTGGAA CAGA-3′;
BRCA1 reverse primer: 5′-AAATATGTGGTCACACTTTGTG GAG-3′;
RPA3 forward primer: 5′-TAATCATGGTGGACATGATG GACTT-3′;
RPA3 reverse primer: 5′-GATTTCTTCATCAAGGGGTTC CATC-3′;
CCND1 forward primer: 5′-CGATTTCATTGAACACTT CCTCTCC-3′;
CCND1 reverse primer: 5′-GAAATGAACTTCACAT CTGTGGCA-3′;
FoxO3 forward primer: 5′-GGCAGCCGAGGAAATGTTCG-3′;
FoxO3 reverse primer: 5′-GTTGTGCCGGATGGAGTTCTTC-3′;
RAD51 forward primer: 5′-TAGAGAAGTGGAGCTAATGG CAATG-3′;
RAD51 reverse primer: 5′-TCTTCCAATTTCTTCACATC GTTGG-3′;
BRIP1 forward primer: 5′-CACTTCATCAACTTGTCAAGAC TCC-3′;
BRIP1 reverse primer: 5′-AATCTGCTGTGTAGTTTCTAA GGGT-3′;
MDC1 forward primer: 5′-CCAGAAAAAGATTTCCCACTA CACC-3′;

MDC1 reverse primer: 5′-GAGGTCTCAGGATTTGAGTAC CATT-3′;
TP53BP1 forward primer: 5′-GTAAAACCTGTAGGGGCAG GAG-3′;
TP53BP1 reverse primer: 5′-TGGGATTCTGTA TACTGCTTGTTGA-3′;
BLM forward primer: 5′-GAGTCTGCGTGCGAGGATTAT-3′;
BLM reverse primer: 5′-CACTGAAGGAAAAGTCTTCGG TAAC-3′;

The sequences of primers 1–10 used for ChIP are listed as follows. Forward (5′-3′): AGGTAGTATCTATAATCACTAAGTT, TGAGGTGCAACAGTTTCATTCCGAA, TTCACACCTGTAATTCCAACACTTT, GGCAGGA AGACTCGCTTGAATCTGG, CACTGCAACCTCCACCTCCCGGGTT, GGCCCATCATAGCTCACTGCAGCCT, CTCTGGCACTTTTCCTCCCTCGCCA, CTAAAGACGAGGTTTCACCACGTTG, CTATCCATCTTCTCGAGCTTCCTCA,TTCCCCC ACCGCCCCCTGAAATCCC; Reverse(5′-3′):AGATCATCAAT TAGATTTCCATAAG,CCATTGCAATGGCCTTATTACTACT, TCAGCCTCCCAAGTAACTGGGATTA,AGCGAGATCACGC CACTGCACTCCA,ACTGCACTCCAGCCTGGGCTACAGA, CAAGTGCCGAAACTGGAAGGTTACA,TAAAAAATA CAAAAATTAGCCAGGT,AAGTGGGAAATGGAGCTAGCG TACG,GACTTAACCGAGTTGCCGTCTTCTG,GTAT CCCCGCCTCCCGGATCCGCCT.

Wound Healing Assay

Cells were grown to confluence in fresh medium supplemented with 10% FBS. Then the medium was changed to FBS-free medium, and the cell monolayers were scraped in a straight line using a p-200 pipette tip to create a "scratch wound". The plates were photographed at 0 and 24 h using a phase contrast inverted microscope.

Transwell Migration Assay

Cell migration was measured by transwell assay (Corning Incorporated, Corning, NY, USA) with 24-well uncoated transwell cell culture chambers. Cancer cells (2×10^4) cultured in serum-free medium (200 μL) were added to the upper chamber. The medium (800 μL) containing 10% FBS was added to the lower chamber. We removed the cells in the upper chamber with a cotton swab after 24 h incubation. Cells on the lower chamber were fixed with 100% methanol for 30 min and then stained with 0.5% crystal violet for 15 min. We observed the migrated cells by inverted microscopy.

Immunofluorescence

Cells were harvested, and fixed in the 4% paraformaldehyde and consequently permeabilized with 0.5% tritonX-100. All Cells were incubated overnight at 4°C with the primary antibodies [anti-RAD51 (Abcam, ab133534) 1/800, or anti-γH2AX (Cell Signaling Technology, 20E3) 1/300]. Secondary Alexa Fluor 594 was used to immunoprecipitate the primary antibody. Finally, Coverslips were mounted with DAPI and visualized with a Zeiss Scope A1 fluorescence microscope. Cells were scored positive for RAD51 and γH2AX foci if more than ten nuclear foci exist. We scored approximately 100 cells.
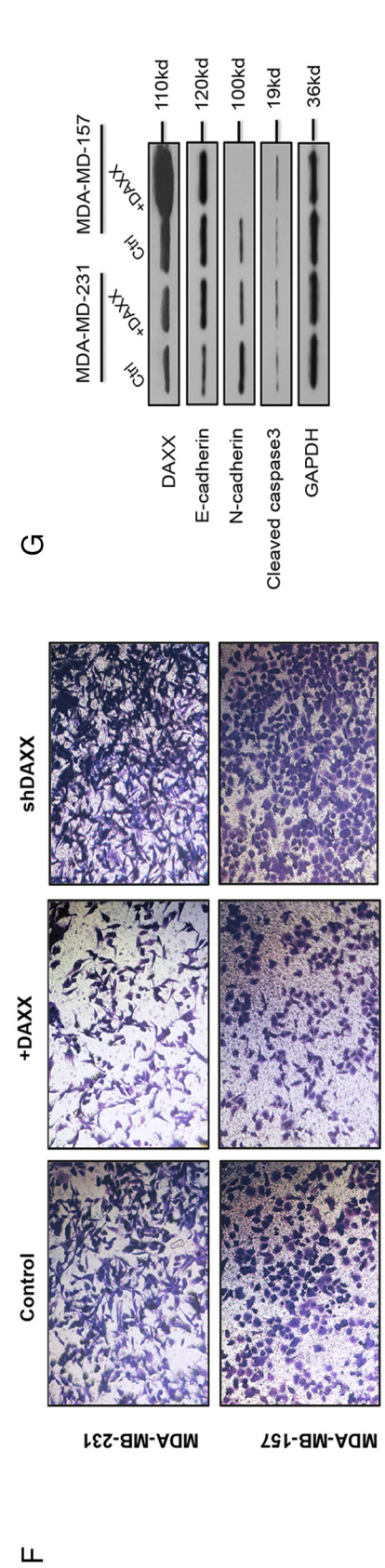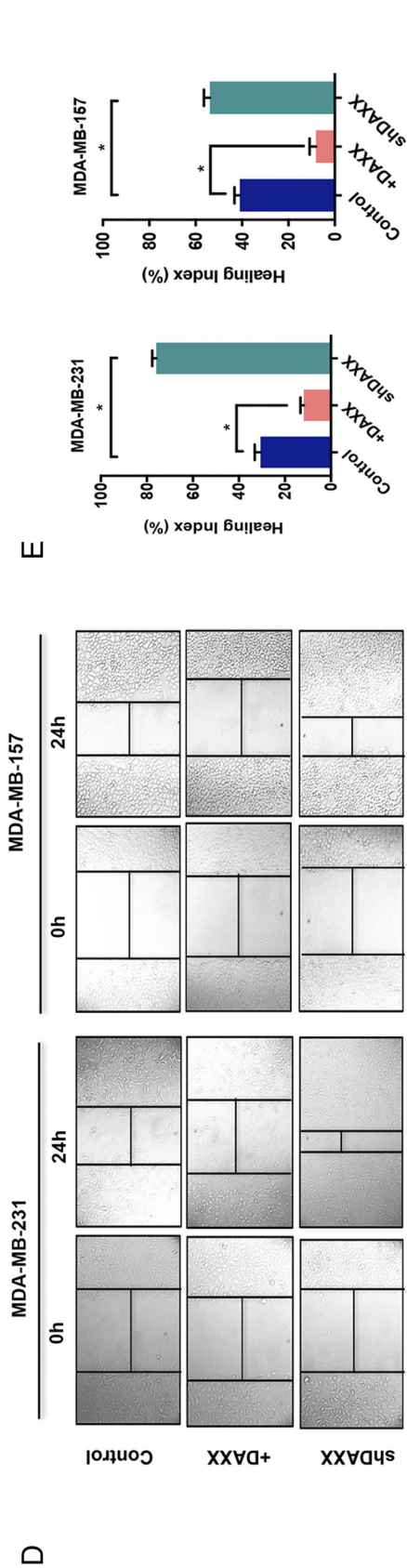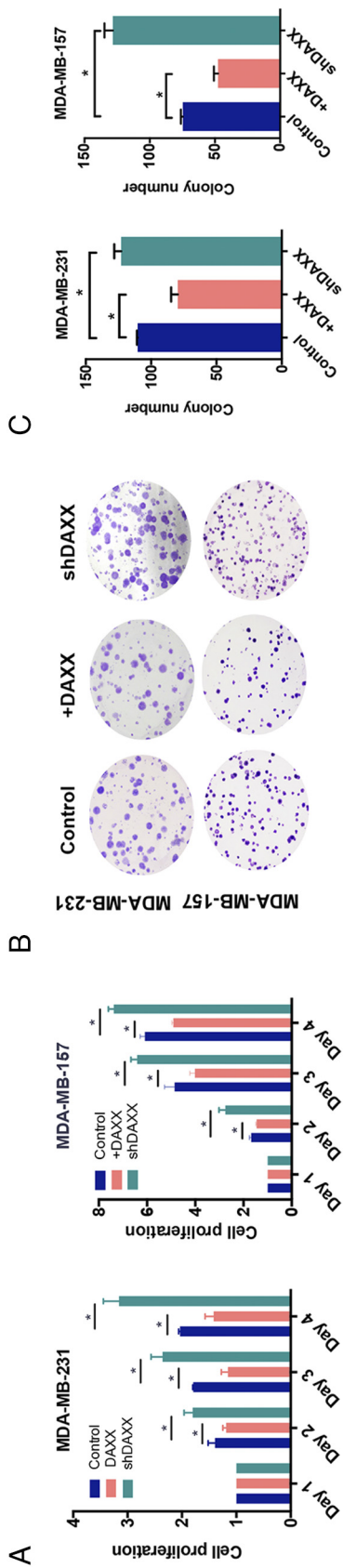
Comet Analysis

The cell suspension was harvested and mixed with 1.2% low melting agarose. We added the mixture over 1% agarose coated fully frosted slides (Thermo-Fischer Scientific). The slides were incubated in lysis buffer overnight at 4°C. The alkaline denaturation was carried out in an electrophoresis chamber for 20 min. Then we run the electrophoresis at 25 V and 300 mA for 20–25 minutes. The slides were stained with PI at dark for 5 minutes. Images were taken with a Zeiss Scope A1 fluorescence microscope. The quantification of tail DNA was measured by CASP software.

Mice and Xenograft Models

We purchased six-week-old female BALB/c mice from the Model Animal Research Center of Nanjing University. All the animal experiments were performed according to the institutional guidelines and approved by the Ethical Review Committee of Comparative Medicine, Jinling Hospital, Nanjing, China. For the effect of DAXX in TNBC tumor growth assay, a total of 5×10^6 MDA-MB-231 and MDA-MB-157 cells, and their derivatives (DAXX overexpression stable cell lines) were injected subcutaneously into nude mice. The tumor volumes were determined every 2 days by measuring the length and width and calculating the tumor volumes with the formula: tumor volume $= 0.5 \times$ length×width^2. After 4 weeks, tumors were removed and weighed. Furthermore, the tumors were used for the immunohistochemical (IHC) staining. For the effect of the combination of RI-1 and ABT888 assay *in vivo*, a total of 5×10^6 MDA-MB-157 cells were injected subcutaneously into nude mice. When the tumor volumes reached $100\text{mm}^3(\pm50)$, mice were randomly grouped and treated by intraperitoneal injection every 3 days for 30 days with RI-1 (50 mg/kg), ABT888 (25 mg/kg), a combination of RI-1 and ABT888, or no treated. Xenografts tumors were harvest 30 days following initial treatments. Tumors were taken 8 hours after the last drug administration for immunofluorescence assay.

Immunohistochemistry

Slides were deparaffinized in a series of xylenes and ethanols. We then fulfilled heat-mediated antigen retrieval with citrate buffer (BioGenex Laboratories, San Ramon, CA). Antibodies used were described in the above sections. Immunostained sections were scanned using a Zeiss microscope (Carl Zeiss, Germany). The staining intensity for DAXX and cleaved caspase3 was scored by a pathologist (B. Yu).

Statistical Analysis

All *in vitro* assays were performed in triplicate. We compared the groups by two-tailed t-tests or analysis of variance using GraphPad Prism statistical programs (GraphPad Prism, San Diego). $P<.05$ was considered significant.

Results

DAXX Inhibits TNBC Cell Proliferation, Migration, and EMT

The dysregulation of DAXX is critically involved in cancer development and progression with a reported controversial role in cancer progression. It functions as either a tumor suppressor or an oncogene in different cancer types. Different cellular contexts and molecular expression profiles may affect the role of DAXX in cancer. However, the role of DAXX in breast cancer, especially in TNBC, remains unknown.

To investigate the role of DAXX in TNBC, we knocked down and overexpressed DAXX by transfecting the plasmids (pLenti-DAXX, pLenti-siDAXX) and matched controls (plenti-Blank) in MDA-MB-231 and MDA-MB-157 cells. We selected the cells by treating with puromycin to develop the stably overexpressing cell lines. The Overexpression of DAXX significantly reduced cell growth in both TNBC cells, and knockdown of DAXX increased the cell viability (Figure 1A). Moreover, DAXX overexpression inhibited colony formation, while silencing DAXX promoted colony formation in both cell lines (Figure 1, B and C). MDA-MB-231 and MDA-MB-157 cells transfected with DAXX-overexpressed plasmid showed significantly decreased cell migration. Conversely, the loss-of-function study in both cell lines revealed that DAXX downregulation accelerated the speed of wound closure (Figure 1, D and E). By performing the transwell assay, we demonstrated that DAXX overexpression could inhibit TNBC cell migration potential, and knockdown of DAXX notably increased the number of migrated cells (Figure 1F). MDA-MB-231 and MDA-MB-157 cells transfected with DAXX-overexpressed plasmids showed an increased expression of the epithelial marker E-cadherin, along with a noticeable decrease in the mesenchymal marker N-cadherin (Figure 1G). Taken together, these data suggest that DAXX is a negative regulator of proliferation, migration and EMT in TNBC cells.

DAXX Inhibits Triple Negative Breast Cancer Tumor Growth In Vivo

To further gain *in vivo* evidence supporting the effect of DAXX on TNBC tumor growth, we next implanted MDA-MB-231 and MDA-MB-157 cells that harbored control and DAXX-overexpressed plasmids subcutaneously into nude mice. As shown in Figure 2, tumors that formed in DAXX-overexpressed group were significantly smaller than those developed in the control group. Besides, tumor growth was slower in the DAXX-overexpressed group, compared with standard control (Figure 2, C and D). Immunohistochemical examination confirmed that higher expression of DAXX was associated with noticeable amounts of apoptotic cells, as shown by immunostaining for cleaved caspase 3 (Figure 2E). Conclusively, these results demonstrated that the overexpression of DAXX could inhibit tumor growth *in vivo* tumor growth of TNBC cells.

Figure 1. DAXX inhibits TNBC cell growth, colony formation, migration and EMT process. A. CCK8 assays for cell proliferation in MDA-MB-231 and MDA-MB-157 cells following the indicated transfections. B. Colony formation assay for the growth in MDA-MB-231 and MDA-MB-157 cells following the indicated transfections. C. Numbers of the colonies were counted, and compared among three groups. D. Wound-healing assay for cell migration ability of MDA-MB-231, MDA-MB-157 cells following the indicated transfections. E. Healing index were measured and compared among groups. F. Transwell experiments for the cell migration capability of MDA-MB-231, MDA-MB-157 cells following the indicated transfections. Cells were allowed to migrate for 24 h. G. Western blotting assay of expression of E-cadherin, N-cadherin, and Cleaved caspase 3 in MDA-MB-231 and MDA-MB-157 cells between the control and DAXX-overexpressing groups. The results are presented as the mean±S.D. *$P<.05$.
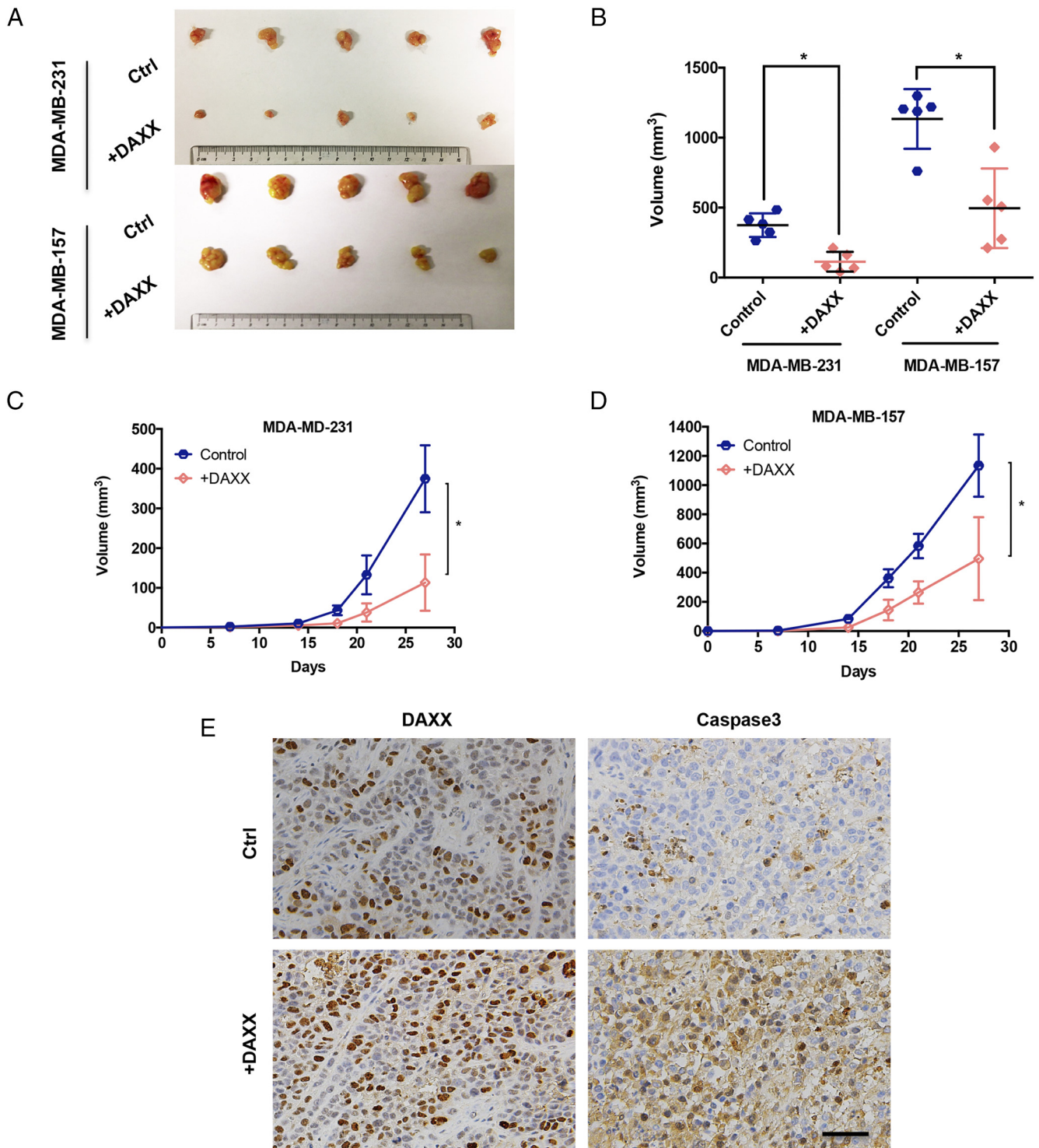
Figure 2. DAXX overexpression reduces TNBC tumor development and growth. MDA-MB-231 and MDA-MB-157 cells, and their derivatives (DAXX overexpression stable cell lines) were injected subcutaneously into nude mice. After 4 weeks, tumors were removed and measured. A and B. Graph depicting the reduction in tumor size observed between control group and DAXX-overexpressing group of MDA-MB-231 and MDA-MB-157 cells. C. Graph depicting the tumor growth curves between two groups in MDA-MB-231 and MDA-MB-157 cells. D. Immunohistochemistry staining for DAXX and cleaved caspase 3 in representative MDA-MB-157-derived tumor tissues. Scale bar, 50 μm. *P<.05.

RAD51 is Responsible for DAXX-Mediated Tumor Suppressive Role In Vitro

Accumulated evidence indicates that DAXX has been significantly implicated in DNA damage response [3,12,17–20]. An interaction between DAXX and the tumor suppressor p53 exists in overexpression experiments. DAXX enhances the intrinsic E3 activity of MDM2 towards p53. In response to DNA damage, DAXX dissociates from MDM2, which contributes to p53 activation [17].
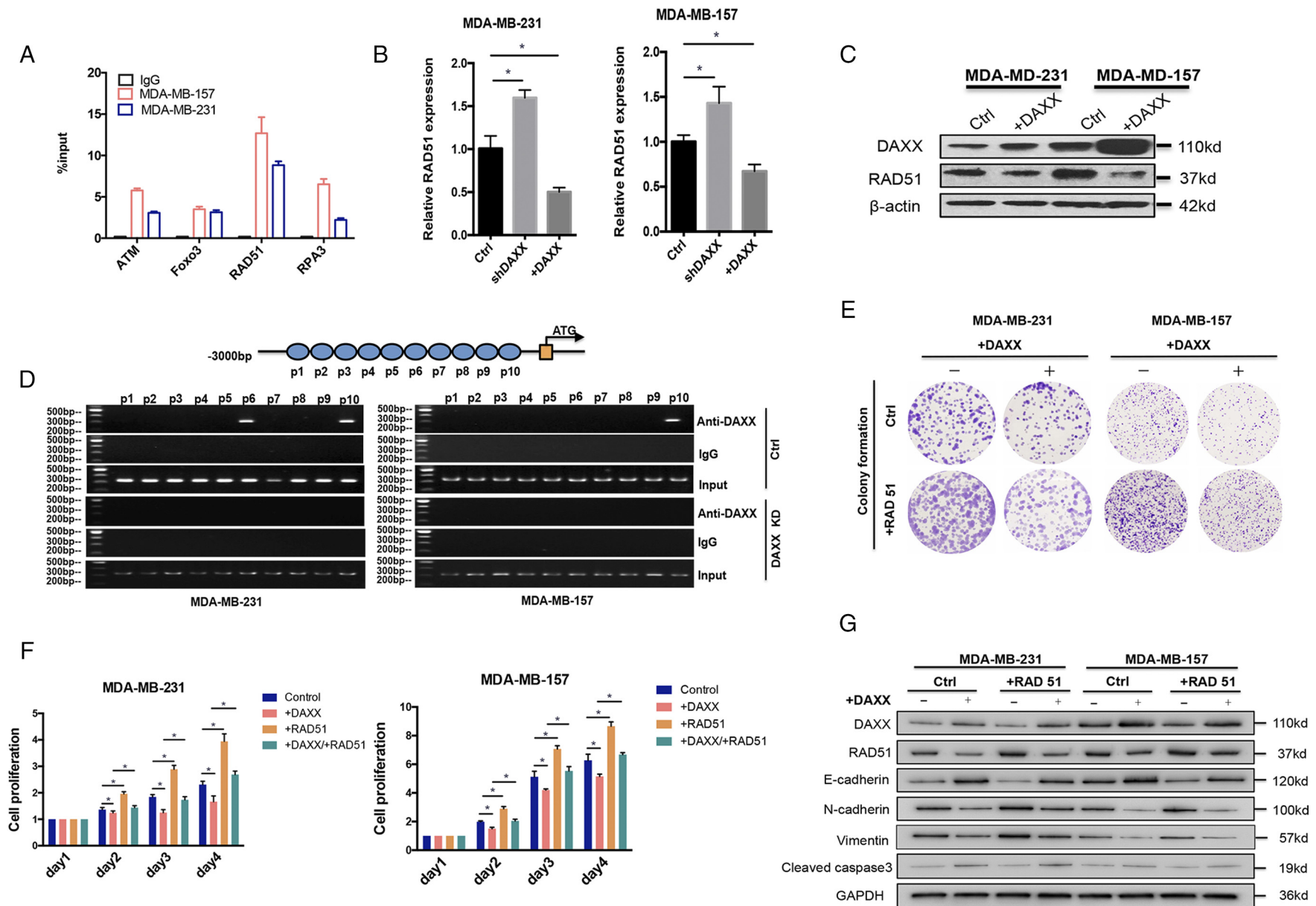
Figure 3. RAD51 is responsible for DAXX-mediated tumor suppressive role in TNBC cells. A. ChIP-qPCR analysis of DAXX on *ATM*, *Foxo3*, *RAD51*, and *RPA3* promoter regions in MDA-MB-231 and MDA-MB-157 cells. B. qRT-PCR analysis of RAD51 expression in MDA-MB-231 and MDA-MB-157 cells following the indicated transfections. C. Western blotting assay of DAXX, and RAD51 expression in MDA-MB-231 and MDA-MB-157 cells between the control and DAXX-overexpressed groups. D. Upper: The primer pair locations with the *RAD51* promoter in MDA-MB-231 and MDA-MB-157 cells. Positive and negative controls are indicated as input and IgG, respectively. E. CCK8 assays for cell proliferation in MDA-MB-231 and MDA-MB-157 cells following the indicated transfections. F. Colony formation assay for cell growth in MDA-MB-231 and MDA-MB-157 cells following the indicated transfections. G. Western blotting assay of expression of DAXX, RAD51, E-cadherin, N-cadherin, Vimentin, and Cleaved caspase 3 in MDA-MB-231 and MDA-MB-157 cells following the indicated transfections.
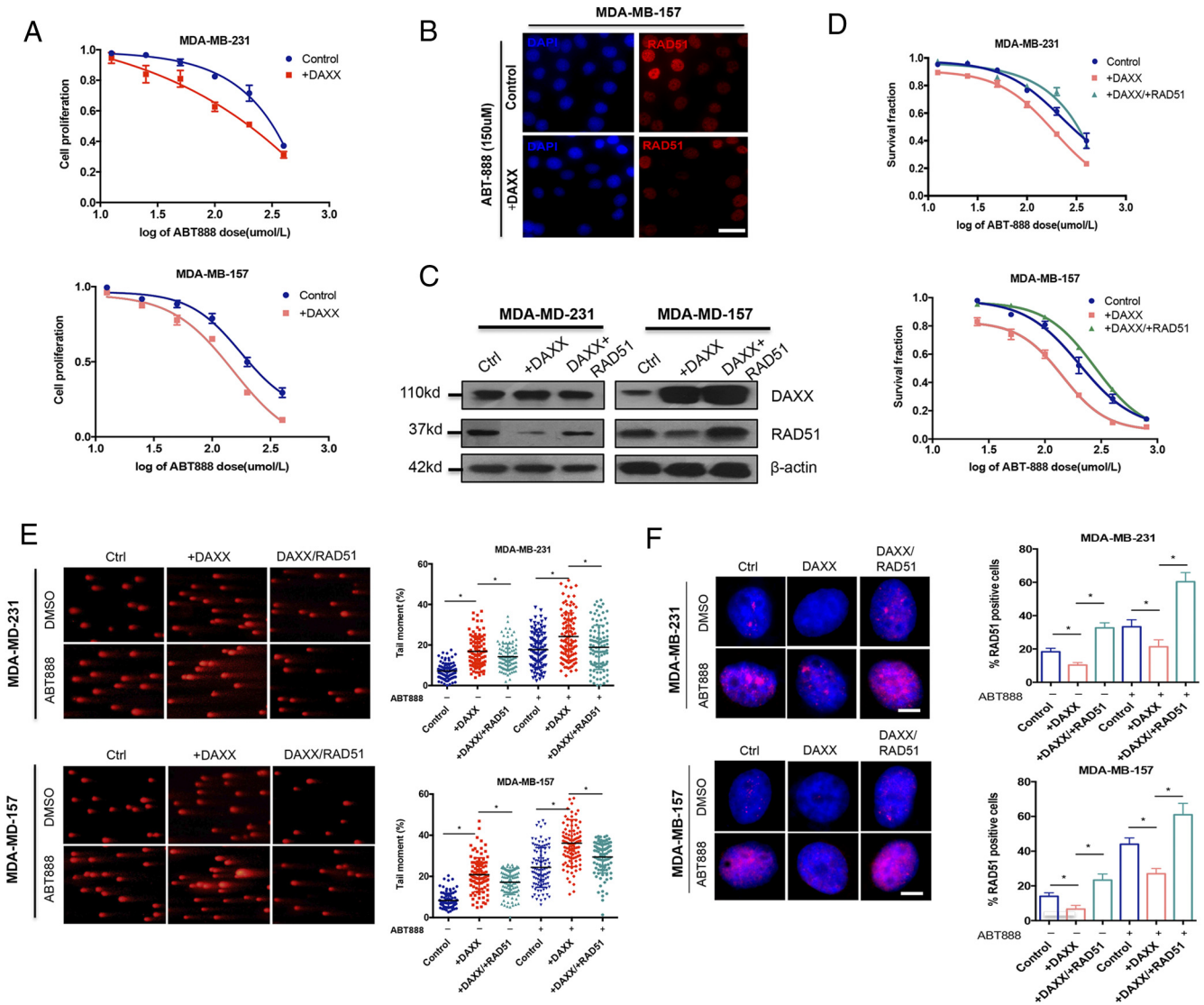
Figure 4. Downregulation of RAD51 is critical for DAXX-mediated PARP inhibitor sensitivity in TNBC. A. MDA-MB-231, MDA-MB-157 cells following the indicated transfections were treated with different concentrations of ABT888 for 72 hours and subjected to CCK8 assays. B. RAD51 expression was detected by immunofluorescent staining in MDA-MB-157 cells following the indicated transfections, treated with or without 150 μM ABT888. *Scale bar,* 20 μm. C. Western blotting assay of expression of DAXX, and RAD51 in MDA-MB-231 and MDA-MB-157 cells following the indicated transfections. D. MDA-MB-231, MDA-MB-157 cells following the indicated transfections were seeded for ABT888 sensitivity assays. Cells were treated for 72 hours. E. The extent of DNA damage treated with DMSO or 150 μM ABT888 measured by comet assays in MDA-MB-231, MDA-MB-157 cells following the indicated transfections. The extent of DNA damage was quantified by the tail moments in comet assay. Bars represent mean values of the tail moment. F. The representative images and quantification of RAD51 immunostaining in MDA-MB-231, MDA-MB-157 cells following the indicated transfections, and treated with DMSO or 150 μM ABT888. *Scale bar,* 5 μm. *$P<.05$.

DAXX could be phosphorylated by ATM rapidly during the DNA damage response and precede p53 activation. Besides, recent studies revealed that DAXX cooperates with ATRX to affect DNA repair synthesis [21]. Given the critical role of DAXX in DNA damage-response signaling, we investigated if genes involved in DNA damage response signaling could participate in DAXX-mediated tumor suppressive effect in TNBC.

By using an online omics tool, we further analyzed of DAXX ChIP-seq data from the ArrayExpress database (Accession: E-GEOD-68656). We enriched the genes anticipating in DNA damage-response signaling. Based on the literature and molecular functions,

genes that have the most significant impact on DNA damage response function were focused. These genes included *BABAM2, ATM, BRCA1, RPA3, CCND1, Foxo3, RAD51, BRIP1, MDC1, TP53BP1* and *BLM.* We further validated the data in TNBC cells. From the ChIP assay in MDA-MB-231 and MDA-MB-157 cells, we observed that DAXX could bind at the promoter region of several DNA damage response-related genes, including *RAD51, ATM, Foxo3,* and *RPA3* (Figure 3A). Since RAD51 functions as a critical DNA recombinase that drives the HR process [22], we focused on the interaction of DAXX with RAD51. To better determine if DAXX binds to the promoter of *RAD51,* we designed ten primers for
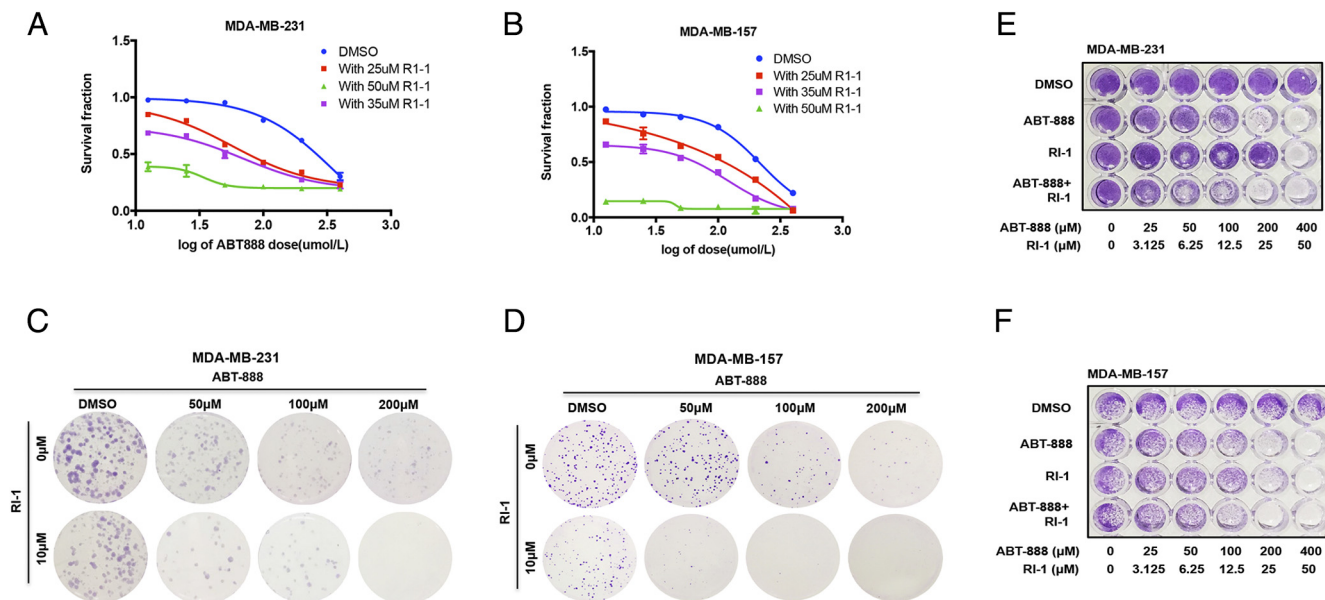
Figure 5. *In vitro* experiments of combined RAD51 and PARP suppression in MDA-MB-231 and MDA-MB-157 cells. A-B. Proliferation curve of MDA-MB-231 (A) and MDA-MB-157 (B) cells treated with different concentrations of ABT888 for 72 hours in combination with DMSO or a constant dose of 25-50 μM RI-1. C-D. 6-well colony formation assays of MDA-MB-231 (C) and MDA-MB-157 (D) Respective images of 6-well colony-formation results in MDA-MB-231 and MDA-MB-157 cells treated with different concentrations of ABT888 in combination with DMSO or 10 μM RI-1. (E-F) Respective images of 96-well colony-formation results in MDA-MB-231 (E) and MDA-MB-157 (F) cells treated with different concentrations of ABT888 and RI-1 (either single or both).

RAD51 promoter in ChIP assay. The gel electrophoresis of ChIP assay showed two binding sites located in primer 6 and 10 in MDA-MB-231 cells, and a binding site found in primer 10 in MDA-MB-157 cells. After knockdown of RAD51 level, no binding site was detected (Figure 3D). Through qRT-PCR and western blotting analysis, RAD51 mRNA and protein levels were found to be significantly decreased when DAXX was overexpressed in both MDA-MB-231 and MDA-MB-157 cells (Figure 3, *B* and *C*). Collectively, these results suggest that DAXX suppresses RAD51 expression through direct binding to the *RAD51* promoter.

Moreover, we performed functional experiments to determine whether DAXX inhibited tumor progression in a RAD51-dependent manner. The plasmid (pCMV-RAD51) and matched controls were transfected into MDA-MB-231 and MDA-MB-157 cells that stably ectopically expressed DAXX or the control. Notably, restoration of RAD51 rescued the inhibitory effect of cell proliferation and colony formation in DAXX-overexpressed cells (Figure 3, *E* and *F*). Moreover, DAXX overexpression failed to suppress the EMT process when RAD51 was ectopically expressed in MDA-MB-231 and MDA-MB-157 cells (Figure 3G). Conclusively, our findings indicate that RAD51 is responsible for DAXX-mediated tumor suppressive role in TNBC cells.

DAXX Increases PARP Inhibitor Sensitivity in BRCA-Proficient TNBC

Inefficient DNA repair mediated by DAXX overexpression suggests the contribution of DAXX to tumor suppression, which also indicates that DAXX-overexpressed cells with HR deficiency might increase sensitivity to PARP inhibition. To address this, we increased DAXX expression in two BRCA-proficient TNBC cell lines, MDA-MB-231, and MDA-MB-157 cells to test for PARP inhibition-induced cell death. Analysis of cell survival after treatment

with ABT888 (veliparib), a potent inhibitor of PARP1 and PARP2, revealed that cells with DAXX overexpression were more sensitive to PARP inhibition compared with standard control (Figure 4A). Furthermore, we treated MDA-MB-157 cells with ABT888 for 24 hours. Decreased expression of RAD51, a critical factor that mediates the HR process, was detected in cells transfected with DAXX plasmid compared with standard control (Figure 4B). These results strongly indicate that DAXX overexpression could increase cellular response to the PARP inhibitor in BRCA-proficient TNBC cells.

Down-regulation of RAD51 is Critical for DAXX-Mediated PARP Inhibitor Sensitivity in TNBC

We further investigated whether the reduction of RAD51 level mediated by DAXX is vital for cellular sensitivity to ABT888 in BRCA-proficient TNBC cells. We transfected plasmids with control or pCMV-RAD51 into MDA-MB-231 and MDA-MB-157 cells that stably expressed DAXX (Figure 4C). The restoration of RAD51 in DAXX-overexpressed cells inhibited DAXX-mediated sensitization to the PARP inhibitor (Figure 4D), suggesting that DAXX-mediated ABT888 sensitivity is primarily a consequence of RAD51 downregulation. We further measured the amount of DNA damage induced by ABT888 in DAXX-overexpressed cells using a comet assay. The extent of DNA damage increased in DAXX-overexpressed group compared with the control group. However, DAXX overexpression failed to amplify DNA damage levels when we ectopically expressed RAD51 in both cell lines (Figure 4E). Furthermore, we detected HR ability by measuring RAD51 foci formation in both MDA-MB-231 and MDA-MB-157 cells. Notably, the restoration of RAD51 rescued the HR ability in DAXX-overexpressed MDA-MB-231 and MDA-MB-157 cells (Figure 4F). Taken together, these findings support the notion that RAD51 is essential for DAXX-mediated PARP inhibitor sensitivity.
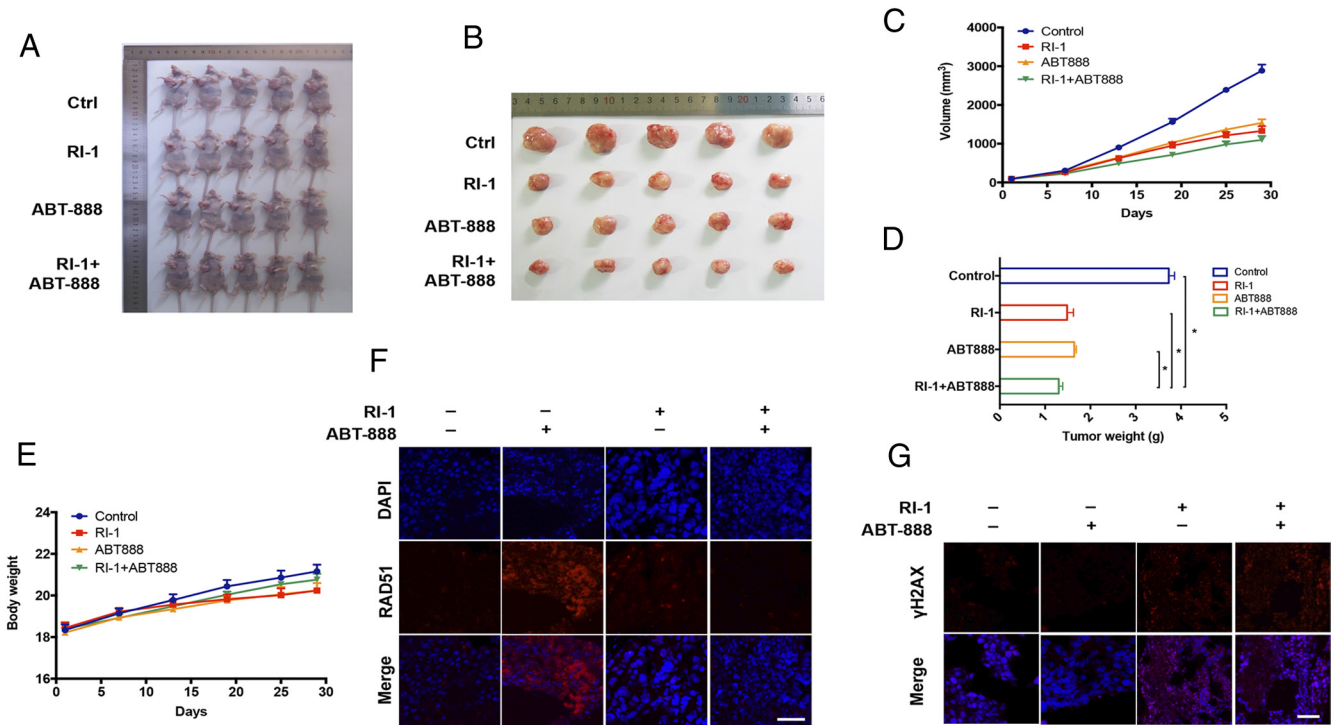
Figure 6. *In vivo* efficacy of combined RAD51 and PARP suppression. Mice bearing MDA-MB-157 xenografts with a tumor volume of 100mm^3($\pm$50), was then randomly grouped and treated by intraperitoneal injection every 3 days for 30 days with RI-1 (50 mg/kg), ABT888 (25 mg/kg), a combination of RI-1 and ABT888, or no treated. Tumors were taken 8 hours after last drug administration for immunofluorescence assay. A. Photo images of tumors in four groups, including groups of no treatment, treated with RI-1, ABT888, or the combination. B. Tumors derived from four groups, after 30 days of initial treatment. C. Growth curves of tumors, tumor volumes were measured at every 2 days by measuring the length and width. D. Tumor weight of tumors, after 30 days of initial treatment. E. Body weight data for MDA-MB-157 xenograft mouse. F-G. immunofluorescence assay was utilized to analyze RAD51 (F) and γH2AX (G) in these four groups. *Scale bar,* 50 μm. *P<.05.

Combined RAD51 and PARP Suppression In Vitro

An essential finding of this study is the elucidation of RAD51 involved in DAXX-modulated DNA repair. DAXX could sensitize TNBC cells to PARP inhibitors through directly repressing RAD51 function. Therefore, enabling to restore RAD51 to lower level may increase the anti-cancer activity of PARP inhibitors. This approach may provide the potential to extend well-tolerated PARP inhibition for the treatment for BRCA-proficient TNBC. TNBC patients have the highest rates of RAD51 overexpression (approximately 33% of cases) [23,24]. Also, small molecular inhibitors now constitute the main component of the pipelines of oncology drug development. Thus we aimed to determine if small molecule inhibitor of RAD51 (RI-1) and ABT888 could create synergistic responses in the TNBC cells.

To evaluate whether HR impairment following RAD51 inhibition conferred an increased sensitivity to PARP inhibition, we tested the *in vitro* activity of the combination of RI-1 and ABT888 in TNBC cells. The RI-1 sensitized cells to ABT888 in a dose-dependent manner (Figure 5, A and B). To further characterize the long-term effects of the combination, we investigated the impact of RI-1 and ABT888 in both 6-well and 96-well colony-formation assays (Figure 5, C–F). The combination of ABT888 and RI-1 was superior to a single agent in inhibiting colony formation in both MDA-MB-231 and MDA-MB-157 cells.

Combination of RI-1 and ABT888 on TNBC in Mouse Xenograft Models

Next, we tested the effect of combined RI-1 and ABT888 in xenograft tumors. In an MDA-MB-157 breast cancer model, mice were randomly assigned to one of four groups to receive vehicle, ABT888, RI-1, or combination of ABT888 and RI-1. The combination of ABT888 and RI-1 resulted in significant inhibition of tumor growth (Figure 6, A and B). Compared with single-agent treatment, the combination of ABT888 and RI-1 showed a slower growth and a decreased tumor weight of tumors (Figure 6, C and D). All treatment protocols were well tolerated. The single drug or combination treatments did not cause body weight loss significantly (Figure 6E). Furthermore, we detected a decrease of RAD51 expression in the combination group (Figure 6F), which suggest that impaired HR ability may occur in the combination group compared with single treatment group. Besides, the expression of γH2AX increased in the combination group (Figure 6G).

Discussion

TNBC makes up about 15% of all breast cancer. The lack of hormone receptors and human epidermal growth factor receptor-2/ Neu expression is highly associated with poor outcome [1,22]. Understanding the underlying molecular factors in TNBC is

essential. DAXX has been found to act as either a tumor suppressor or an oncogene in different cancer types. However, recent studies demonstrated that DAXX could serve as a tumor suppressor by inhibiting several oncogenes in many cancer types, including cancers of the lung, colon, and pancreas. However, no study has investigated the role of DAXX in breast cancer, especially in TNBC. Our study provides new evidence that, in TNBC, DAXX continues to function as a tumor suppressor. DAXX has a significant inhibitory effect on TNBC cell proliferation and cell migration. Besides, from the *in vivo* observation, there was a remarkable reduction of tumor formation in DAXX-overexpressed groups. Our *in vivo* results with TNBC cells are consistent with the recently reported tumor suppressor function of DAXX in PanNETs. Our research revealed the suppression of DNA repair function by DAXX as a mechanism for its role. ChIP analysis determined that DAXX could be bound to *RAD51* promoter in MDA-MB-231 and MDA-MB-157 cells. Protein and RNA analyses revealed the RAD51 gene transcription was suppressed by DAXX binding.

Among many functions, DAXX can act as an H3.3-specific histone chaperone and facilitates H3.3 deposition when cooperates with ATRX. DAXX/ATRX/H3.3 complex was reported to induce transcriptional repression through H3.3/H3K9me3 pathway, resulting in up-regulation of DAXX target genes [9]. Moreover, DAXX has been identified to bind to the c-met promoter and repress its transcription *via* HDAC2 recruitment [5]. In macrophages, DAXX could interact with HDAC1 and bind to the promoter of IL-6 to repress its transcription through HDAC1-mediated histone deacetylation [23]. Currently, we identified that DAXX was bound to the RAD51 promoter. However, our further work is required to determine the exact mechanistic details of repression, whether the precise mechanism involves DAXX/ATRX/H3.3 complex or HDAC recruitment. Overall, our current study serves as proof of concept unraveling the role of DAXX in TNBC tumorigenesis and its negative regulation of DNA damage repair.

The genetic spectrum of TNBC, as a type of aggressive breast cancer, shows a high level of genetic defects, which is distinct from other types of breast cancer. To keep the consistent proliferation, cancer cells that harbor genetic defects display a higher dependency on a particular DDR target or pathway for survival [16,24]. In the present study, we identified that DAXX could directly bind to the promoter region of *RAD51* and impair DNA damage repair. To this extent, DAXX-mediated HR defects directly break the protection mechanism of tumor cells that greatly depend on remaining DDR pathways for survival. This mediated HR defects could potent the cellular response to PARP inhibition. As a single agent, the PARP inhibitor has a minimal impact on growth and apoptosis in BRCA proficient breast cancer. However, as shown in our present study, DAXX, which could downregulate RAD51, increased the effect of PARP inhibitors in BRCA-proficient TNBC cells, including MDA-MB-231 and MDA-MB-157 cells.

More importantly, the treatment combination of both RAD51 and PARP inhibitors was effective in BRCA-proficient TNBC cells. *In vivo,* using an MDA-MB-231 xenograft mouse model, we found that the combination of both inhibitors decreased the tumor growth in combinatorial arm compared with single arm treated with ABT888. Therefore, targeting of RAD51 in combination with PARP inhibition represents a mechanism by which to produce synthetic lethality in tumors with BRCA-proficient. Ashworth's group firstly identified the utility of downregulating RAD51 combined with PARPi in HR proficient tumors [25]. Impressively, siRNA knockdown of RAD51 in combination with PARPi (KU0058948) achieved a 1000-fold decrease in the viability of Hela cells. This therapeutic effect is comparable to what can be achieved

by single PARPi in BRCA-deficient tumors. These previous results provide a rationale for further investigation of RAD51 inhibition combined with PARPi in treating BRCA-proficient tumors, especially for breast cancer. Targeting RAD51 is clinically relevant, but some concerns exist. RAD51 performs an essential function in mammalian cells and loss of RAD51 results in an early embryonic lethal effect [26]. Due to the dangers of genomic instability induced by aberrant chromosomal recombination, RAD51 expression is tightly controlled in normal tissues [27]. RAD51 expression is tumor-specific, and the majority of cancer cells, including prostate, breast, and lung cancer cells, overexpress RAD51 [28,29]. Cancer cells with the overexpression of RAD51 have a selective advantage due to RAD51's effect to support rapidly dividing cancer cells and eliminate DNA replicate stress [30]. Therapies targeting RAD51, including RAD51 promoter-targeted gene therapies, RAD51 inhibitor B02, or RI-1 have been investigated with no obvious toxicity observed in mice, nor any detectable morphological change in kidneys and livers [30,31]. Taken together, our data suggest that targeting RAD51 may be well tolerated as a single agent or be used to sensitize cancer cells to PARP inhibition.

Conclusions

In summary, we found, for the first time to the best of our knowledge, that DAXX directly regulates RAD51 expression to impact the DNA damage-repair response in TNBC cells. Additionally, DAXX-mediated DNA damage repair defects could enhance the therapeutic effect of PARPi in BRCA-proficient TNBC cells. Dual inhibition of RAD51 and PARP exerts a greater therapeutic potential in TNBC cells bearing positive BRCA, which provides an effective strategy for better application of PARP inhibition to the treatment of TNBC.

Ethics Approval and Consent to Participate

All the animal experiments were performed according to the institutional guidelines and approved by the Ethical Review Committee of Comparative Medicine, Jinling Hospital, Nanjing, China.

Consent for Publication

Not applicable.

Availability of Data and Materials

Please contact author for data requests.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contribution

X.G. designed the study; YS. wrote the main manuscript text; Y.S. and J.J. conducted most experiments; X.W. and W.J. Critical revision of the article for important intellectual content; All authors reviewed the manuscript.

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