BRIT1 regulates p53 stability and functions as a tumor suppressor in breast cancer

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In humans, the gene encoding the BRCA1 C terminus-repeat inhibitor of human telomerase expression 1 (BRIT1) protein is located on chromosome 8p23.1, a region implicated in the development of several malignancies, including breast cancer. Previous studies by our group and others suggested that BRIT1 might function as a novel tumor suppressor. Thus, identifying the molecular mechanisms that underlie BRIT1's tumor suppressive function is important to understand cancer etiology and to identify effective therapeutic strategies for BRIT1-deficient tumors. We thus investigated the role of BRIT1 as a tumor suppressor in breast cancer by using genetic approaches. We discovered that BRIT1 functions as a post-transcriptional regulator of p53 expression. BRIT1 regulates p53 protein stability through blocking murine double minute 2-mediated p53 ubiquitination. To fully demonstrate the role of BRIT1 as a tumor suppressor, we depleted BRIT1 in normal breast epithelial cells. We found that knockdown of BRIT1 caused the oncogenic transformation of normal mammary epithelial cells. Furthermore, ectopic expression of BRIT1 effectively suppressed breast cancer cell proliferation and colony formation *in vitro* and tumor growth *in vivo*. Taken together, our study provides new insights into the biological functions of BRIT1 as a tumor suppressor in human breast cancer.

Introduction

Breast cancer 1 (BRCA1) C terminus-repeat inhibitor of human telomerase expression 1 (BRIT1) is a gene initially identified by a functional genomic screen as a novel repressor of human telomerase and later matched to a putative disease gene called microcephalin 1 (MCPH1) (1,2). Our previous studies further showed that expression of BRIT1 is indeed decreased in several types of human cancers. We found significant decreases in BRIT1 gene copy number in 72% of 54 breast cell lines and 40% of 87 advanced epithelial ovarian cancers. In one of the 10 cancer specimens studied, we also identified a BRIT1

gene deletion that led to the loss of BRIT1 function in DNA damage response (DDR) (3). More recently, a study of breast cancer samples from 319 patients showed that BRIT1 protein expression was reduced in 29% of breast cancers, particularly in higher grade tumors (4). Another study conducted in epithelial ovarian cancers found that low expression of BRIT1 protein correlated with low patient survival (5). Collectively, our studies and studies from other groups suggest that BRIT1 may function as a novel tumor suppressor. Thus, identifying the molecular mechanisms that underlie BRIT1's tumor suppressive function is important not only to understand cancer etiology but also to identify effective therapeutic strategies for BRIT1-deficient tumors.

To understand its biological function, we analyzed the protein structure of BRIT1 and found that human BRIT1 protein contains three BRCA1 C terminus (BRCT) domains: one in its N terminus and two in its C terminus (6–10). BRCT domains are conserved in many important molecules involved in DDR and tumor suppression, such as BRCA1, mediator of DNA damage checkpoint protein 1 (MDC1) and Nijmegen breakage syndrome 1 (11). DDR is an evolutionarily conserved pathway in cells to detect, signal and repair damaged DNA and thereby to assure the integrity of their genome. Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) are two central kinases in the DDR pathway that can phosphorylate and activate a variety of downstream proteins for cell cycle arrest, DNA repair or apoptosis if the damage is irreparable (12–15). It has been well established that an intact and effective DDR is essential for the maintenance of genomic stability and that the DDR acts as a critical barrier to suppress the development of cancer in humans (16). As BRIT1 contains multiple BRCT domains, predominant in DDR proteins, we and other research groups explored the function of BRIT1 in DDR and found that BRIT1 functions as a key regulator of the DNA damage signalling pathway (3,6,8). Depletion of BRIT1 inhibits the recruitment of phosphorylated ATM to double-stranded DNA break ends and subsequently impairs the phosphorylation and recruitment of many downstream molecules of the ATM pathway, including p53-binding protein 1, Nijmegen breakage syndrome 1, BRCA1 and BRCA2, to the DNA damage sites (3). Moreover, BRIT1 plays multiple roles in cell cycle control, chromosome structure modification and homologous recombination-mediated-DNA repair (7,9,10,17–23). It is thus reasonable to propose that BRIT1 deficiency would lead to genomic instability, which could in turn contribute to malignant transformation and tumorigenesis.

Therefore, in this study, we investigated the role and mechanism of action of BRIT1 as a tumor suppressor in breast cancer. Surprisingly, we found that BRIT1 interacts with a master tumor suppressor p53 and is involved in controlling p53 protein stability by regulating the murine double minute 2 (MDM2)-mediated ubiquitination process. Consistent with reduced p53 levels in BRIT1-depleted cells, we found that BRIT1 deficiency promotes cell proliferation, anchorage-independent growth and survival ability in normal breast epithelial cells (MCF10A). In addition, BRIT1 overexpression represses proliferation and survival ability of MCF7 human breast cancer cells, as demonstrated by both *in vitro* cell culture studies and *in vivo* xenograft tumorigenic analyses. Together, our study reveals BRIT1 as a previously unknown regulator of p53 and identifies a new mechanism underlying the tumor suppression function of BRIT1 in human breast cancer.

Materials and methods

Cell culture

Osteosarcoma (U-2OS) cells and breast cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The U-2OS cells were maintained in McCoy's 5A medium (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS). MCF10A cells were maintained in mammary epithelial cell growth medium containing insulin, hydrocortisone,

Abbreviations: ATM, ataxia telangiectasia mutated; BRCA1, breast cancer 1; BRCT, BRCA1 C terminus; BRIT1, BRCA1 C terminus-repeat inhibitor of human telomerase expression 1; CHK2, checkpoint kinase 2; CHX, cycloheximide; DDR, DNA damage response; FBS, fetal bovine serum; MDM2, murine double minute 2; MTT, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide; shRNA, small hairpin RNA; siRNA, small interfering RNA; TBST, Tris-buffered saline–0.1% Tween-20; UV, ultraviolet.

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epidermal growth factor and bovine pituitary extract (Lonza, Basel, Switzerland) supplemented with 5% horse serum. MCF7 cells were maintained in Dulbecco's modified Eagle's medium (Cellgro) supplemented with 15% FBS. Cells were incubated at 37°C in a humidified incubator with 5% CO₂.

Plasmids and cell culture transfection

The p3×FLAG-CMV vector encoding full-length BRIT1 was previously generated in our lab. The deletions of BRIT1 were generated from FLAG-BRIT1 plasmids via PCR using primers with restriction sites and subcloned into the N-terminal p3×FLAG-CMV plasmids in frame. MDM2 wild-type and deletion plasmids Δ9, Δ58–89, Δ212–296 and Δ295–417 were kindly provided by Dr Karen Vousden (The Beatson Institute for Cancer Research). The identity of the plasmids was confirmed by sequencing at The University of Texas MD Anderson Cancer Center DNA Core Sequencing Facility. Cell culture transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), FuGENE 6 (Roche) and Oligofectamine (Invitrogen) following the manufacturers' protocols.

RNA interference

BRIT1 small interfering RNA (siRNA) sequence, control siRNA and the procedures for BRIT1 transient knockdown were all described previously (3). On-target smart pool siRNA against p53 and non-target control siRNA were purchased from Dharmacon Research (Thermo Fisher Scientific, Lafayette, CO). BRIT1 stable knockdown was achieved by RNA interference using a lentiviral vector-based MISSION small hairpin RNA (shRNA) (Sigma-Aldrich, St Louis, MO). Lentiviral particles corresponding to the MISSION shRNA BRIT1-target set and the MISSION non-target shRNA control were used. Specificity and efficacy of the shRNA BRIT1 procedure were controlled by western blotting after transduction and puromycin selection in MCF10A cells.

Antibodies and reagents

Rabbit anti-BRIT1 antibody was generated as described previously (3). Anti-FLAG M2 affinity gel, anti-FLAG M2 and anti-β-actin were purchased from Sigma-Aldrich. Anti-p53 (DO-1), anti-MDM2 (SMP14) and anti-p53-HRP (FL-393) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Geneticin (G418) was purchased from Roche Applied Science. Puromycin was purchased from Sigma-Aldrich. Cycloheximide (CHX) was obtained from Sigma-Aldrich and used at a concentration of 10 µg/ml. MG132 (carbobenzoxycarbonyl-L-leucyl-L-leucyl-L-leucine) was obtained from EMD Biosciences (Billerica, MA) and used at a concentration of 10 µM.

Immunoblotting and immunoprecipitation

Cells were washed in phosphate-buffered saline, and whole cellular extracts were prepared with urea buffer (8 M urea, 50 mM Tris-HCl pH 7.4 and 150 mM 2-mercaptoethanol) or modified radioimmunoprecipitation assay buffer for 30 min on ice. Lysates were cleared by centrifugation and proteins were separated by gel electrophoresis. Membranes were blocked in Tris-buffered saline–0.1% Tween-20 (TBST) with 5% (w/v) non-fat, dry milk for 1 h at room temperature. Membranes were then incubated with primary antibodies diluted in phosphate-buffered saline and 5% bovine serum albumin for 2 h at room temperature. Subsequently, membranes were washed with TBST and incubated with horseradish peroxidase secondary antibody (1:5000) (Sigma-Aldrich) diluted in TBST with 5% non-fat, dry milk. Membranes were washed in TBST and bound antibody was detected by enhanced chemiluminescence (GE Healthcare, Pittsburgh, PA).

U-2OS cells were transiently cotransfected with FLAG-BRIT1 wild-type plasmids and MDM2 wild-type/deletions plasmids or with MDM2 wild-type plasmids and FLAG-BRIT1 wild-type/deletions plasmids. Cellular proteins were extracted in radioimmunoprecipitation assay buffer and immunoprecipitated with anti-FLAG M2 affinity gel (Sigma-Aldrich) overnight. Bead-bound immunocomplexes were eluted with 3×FLAG peptide (Sigma-Aldrich) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For reciprocal immunoprecipitation, the whole cellular extracts were prepared in RIPA buffer and were subjected to incubation with antibody for 2 h followed by incubation with Protein A/G PLUS Agarose beads (Santa Cruz Biotechnology) overnight at 4°C. The precipitates were washed three times with RIPA buffer, eluted in 3× loading buffer by boiling at 95°C for 5 min and resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting.

In vitro proliferation assay, colony-forming assay and soft agar assay

To measure cell proliferation, 500–1000 cells were plated in 96-well plates and 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) substrate (2 mg/ml) (Sigma-Aldrich) was added to the culture medium. Four hours later, the optical density was measured spectrophotometrically at 570 nm. For the colony-forming assay, 250–500 cells were

seeded in 6-well plates and 0.001% crystal violet was used to stain the colonies 10–14 days later. The forming colonies were counted and analyzed. For the soft agar assay, cells were suspended in corresponding medium containing 0.5% low-melting agarose (GenePure LE, ISC BioExpress, Pittsburgh, PA) and 10% FBS. Then cells were seeded onto a coating of 1% low-melting agarose in medium containing 10% FBS. Colonies were scored 3–4 weeks after preparation. Colonies larger than 0.1 mm in diameter were scored as positive.

Reverse transcriptase-PCR

Complementary DNA was transcribed using SuperScript III RT (Invitrogen) following the manufacturer's instructions. p53 was amplified by PCR using the primers 5'-GCGCACAGAGGAAGAGAATC-3' (forward) and 5'-CCTCATTAGCTCTCGGAAC-3' (reverse).

Tumor growth in nude mice

All animal studies were conducted in compliance with animal protocols approved by the MD Anderson Institutional Animal Care and Use Committee. Before injecting the mice with MCF7 cells, we subcutaneously implanted 6-week-old female nude mice with 0.36 mg of 17β-estradiol 60-day release pellets (SE121; Innovative Research of America, Sarasota, FL). Then mice were injected in the mammary glands with 2×10^6 cells from various cell lines in 100 µl of culture medium. Each cell line was tested in 10 mammary glands of 5 different animals. After 2–3 weeks, tumors were measured every 3–5 days. Volume was calculated as $W^2 \times L \times 0.52$.

BrdU incorporation assay

BrdU cell proliferation assay kit was used to detect proliferative activity of cells based on the commercial protocol from Calbiochem. Briefly, cells were transfected with indicated plasmids. The next day cells were seeded in 96-well plates at 10^4 cells/well. Forty-eight hours later, BrdU labelling reagent was added and incubated for 2 h. Immunofluorescent signals of BrdU were analyzed and calculated to detect proliferative cells.

Apoptosis assay

Apoptotic cells were detected by FITC Annexin V Apoptosis Detection Kit (BD Pharmingen) according to manufacturer's instruction. Briefly, cells were transfected with indicated plasmids. The next day cells were exposed to ultraviolet (UV) (50 J/m²). Cells were harvested for apoptosis analysis 72 h after UV treatment. Cells in late apoptosis were analyzed, which were both FITC Annexin V and propidium iodide positive. Basically cells were washed with cold phosphate-buffered saline and then resuspend in 1× binding buffer at a concentration of 10^6 cells. One hundred microliters of cell suspension was transferred to a 5 ml culture tube and added 5 µl FITC Annexin V and 5 µl propidium iodide. Cells were incubated 15 min before flow cytometry analysis.

Data analysis

All data were shown as mean ± SD. Difference among groups was analyzed by Student–Newman–Keuls (SNK)-*q* test using SPSS 12.0 for Windows software. Statistical significant was defined as **P* ≤ 0.05 and ***P* ≤ 0.01. Quantification of protein levels was done using NIH Image J software.

Results

BRIT1 regulates p53 activity and protein stability

Our previous studies determined that BRIT1 functions as an early DNA damage responsive protein and regulates DDR signalling at multiple levels (4,9). As p53 is the central regulator of cellular responses to DNA damage, we examined the effects of BRIT1 knockdown on p53 activation in response to DNA damage stimuli. We first depleted BRIT1 expression by using BRIT1-specific siRNA in non-tumorigenic breast epithelial MCF10A cells. Then we established three stable BRIT1 knockdown MCF10A cell lines (BRIT1 KD1, BRIT1 KD2 and BRIT1 KD3) using lentiviral infection as mentioned previously. Both transient BRIT1 and stable BRIT1 knockdown MCF10A cells were treated with UV radiation (50 J/m²) or γ radiation (10 Gy). Cell lysates were harvested at indicated time points and the protein levels of BRIT1 and p53 were analyzed. We found that both of these DNA damage signals induced p53 expression in control cells. However, this induction of p53 was markedly reduced in BRIT1-deficient cells. Notably, we observed that the basal level of p53 expression was also reduced in BRIT1-deficient cells (Figure 1). In addition to regulating p53 activation in response to DNA damage, more interestingly, these

Fig. 1. BRIT1 deficiency impairs DNA damage-induced p53 expression levels. (A and B) MCF10A cells were transiently transfected with BRIT1 siRNA and treated with UV radiation (50 J/m²) or γ radiation (10 Gy) as indicated. Cell lysates were harvested 3 and 6 h later and analyzed by immunoblotting. (C and D) BRIT1 stable knockdown MCF10A cells (BRIT1 KD1, BRIT1 KD2 and BRIT1 KD3) were treated with UV (50 J/m²) or γ radiation (10 Gy) as indicated. Cell lysates were harvested 3 and 6 h later and analyzed by immunoblotting.

results indicated that BRIT1 might be directly involved in controlling p53 expression in the absence of DNA damage signalling.

We then tested the regulation of p53 in BRIT1-deficient cells without DNA damage stimulation. Our data showed that BRIT1 deficiency reduced p53 protein expression levels (Figure 2A and B). To further confirm this observation, a stable BRIT1-overexpressing U-2OS cell line was established by FLAG-tagged BRIT1 expression vector transfection and selection (Figure 2C). Consistently, we found that p53 protein expression was significantly increased in the BRIT1-overexpressing U-2OS cells and was reduced in the BRIT1 knockdown MCF10A cells. Moreover, BRIT1 depletion did not affect p53 messenger RNA expression levels in MCF10A or other cell lines used in this study, suggesting that BRIT1's control of p53 is independent of transcriptional regulation (Figure 2D and Supplementary Figure 1, available at *Carcinogenesis* Online).

To determine whether the p53 protein level was regulated at the post-translational level, we compared p53 protein turnover between control and BRIT1 knockdown cells in the presence of CHX, which blocks *de novo* protein synthesis. As shown in Figure 2E and F, transient depletion of BRIT1 by specific BRIT1 siRNA resulted in decreased p53 protein half-life from 105 to 65 min. In the stable BRIT1 knockdown cells, p53 protein half-life was reduced from 105 to <35 min (Figure 2G and H). Inversely, stable overexpression of BRIT1 in U-2OS cells increased the half-life of p53 from 30 to 75 min (Figure 2I and J). These data indicate that BRIT1 might regulate p53 expression by stabilizing its protein expression.

BRIT1 interacts with p53 and MDM2 and regulates p53 ubiquitination

To understand the potential mechanism underlying BRIT1's regulation of p53 protein stability, we conducted a biochemical analysis to test whether BRIT1 interacts with p53. We found that BRIT1 interacted with p53 when BRIT1 was ectopically expressed in U-2OS cells (Figure 3A). Interestingly, BRIT1 also interacted with the negative regulator of p53, MDM2 (Figure 3B). Reciprocally, BRIT1 could also be pulled down when an antibody against p53 or MDM2 was used for immunoprecipitation in MCF10A cells (Figure 3C and D), a finding that supports the physical interactions among these three proteins in cells. *In vitro* analysis of the interaction between these proteins showed that BRIT1 interacts with both p53 and MDM2 independently of each other (Supplementary Figure 2, available at *Carcinogenesis* Online). The interactions of BRIT1–p53–MDM2 suggest that BRIT1 might regulate p53 stability by blocking MDM2-mediated p53 ubiquitination and degradation.

To test this hypothesis, firstly, we treated control and BRIT1 transient knockdown or stable knockdown MCF10A cells with a 10 μ M concentration of the proteasome inhibitor MG132. Our results showed that MG132 treatment cancelled the regulatory effect of BRIT1 on p53 levels (Figure 4A and B), indicating that BRIT1 regulates p53 protein stability through the proteasomal pathway and protects p53 from proteasome-dependent degradation.

Moreover, we found that p53 polyubiquitination increased in the absence of BRIT1 in MCF10A cells and that the treatment of cells

Fig. 2. BRIT1 deficiency reduces p53 protein expression levels and BRIT1 is required for p53 stabilization. (A) Expression of BRIT1 and p53 protein in control MCF10A cells and BRIT1 transient knockdown MCF10A cells (BRIT1 siRNA). Control, non-target siRNA included as a negative control. (B) Expression of BRIT1 and p53 protein in control MCF10A cells and BRIT1 stable knockdown MCF10A cells by lentiviral transduction (BRIT1 KD1, BRIT1 KD2 and BRIT1 KD3). Control, non-target lentiviral included as a negative control. (C) Expression of BRIT1 and p53 protein in control (3-FLAG) U-2OS cells and BRIT1 stable-overexpressing U-2OS cells (BRIT1-FLAG). (D) Expression of p53 messenger RNA in control MCF10A cells and BRIT1 stable knockdown MCF10A cell by lentiviral transduction (BRIT1 KD1, BRIT1 KD2 and BRIT1 KD3). Control, non-target lentiviral included as a negative control. (E) MCF10A cells were transiently transfected with BRIT1 siRNA and treated with 10 μ g/ml CHX for the indicated periods of time to inhibit protein synthesis. Lysates were harvested from the cells and analyzed by western blotting. (F) Quantification of p53 protein levels. The analysis was done using NIH Image J software. (G) BRIT1 stable

Fig. 3. BRIT1 interacts with p53 and MDM2. (A and B) U-2OS cells were transfected with 3-FLAG or BRIT1-FLAG expression vectors as indicated. Forty-eight hours later, cells lysates (3 mg) were immunoprecipitated by anti-FLAG M2 affinity gel and analyzed by western blotting. The input was 3% of that used in the immunoprecipitation. (C) MCF10A cell lysates were immunoprecipitated with anti-p53 or preimmune immunoglobulin G and immunoblotted with anti-BRIT1 or anti-p53-HRP antibody. The input was 3% of that used in the immunoprecipitation. HRP, horseradish peroxidase. (D) MCF10A cell lysates were immunoprecipitated with anti-MDM2 or preimmune immunoglobulin G and immunoblotted with anti-BRIT1 or anti-MDM2 antibody. The input was 3% of that used in the immunoprecipitation.

with the proteasome inhibitor MG132 caused a robust increase of polyubiquitinated p53 protein (Figure 4C), thus suggesting a role of BRIT1 in counteracting p53 ubiquitination. Interestingly, we also discovered that BRIT1 facilitated MDM2 autoubiquitination (Figure 4D), indicating that BRIT1 might also alter the function of MDM2 by inducing autoubiquitination of MDM2 molecules. This is supported by data showing that BRIT1 overexpression caused a decrease in MDM2 expression levels, which is rescued by addition of MG132 (Supplementary Figure 3, available at *Carcinogenesis* Online). It is possible that the dysregulated ubiquitination of MDM2 in BRIT1-deficient cells might further facilitate the stabilization of p53 by altering MDM2 and its substrates. Our data to this point support a model where BRIT1 interacts with both p53 and MDM2, thus blocking MDM2-mediated p53 degradation (Figure 4E). To further support this model, we show that in the absence of BRIT1, MDM2–p53 interaction is increased, whereas increasing BRIT1 expression leads to a direct decrease in MDM2–p53 interaction, concomitant with increased BRIT1–MDM2 interaction (Supplementary Figures 4 and 5, available at *Carcinogenesis* Online).

Interaction between BRIT1 and MDM2 is required for p53 stabilization

To test whether BRIT1 and MDM2 interaction is required for p53 stabilization, we mapped the structure determinants of the interaction between BRIT1 and MDM2. FLAG-tagged wild-type BRIT1 or a series of BRIT1 deletion mutants (Figure 5D) was transfected into U-2OS cells with wild-type MDM2. Immunoprecipitation analysis with anti-FLAG M2 affinity gel showed that MDM2 was coprecipitated with wild-type BRIT1, but MDM2's binding to BRIT1 with C-terminal deletions was much weaker than its binding to wild-type BRIT1 or BRIT1 with N-terminal deletions (Figure 5A). BRIT1–MDM2 interaction was significantly reduced when BRCT2 and BRCT3 domains were both deleted. These data indicate that the C-terminal BRCT domains of BRIT1 are required for BRIT1's binding with MDM2.

Conversely, we also sought to map the BRIT1 binding domain on MDM2. Cotransfection was performed in U-2OS cells with wild-type MDM2 or MDM2 mutants lacking different parts of the amino acid sequences as indicated (Figure 5E). Cell lysates were immunoprecipitated by anti-FLAG M2 affinity gel and analyzed by western blotting. We found that the interaction between BRIT1 and MDM2 was significantly reduced when MDM2 lacks the central region that contains a zinc finger structure (MDM2 Δ 295–417) (Figure 5B).

To formally test whether the BRIT1–MDM2 interaction could affect the p53 protein level, we transiently transfected U-2OS cells with wild-type BRIT1 or BRIT1 deletion mutants, which lack the essential binding domain for MDM2. As expected, the p53 protein expression level was significantly reduced when the BRIT1 C-terminal domain was deleted (Figure 5C). Importantly, to conclude that this reduction in p53 is directly related to a decrease in the ability of MDM2 to bind p53 caused by wild-type BRIT1, we show that wild-type BRIT1, but not BRIT1 Δ 2, 3, reduces MDM2 binding to p53 and stabilizes p53 protein expression (Supplementary Figures 6 and 7, available at *Carcinogenesis* Online). These results show that the interaction between BRIT1 and MDM2 is indeed required for the regulation of p53 by BRIT1.

BRIT1 functions as a tumor suppressor gene in human breast cancer by both p53-dependent and p53-independent manners

To assess whether BRIT1 is a breast tumor suppressor gene, we determined whether loss of BRIT1 expression would malignantly transform normal mammary epithelial cells. Three stable BRIT1 knockdown MCF10A cell lines (BRIT1 KD1, BRIT1 KD2 and BRIT1 KD3) were established by lentiviral transduction and antibiotics selection. Using these three BRIT1 knockdown cell lines, we found that BRIT1 knockdown promoted proliferation of MCF10A cells (Figure 6A) and their ability to grow in soft agar (Figure 6B). Consistently, BRIT1 knockdown increased the survival ability of MCF10A cells, as indicated by colony formation assays (Figure 6C). These results demonstrate that loss of BRIT1 is sufficient to induce cellular transformation

← knockdown MCF10A cells (BRIT1 KD1, BRIT1 KD2 and BRIT1 KD3) were treated with CHX as indicated. Control, non-target lentiviral included as a negative control. (H) Quantification of p53 protein levels. (I) Control (3-FLAG) and BRIT1 stable-overexpressing U-2OS cells (BRIT1-FLAG) were treated with CHX as indicated. (J) Quantification of p53 protein levels.

Fig. 4. p53 ubiquitination is accelerated in the absence of BRIT1. (**A** and **B**) The proteasome inhibitor MG132 effectively rescues the expression level of p53 in BRIT1 knockdown cells. Mock, control, BRIT1 transient knockdown MCF10A cells (BRIT1 siRNA) or BRIT1 stable knockdown MCF10A cells (BRIT1 KD1, BRIT1 KD2 and BRIT1 KD3) were treated with or without 10 μ Mol/l MG132 for 6 h and probed for p53 expression by western blotting. (**C**) Endogenous p53 ubiquitination. Control or BRIT1 stable knockdown MCF10A cells (BRIT1 KD1 and BRIT1 KD3) were treated with or without 10 μ Mol/l MG132 for 6 h and then harvested and analyzed. p53 levels were normalized prior to immunoprecipitation by loading proportionally different amounts of cell extracts. (**D**) Endogenous MDM2 ubiquitination. Control or BRIT1 stable knockdown MCF10A cells (BRIT1 KD1 and BRIT1 KD3) were treated with or without 10 μ Mol/l MG132 for 6 h and then harvested and analyzed. (**E**) Schematic model of BRIT1 regulates p53 protein stability through blocking MDM2-mediated p53 ubiquitination.

of immortalized normal breast epithelial cells, strongly supporting BRIT1's role as a tumor suppressor in breast cancer.

To further confirm that BRIT1 functions as a tumor suppressor gene in breast cancer cells, we established a stable BRIT1-overexpressing breast cancer cell line (MCF7 BRIT1) by transfecting cells with FLAG-tagged BRIT1 expression vector (Figure 6E). Overexpression of BRIT1 repressed the proliferation and the survival abilities of MCF7 cells (Figure 6D and F). Consistent with the *in vitro* assays, results of our *in vivo* tumorigenic analysis showed that BRIT1 overexpression reduced tumor formation of MCF7 breast cancer cells in nude mice (Figure 6J). We then further tested whether overexpression

of BRIT1 led to reduced proliferative activity of MCF7 cells and/or increase apoptosis-induced by DNA damage stimuli. As shown in Supplementary Figure 8, available at *Carcinogenesis* Online, our data indicate that BRIT1 overexpression indeed suppressed BrdU incorporation and induces apoptosis after UV treatment. Taken together, these results show that BRIT1 indeed has a functional role in suppressing cellular transformation and tumorigenic potential during the development of breast cancer.

After determining that BRIT1 regulates the stability of p53 protein, we investigated whether the tumor suppressive function of BRIT1 is dependent on its regulation of p53. Control MCF7 cells

Fig. 5. BRIT1-MDM2 interaction is required for p53 stabilization. (A) Mapping the MDM2 binding domain on BRIT1. U-2OS cells were transfected as indicated. 3-FLAG expression vector as negative control. Cells lysates (2 mg) were immunoprecipitated by anti-FLAG M2 affinity gel and analyzed by immunoblotting. MDM2 interacts with the BRIT1 C-terminal domain. (B) Mapping the BRIT1 binding domain on MDM2. U-2OS cells were transfected as indicated. 3-FLAG expression vector as negative control. Cells lysates (2 mg) were immunoprecipitated by anti-FLAG M2 affinity gel and analyzed by immunoblotting. BRIT1 interacts with the MDM2 C-terminal domain. (C) U-2OS cells were transfected with 3-FLAG, BRIT1 wild-type and deletion mutants. Cell lysates were analyzed with anti-p53 antibody by western blotting. (D) Schematic diagram of the BRIT1 protein and BRIT1 deletions. (E) Schematic diagram of the MDM2 protein and MDM2 deletions. NLS, nucleolar localization signal; NES, nuclear export signal.

with wild-type p53 and MCF7 cells with p53 knockdown were used to establish stable BRIT1-overexpression cells by transfecting with FLAG-tagged BRIT1 and selecting with G418. Overexpression of BRIT1 was confirmed by western blotting (Figure 6G). Our data show that BRIT1 has the ability to repress the proliferation and survival abilities of MCF7 cells with p53 knockdown (Figure 6H and I), albeit to a lesser extent than it suppressed the proliferation and the

survival abilities of control MCF7 cells with wild-type p53. We also injected these cell lines into nude mice and observed similar effects in the *in vivo* tumorigenic analysis (Figure 6K). BRIT1 suppressed tumor formation to a greater extent in MCF7 cells with wild-type p53. In summary, these *in vitro* and *in vivo* analyses indicate that BRIT1 might suppress cell transformation through both p53-dependent and p53-independent pathways.

Fig. 6. BRIT1 functions as a tumor suppressor in breast cancer and partially depends on the regulation of p53. (A) BRIT1 knockdown enhances the proliferation of MCF10A cells. Control or BRIT1 stable knockdown MCF10A cells (BRIT1 KD1, BRIT1 KD2 and BRIT1 KD3) were seeded in a 96-well plate at 1×10^3 cells/well. Cell proliferation was measured by MTT assay for 4 days. (B) BRIT1 knockdown induces anchorage-independent growth of MCF10A cells. Viable colonies of MCF10A clones were counted. (C) BRIT1 knockdown enhances the survival ability of MCF10A cells. Control or BRIT1 stable knockdown MCF10A cells (BRIT1 KD1, BRIT1 KD2 and BRIT1 KD3) were seeded in a 6-well plate at 250 cells/well. Viable colonies were stained by violet crystal and counted. (D) BRIT1 stable-overexpressing MCF7 cells pool was established by FLAG-tagged BRIT1 expression vector transfection and G418 selection. BRIT1 overexpression and p53 protein level were analyzed by western blotting. (E) BRIT1 overexpression suppresses the proliferation of MCF7 cells. Control or BRIT1 stable-overexpression MCF7 cells pool were seeded in a 96-well plate at 1×10^3 cells/well. Cell proliferation was measured by MTT assay for 5 days. (F) BRIT1 overexpression represses the survival ability of MCF7 cells. Control or BRIT1 stable-overexpressing MCF7 cells pool were seeded in a 6-well plate at 250 cells/well. Viable colonies were stained by violet crystal and counted. (G) MCF7 p53-knockdown cells were used to establish a BRIT1-overexpressing cells pool by transfection with FLAG-tagged BRIT1 and selection with G418. Overexpression of BRIT1 was confirmed by western blotting. (H) MTT assay was performed in control MCF7 p53-knockdown cell and BRIT1-overexpressing MCF7 p53-knockdown cell pool. (I) Colony formation assay was performed in control MCF7 p53-knockdown cell and BRIT1-overexpressing MCF7 p53-knockdown cell pool. (J) BRIT1-overexpressing or vector control MCF7 cells pool with wild-type p53 were injected into the fatty pads of nude mice and tumor volumes were measured. (K) BRIT1-overexpressing or vector control MCF7 cells pool with p53 knockdown were injected into the fatty pads of nude mice and tumor volumes were measured. p53-KD, p53 knockdown. * $P \leq 0.05$ and ** $P \leq 0.01$.

Discussion

In humans, BRIT1 is located on chromosome 8p23.1, a region implicated in the development of several malignancies, including breast, ovarian and prostate cancers (24–31). Previous findings from our studies and those of others have identified aberrations of BRIT1 in a variety of human cancer specimens including breast cancer, suggesting that BRIT1 deficiency could lead to the development of cancers (3–5). In this study, we identified BRIT1 as a novel regulator of p53 stability, a finding that enables a mechanistic understanding of the tumor suppressor function of BRIT1. We found that BRIT1 interacts with p53 and MDM2. The interaction between BRIT1 and MDM2 potentially blocks the binding of p53 to MDM2 and thereby reduces p53 ubiquitination mediated by MDM2 and stabilizes p53. More importantly, we provide critical experimental evidence that strongly supports the role of BRIT1 as a tumor suppressor. We showed that BRIT1 indeed functions as a tumor suppressor in human breast cancer. By using genetic approaches to alter the expression level of BRIT1 in MCF10A and MCF7 cells, we found that BRIT1 plays a critical role in suppressing cellular transformation activity *in vitro* and tumorigenicity *in vivo*. Our study also provides new insights into how BRIT1 deficiency might contribute to the development of human cancer. Previous studies have shown that BRIT1 plays multiple roles in maintaining genomic instability through regulating DDR, the DNA repair process, checkpoint activation and centrosome stability, all of which are well-known mechanisms to prevent cancer initiation and progression (32–35). Moreover, BRIT1 functions as a transcriptional regulator of many important genes involved in tumorigenesis. For example, BRIT1 is a transcriptional activator of tumor suppressors BRCA1 and checkpoint kinase 1 through its interaction with E2F transcription 1 (36). BRIT1 also transcriptionally represses human telomerase expression and prevents cancer cell immortalization (1). Our data reveal that BRIT1 can function as a post-transcriptional regulator of p53 expression, regulating p53 protein stability through blocking MDM2-mediated p53 ubiquitination. Thus, our study adds new information to the biological functions of BRIT1 as a tumor suppressor. Consistent with multiple mechanisms underlying BRIT1's function in preventing cancer, we observed that BRIT1 suppresses cellular oncogenic transformation and xenograft tumor formation in both p53-dependent and p53-independent pathways. In cells with wild-type p53, BRIT1 has a much stronger tumor suppression effect than in cells with p53 depletion.

The p53 tumor suppressor protein serves as a genome guardian and functions mainly as a transcription factor by transactivating or repressing a large group of target genes that regulate cell cycle arrest, apoptosis and DNA repair to maintain a balance between cell growth and arrest in response to stimulations such as DNA damage (37–42). However, p53 is normally expressed at low levels so that it does not disrupt the cell cycle or cause the cell to undergo untimely death. Such low concentrations of p53 are achieved through the operation of a negative feedback loop of p53 and MDM2. MDM2 is a p53 transcriptional target, which ubiquitinates p53, thus marking it for proteasome-mediated degradation (43–45). More importantly, p53 is stabilized and activated upon exposure to DNA-damaging agents or oncogene activation by a series of post-translational modifications that free it from MDM2, resulting in cell cycle arrest, senescence or cell death (46,47).

Owing to the complexity of controlling p53 protein stability, many previous studies have demonstrated that multiple proteins and post-translational modifications were responsible for p53 stabilization and activation. p53 can be phosphorylated and stabilized by the phosphoinositide-3-like protein kinase family members, ATM, ATR and DNA-dependent protein kinase. p53 can also be phosphorylated by checkpoint kinase 2 (CHK2), which itself functions as a substrate of ATM in response to DNA damage. The phosphorylation of p53 by these DDR kinases lead to reduced binding of p53 to MDM2, which in turn allows the replacement of ubiquitin moieties by acetylation, resulting in p53 stabilization and full activation (14,48,49). Thus, preventing the ubiquitination of p53 by reducing its interaction with

MDM2 is the major mechanism for control of the p53 protein. On the other hand, others have shown that decreasing p53 stability can be achieved via complex formation with MDM2. This was observed when CK1 was found to directly associate with MDM2, promoting MDM2 binding to p53 and promoting its ubiquitination and degradation (50). Our data identify a new mechanism, suggesting that BRIT1 might bind MDM2 and block the interaction between MDM2 and p53 through its C-terminal BRCT domain, reducing the binding of MDM2 to p53 while facilitating MDM2 autoubiquitination and p53 stabilization. Since BRCT domains commonly target phosphorylated sequences, it is possible that MDM2–BRIT1 interaction is dependent on MDM2 phosphorylation within the region of BRIT1 binding. This newly discovered mechanism in the regulation of p53 stabilization by BRIT1 supports BRIT1's function in tumor suppression.

Our studies indicate that BRIT1 might function as a p53 regulator at multiple levels. In the presence of DNA damage lesions, BRIT1 functions as an early DDR protein. Its deficiency may lead to defective DDR signalling, which prevents p53 activation and stabilization induced by damaged DNA. In the absence of DNA damage stimuli, BRIT1 can regulate p53 through its C-terminal BRCT domain, thereby reducing MDM2-mediated p53 ubiquitination. In this study, we also observed that BRIT1 deficiency leads to reduced MDM2 autoubiquitination. It is possible that in addition to physically blocking MDM2–p53 interaction, BRIT1 may regulate the function of MDM2 by modulating its post-translational modifications such as autoubiquitination, which might alter E3-ligase activity or the substrate specificity/binding affinity of MDM2. We will investigate this possibility in future studies.

In clinical specimens from different stages of human cancers such as those in the bladder, breast, lung and colon, it has been shown that early precursor lesions commonly express markers of an activated DDR, including phosphorylated ATM and CHK2 and phosphorylated histone H2AX and p53. Other studies have proposed that early in tumorigenesis (before genomic instability and malignant conversion), human cells activate an ATR/ATM-regulated DDR network that delays or prevents cancer. Thus, mutations compromising this checkpoint, including defects in the ATM–CHK2–p53 pathway, might allow cell proliferation, survival ability, increased genomic instability and tumor progression (51,52). As discussed above, BRIT1 deficiency results in defective DNA response and a defective ATM–CHK2–p53 signalling pathway. BRIT1 can also impair p53 expression in the absence of DNA damage signalling. These two mechanisms may cooperatively accelerate the evolution of early precancerous lesions into cancers. Therefore, BRIT1 deficiency could be an effective target for both cancer prevention and therapy.

In the clinic, the functional status of p53 has been associated with the cancer's prognosis, progression and response to therapy. Tumor cells containing wild-type p53 are usually more sensitive to radiotherapy or chemotherapy than are those bearing mutant p53 (53,54). Although BRIT1 deficiency has been shown to cause DNA repair defects, the breast tumors in cancer patients with BRIT1 gene deficiency may not be sensitive to radiotherapy or chemotherapy because of the disruption of p53 expression. Thus, it will be critical to conduct future studies to evaluate the correlation between BRIT1 and p53 expression status with the clinical prognosis and tumor response to radiotherapy or chemotherapy, in order to identify effective targeted therapeutics for BRIT1-deficient tumors.

Supplementary material

Supplementary Figures 1–9 can be found at <http://carcin.oxfordjournals.org/>

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