# BRIT1 regulates early DNA damage response, chromosomal integrity, and cancer

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## Summary

***BRIT1*, initially identified as an hTERT repressor, has additional functions at DNA damage checkpoints. Here, we demonstrate that BRIT1 formed nuclear foci minutes after irradiation. The foci of BRIT1 colocalized with 53BP1, MDC1, NBS1, ATM, RPA, and ATR. BRIT1 was required for activation of these elements, indicating that BRIT1 is a proximal factor in the DNA damage response pathway. Depletion of BRIT1 increased the accumulation of chromosomal aberrations. In addition, decreased levels of BRIT1 were detected in several types of human cancer, with BRIT1 expression being inversely correlated with genomic instability and metastasis. These results identify BRIT1 as a crucial DNA damage regulator in the ATM/ATR pathways and suggest that it functions as a tumor suppressor gene.**

## Introduction

Maintenance of genomic integrity in the face of mutagenic effects of DNA damage relies on flawless execution of genome surveillance pathways, checkpoints that coordinate cell cycle progression with DNA repair (Nyberg et al., 2002; Zhou and Elledge, 2000). In response to DNA damage, mammalian cells initiate a cascade of phosphorylation events mediated by two phosphatidylinositol-3-related kinases, ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3-related), which phosphorylate and activate a variety of molecules to execute the DNA damage response (Osborn et al., 2002; Shiloh, 2001, 2003). ATM is activated primarily by double-strand breaks (DSBs) induced by ionizing radiation, whereas ATR also responds to ultraviolet (UV) radiation or stalled replication forks (Osborn et al., 2002). The current model of the DNA damage response describes a linear progression beginning with sensors that convey the initial damage signal to mediators and transducers, which in turn transmit the signal to numerous effectors. During the past few years, many studies have been conducted on how damage signals coordinately execute cellular responses to DNA damage; however, much less is known about the mechanisms that initiate the early events prompted by DNA damage that precede the spread of the damage signal throughout the cell.

Sensors and early mediators are molecules that, after DNA damage, are promptly recruited to the damaged DNA structure and phosphorylated by ATM, ATR, or both; they then in turn transmit the signals to downstream transducers and effectors (Sancar et al., 2004). The Mre11/Rad50/NBS1 complex, MDC1, 53BP1, RPA, and Rad17 are all components of the early DNA damage response complex; defects in these molecules disrupt the dedicated DNA damage response and lead to genomic instability and cancer development (DiTullio et al., 2002; Gorgoulis et al., 2005; Kang et al., 2005; Wang et al., 2002, 2005a; Ward et al., 2003).

We previously identified *BRIT1* (BRCT-repeat inhibitor of hTERT expression) in a genetic screen for transcriptional repressors of hTERT, the catalytic subunit of human telomerase (Lin and Elledge, 2003). The sequence of *BRIT1* was derived from a hypothetical protein that was later matched to a putative disease gene called microcephalin (*MCPH1*), one of at least six loci implicated in the autosomal recessive disease primary microcephaly (Jackson et al., 2002). Recently, we and others showed that BRIT1 is required for DNA damage-induced intra-S and

### SIGNIFICANCE

**The human genome is constantly challenged by endogenous and environmental factors that can alter its structure and corrupt its encoded message. A signaling network of checkpoint pathways has evolved to respond to these challenges to maintain genomic integrity. Our studies indicate that BRIT1 functions as a proximal factor in the DNA damage checkpoints that control multiple damage sensors and early mediators. Disruption of BRIT1 function abolishes DNA damage responses and leads to genomic instability. Furthermore, aberrations of BRIT1 have been identified in several cancer lineages that link its deficiency to cancer initiation and progression. Thus, BRIT1 may function as a tumor suppressor, and as such, further understanding of its function may well contribute to novel, effective therapeutic approaches for cancer.**

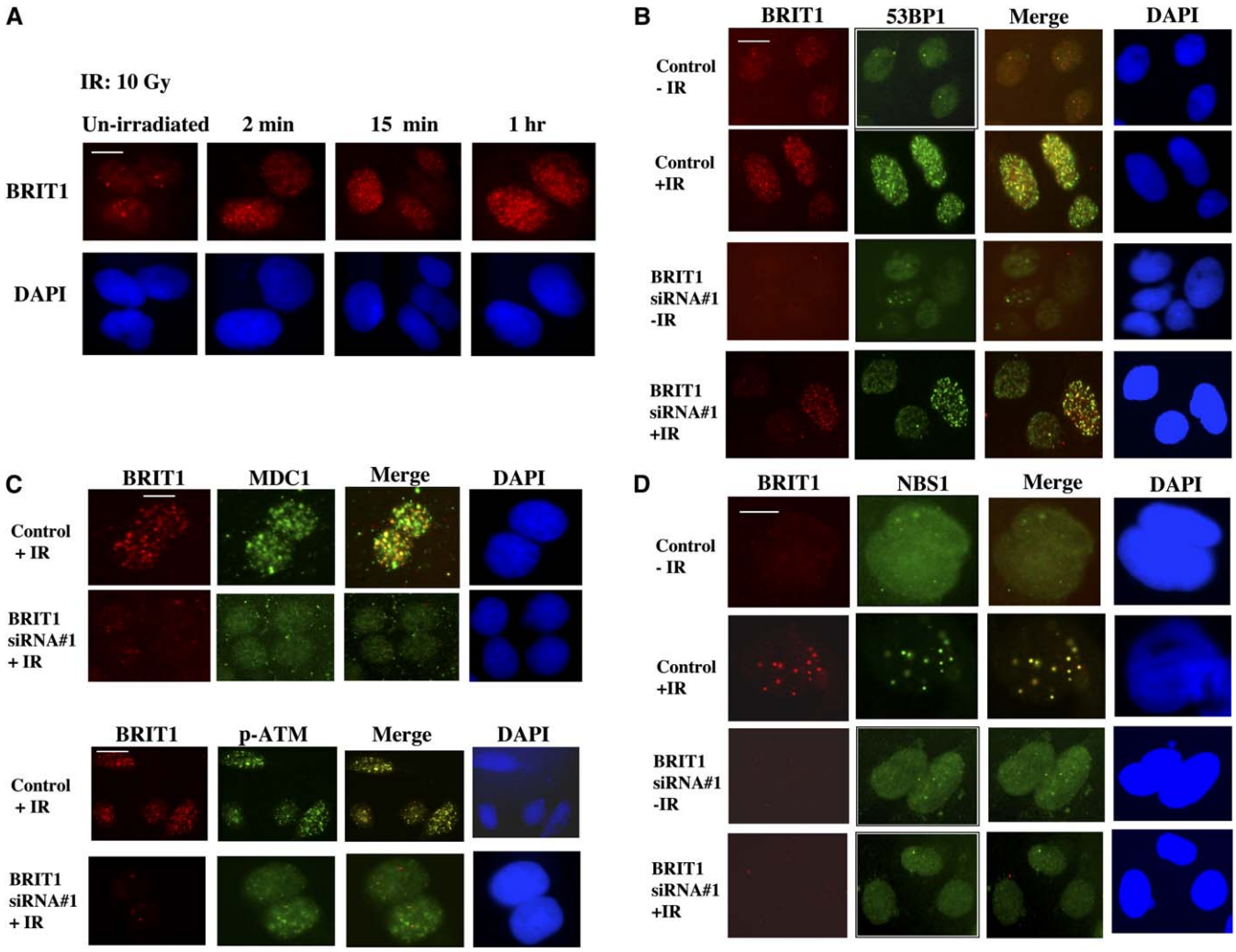



**Figure 1.** BRIT1 forms IRIF immediately after IR and is required for NBS1, 53BP1, MDC1, and p-ATM IRIF formation

**A:** U2OS cells were analyzed for IRIF formation at the indicated times after irradiation (10 Gy).

**B:** U2OS cells were transfected with control (luciferase) or BRIT1 siRNA#1 twice as described; 48 hr after the second transfection, cells were treated or not treated with 10 Gy of ionizing radiation, and 1 hr later they were fixed and stained with antibodies to BRIT1 and 53BP1. Cells were then washed and stained with rhodamine- or FITC-conjugated antibodies; nuclei were visualized by DAPI staining. The 53BP1 foci were abolished in cells in which BRIT1 was completely knocked down; the cells with incomplete knockdown of BRIT1 did show 53BP1 foci (bottom panel).

**C:** U2OS cells were transfected and treated as described in **B** except that the antibodies used were to BRIT1 and MDC1 (top) or BRIT1 and p-ATM (bottom).

**D:** U2OS cells were transfected and treated as described for **B** except that the antibodies used were to BRIT1 and NBS1. Scale bars, 20 μm.

$G_2$/M checkpoints and that this requirement may result in part from its regulation of the expression of BRCA1 and Chk1 (Lin et al., 2005; Xu et al., 2004). We also demonstrated that BRIT1 is a chromatin binding protein that forms irradiation-induced nuclear foci (IRIF), which colocalize with γ-H2AX (Lin et al., 2005). These observations suggested that BRIT1 may have a direct role in transmitting DNA damage signals.

In this study, we report that BRIT1 colocalizes with several DNA damage sensors and early mediators upon DNA damage and is required for IRIF formation, chromatin binding, and phosphorylation of these molecules in the ATM/ATR pathways. Furthermore, BRIT1 is required for chromosomal integrity in human cells, as demonstrated by reductions in gene copy numbers and expression in several cell lineages that correlate with tumor behavior. Thus, we propose that BRIT1 functions as an upstream component of the DNA damage pathway that recruits sensors and mediators to damaged DNA loci contributing to genomic stability, suggesting that BRIT1 may be an as yet unidentified tumor suppressor gene in multiple cancer lineages.

## Results

### BRIT1 is required for the formation of irradiation-induced nuclear foci by NBS1, 53BP1, MDC1, and phosphorylated ATM

When DNA is damaged in cells, many DNA damage-signaling proteins are recruited to the damaged loci and form discrete nuclear foci (IRIF) (Paull et al., 2000; Rouse and Jackson, 2002). The order and timing of these events are thought to be critical for checkpoint response and DNA repair (Stewart et al., 2003). Also, a hierarchy of proteins seems to be involved in the assembly of IRIF, and the resultant hierarchy of foci formation provides

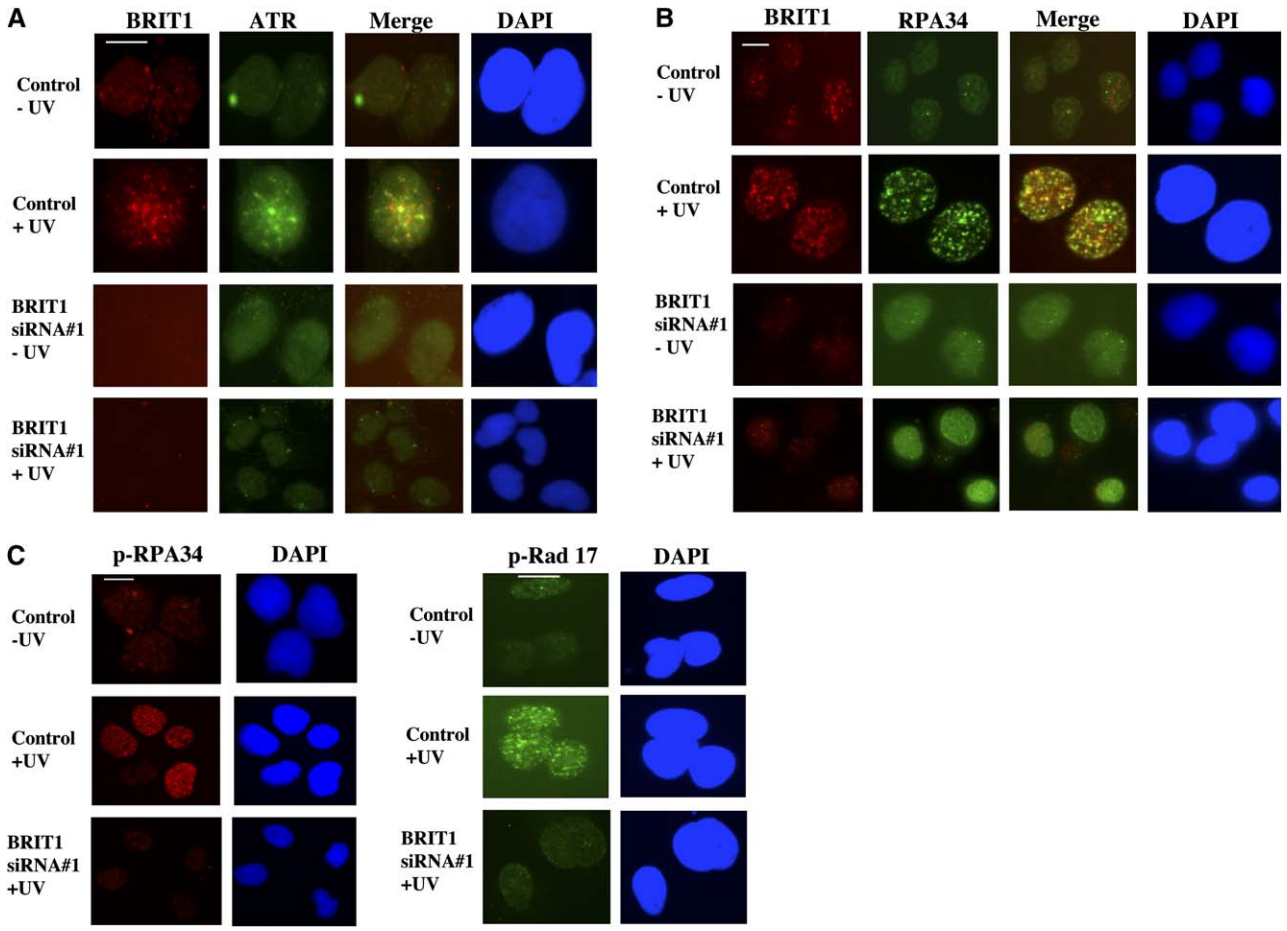



**Figure 2.** BRIT1 is required for UV-induced formation of ATR, RPA, and p-Rad17 foci

U2OS cells were transfected with control (luciferase) or BRIT1 siRNA twice; at 48 hr after the second transfection, cells were treated or not treated with 50 J/m$^2$ of UV radiation. One hour after irradiation, cells were fixed and costained with antibodies to BRIT1 and ATR (**A**), RPA34 (**B**), p-RPA34 (**C**, left panel), and p-Rad17 (**C**, right panel). Cells were then washed and stained with rhodamine- or FITC-conjugated antibodies. Nuclei were visualized by DAPI staining. Scale bars, 20 μm.

a means of ordering the molecular events ensuing from DNA damage detection and signal transduction (Petrini and Stracker, 2003). To dissect the role of BRIT1 in checkpoint signaling and its position within the signaling pathway, we first studied how quickly BRIT1 formed IRIF following DNA damage and the hierarchy between recruitment of BRIT1 and other key checkpoint regulators.

As shown in Figure 1A, BRIT1 formed IRIF very promptly; the BRIT1 foci could be detected as early as 2 min after ionizing irradiation. This result placed BRIT1 very upstream of the DNA damage signaling. Previously, we demonstrated that BRIT1 colocalized with γ-H2AX (Lin et al., 2005), one of the earliest molecules to respond to DNA damage (Paull et al., 2000). However, depletion of BRIT1 by small interfering RNA (siRNA) did not abolish the formation of γ-H2AX foci, suggesting that γ-H2AX foci formation was not regulated by BRIT1 (data not shown). Thus, BRIT1 may function in parallel with or downstream of γ-H2AX.

Based on current model, after ionizing irradiation, γ-H2AX marks the chromatin region at or near the DNA damage site and serves as a platform for the recruitment of DNA checkpoint signaling and repair factors including 53BP1, MDC1, MRN (MRE11-RAD50-NBS1), and BRCA1 (Carney et al., 1998; Celeste et al., 2002; Goldberg et al., 2003; Lou et al., 2003; Paull et al., 2000; Rappold et al., 2001; Scully et al., 1997; Stewart et al., 2003; van den Bosch et al., 2003; Wang et al., 2002; Xu and Stern, 2003; Zhong et al., 1999). The appearance of 53BP1, MDC1, and NBS1 foci seems to be coincident with formation of the γ-H2AX foci (Thompson and Limoli, 2003). To determine whether BRIT1 functions in the early damage response, we tested whether BRIT1 colocalized with these proteins after ionizing irradiation and whether BRIT1 expression was required for the formation of IRIF containing these early response elements.

We first analyzed IRIF formation using immunofluorescence staining with specific antibodies to BRIT1, 53BP1, MDC1, phospho- (p-) ATM (p-S1981), and NBS1. After ionizing irradiation, numbers of BRIT1 foci sharply increased and colocalized with 53BP1 foci (Figure 1B), MDC1 and p-ATM foci (Figure 1C), and NBS1 foci (Figure 1D). Moreover, depletion of BRIT1 by BRIT1-specific siRNA, but not a control luciferase siRNA, abolished the formation of the IRIF containing each of these molecules. These results were confirmed using two additional BRIT1 siRNAs (not presented), the specificity of which has been demonstrated previously (Lin et al., 2005). Moreover, the foci formation could be restored when siRNA-resistant BRIT1 was ectopically expressed (Figure S1 in the Supplemental

Data available with this article online shows the rescue of MDC1 foci as one of the examples). These results suggest that BRIT1 is likely to function upstream of 53BP1, MDC1, and the MRN complex and is required for the recruitment of active p-ATM to the damaged loci, through direct binding or indirectly through bridging via 53BP1, MDC1, or NBS1.

### BRIT1 is required for the formation of UV-induced ATR, RPA, and p-Rad17 foci

To determine if BRIT1 plays a parallel role in regulation of the ATR pathway, we used coimmunostaining of BRIT1 protein with three known sensors or early mediators in the ATR pathway—ATR, RPA, and Rad17 (Unsal-Kacmaz et al., 2002; Zou and Elledge, 2003; Zou et al., 2003; Sancar et al., 2004).

We found that BRIT1 formed discrete foci after UV irradiation (Figure 2), which colocalized with ATR and RPA (Figures 2A and 2B). siRNA knockdown showed that BRIT1 was required for the formation of ATR and RPA foci (Figures 2A and 2B), indicating that BRIT1 is required for proper ATR and RPA translocation after UV irradiation. The rescue of RPA foci was also demonstrated when siRNA-resistant BRIT1 was ectopically expressed (Figure S2). The RPA foci, however, could not be restored when either BRCA1 and/or Chk1 was ectopically expressed, indicating that BRIT1 regulated RPA foci through a mechanism independent of its regulation on BRCA1/Chk1 expression (Figure S2).

Both RPA and Rad17, another checkpoint protein that serves as a sensor after UV exposure, bind to the damaged DNA and are phosphorylated by ATR (Zou et al., 2002; Zou and Elledge, 2003). BRIT1 was also required for the phosphorylation of both RPA and Rad17 (Figure 2C), compatible with a lack of recruitment of RPA and Rad17 to the damaged DNA loci or of the failure of recruitment of ATR to the complex.

Together, these findings implicate BRIT1 in signaling upstream of foci formation in both the ATM and ATR pathways.

### BRIT1 is required for the association of MDC1, 53BP1, p-NBS1, RPA, and p-Rad17 with chromatin

DNA damage results in enhanced binding of checkpoint regulators including MDC1, 53BP1, NBS1, RPA, and Rad17 to chromatin (Liu et al., 2001; Peng and Chen, 2005; Zou et al., 2002, 2003; Zou and Elledge, 2003). The association of these factors with chromatin is thought to take place in parallel with their recruitment into IRIF on damaged DNA.

Because BRIT1 is a chromatin binding protein (Lin et al., 2005) and is required for the recruitment of sensors and early mediators to damaged DNA loci, we expected that BRIT1 depletion would also abolish the damage-induced binding of these molecules to chromatin, as assessed by chromatin fractionation analysis (Lin et al., 2005). Indeed, depletion of BRIT1 by two specific BRIT1 siRNAs in U2OS cells markedly reduced both basal and ionizing irradiation-induced association of MDC1, 53BP1, and p-NBS1 with chromatin (Figure 3A), suggesting that BRIT1 is required for surveillance of the DNA structure by these sensors and early mediators. MDC1, 53BP1, and p-NBS1 associated at a low level with chromatin in the absence of exogenous genome stress, suggesting that endogenous genomic stress takes place during cell culture. In contrast to its effects on chromatin binding, BRIT1 knockdown did not change total levels of MDC1, 53BP1, or NBS1 (Figure S3 and Lin et al., 2005). Thus, the effects of BRIT1 knockdown on recruitment of these molecules to chromatin probably resulted from changes in the access or affinity of these molecules to the chromatin structure. We used an anti-Orc2 antibody as the loading control because the association of Orc2 to chromatin is not affected by DNA damage (Zou et al., 2002).

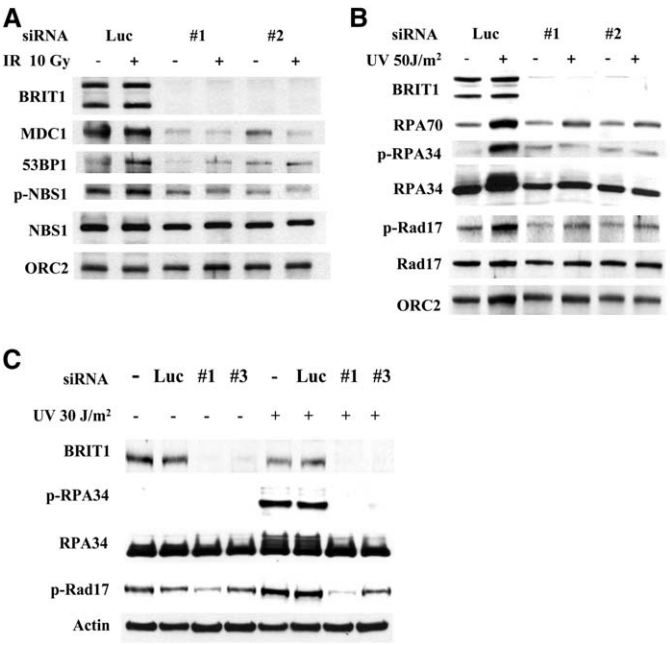



**Figure 3.** BRIT1 is required for the association of MDC1, 53BP1, p-NBS1, RPA, and p-Rad17 with chromatin and for the UV-induced phosphorylation of RPA and Rad17

**A:** U2OS cells were transfected twice with luciferase or with two different BRIT1 siRNAs; at 48 hr after the second transfection, cells were treated or not treated with 10 Gy of ionizing radiation. Two hours after irradiation, chromatin-enriched sediments were subjected to Western blot analysis and probed with antibodies against BRIT1, MDC1, 53BP1, p-NBS1, NBS1, and ORC2.

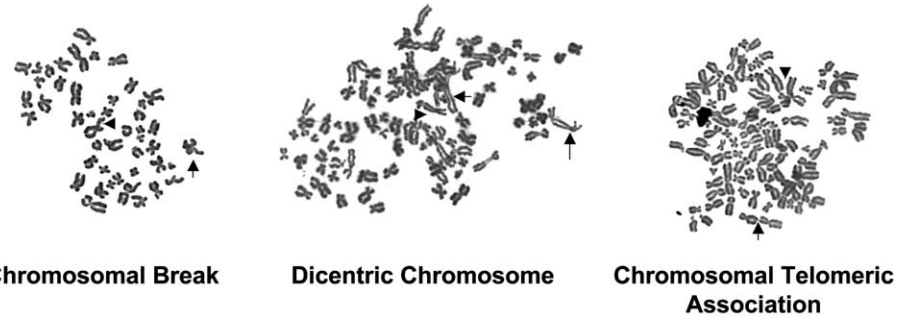
**B:** U2OS cells were transfected as described for **A** and treated with 50 J/m$^2$ UV radiation; 2 hr later, chromatin-enriched fractions were subjected to Western blotting and probed with antibodies against BRIT1, RPA70, p-RPA34, RPA34, p-Rad17, Rad17, and ORC2.

**C:** U2OS cells were treated as described above except that cells were exposed to 30 J/m$^2$ of UV; 8 hr after irradiation, whole-cell lysates were collected and subjected to Western blot analysis and probed with antibodies against BRIT1, p-RPA34, RPA34, p-Rad17, and actin.

BRIT1 depletion also markedly decreased UV-induced binding of two subunits of the RPA molecule (RPA70 and RPA34) and of p-Rad17 to chromatin (Figure 3B). Following DNA damage in the absence of BRIT1 depletion, chromatin bound RPA34 was likely phosphorylated by ATR, as evidenced by the formation of a heavy band upon probing with a p-RPA34 antibody and by the band shift of total RPA34. These results implicate BRIT1 in the recruitment of DNA damage sensors or early mediators in the UV-triggered ATR pathway. We did not detect significant changes in ATR binding to chromatin in BRIT1 knockdown cells after UV irradiation (data not shown), which is consistent with previous reports that UV induces foci formation, but not chromatin binding, of ATR (Zou et al., 2002).

### BRIT1 is required for the UV-induced phosphorylation of RPA34 and Rad17

The DNA damage checkpoint is mediated by a cascade of protein phosphorylation events. Sensors and mediators are



Chromosomal Break | Dicentric Chromosome | Chromosomal Telomeric Association

**Figure 4.** BRIT1 deficiency leads to chromosomal aberrations

HMEC cells were mock transfected or transfected with luciferase or one of three different BRIT1 siRNAs twice; 72 hr later, cells were analyzed by metaphase spreads. The top panels show examples of abnormal chromosomes, and the bottom panel summarizes percentages of cells containing chromosomal aberrations.

| Samples | No. of cells analyzed | % normal diploid cells | % cells with aberrations | % Polyploidy | % cells with fusions | No. of fusions per metaphase |
|---|---|---|---|---|---|---|
| HMEC control | 34 | 88.2 | 0 | 11.8 | 0 | 0.0 |
| Control siRNA | 33 | 69.7 | 0.00 | 11.2 | 0.00 | 0.00 |
| siRNA #1 | 33 | 75.7 | 21.2 | 9.1 | 18.2 | 0.18 |
| siRNA#2 | 33 | 75.7 | 24.2 | 12.1 | 18.2 | 0.27 |
| siRNA#3 | 34 | 73.5 | 26.5 | 2.9 | 26.5 | 0.32 |

recruited to DNA damage sites (chromatin) and then phosphorylated by ATM or ATR to transmit signals to downstream mediators, resulting in execution of DNA damage responses. Because BRIT1 depletion blocked the recruitment of those sensors or mediators to foci and to chromatin, we next sought to determine whether damage-induced phosphorylation of these factors by ATM or ATR would consequently be abolished by BRIT1 depletion. Indeed, BRIT1 depletion abolished the UV-induced phosphorylation of RPA34, as evidenced by the appearance of a band upon treatment with an RPA34-Ser4/Ser8 p-antibody and by the band shift of total RPA34 (Figure 3C). BRIT1 depletion also abolished the phosphorylation of Rad17 (Figure 3C). This effect could represent the failure of recruitment of RPA34 and Rad17 to damaged DNA loci and/or the failure of ATR to get access to phosphorylate RPA34 and Rad17.

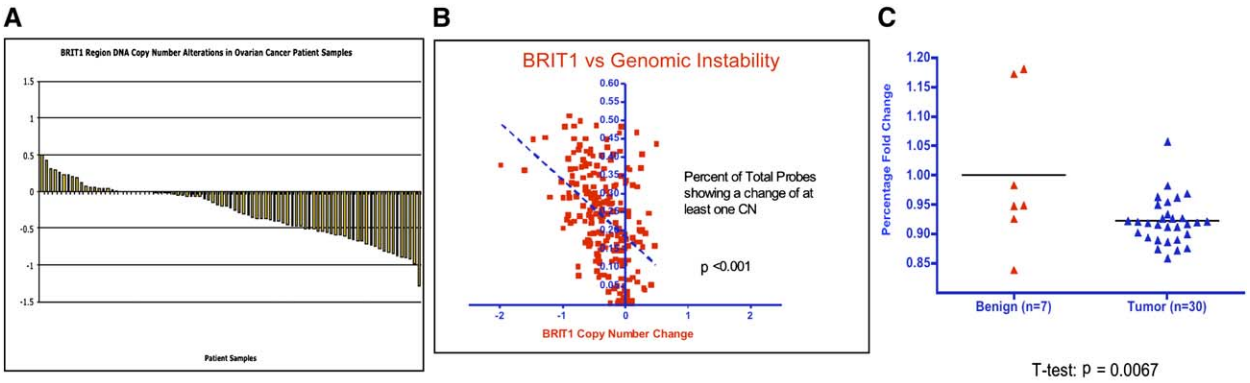
### BRIT1 deficiency increases the frequency of chromosomal aberrations

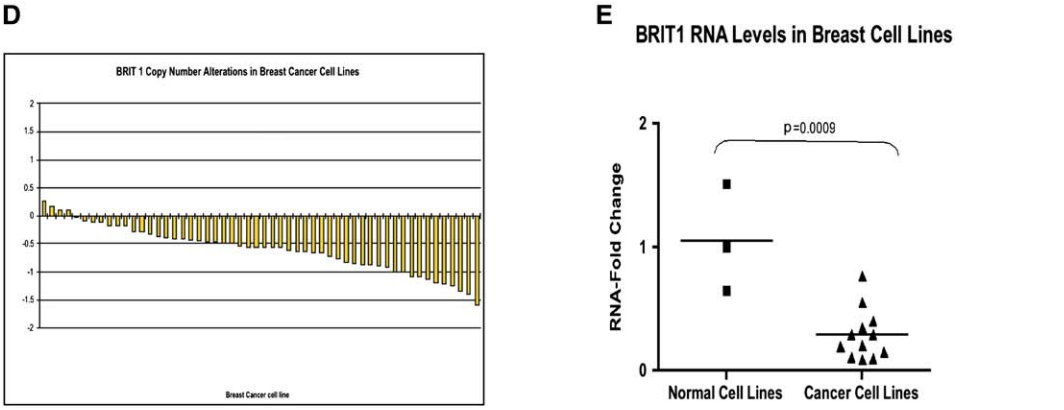
Intact DNA damage pathways are crucial for maintaining genomic integrity. Deficiencies of γ-H2AX (Celeste et al., 2003), NBS1 (Difilippantonio et al., 2005), and RPA (Wang et al., 2005a) have all been shown to increase the accumulation of chromosome aberrations. Because our findings clearly demonstrated that BRIT1 has an important role in regulating the recruitment of several essential checkpoint regulators to damaged DNA, we assessed whether depletion of BRIT1 in cells would lead to an increase in chromosome aberrations and genomic instability. To test this hypothesis, we transfected normal human mammary epithelial cells (HMECs) with control siRNA or with three specific BRIT1 siRNAs. At 72 hr after the second transfection, cells were treated with colcemid and the chromosomes were analyzed by metaphase spreads. BRIT1-depleted HMECs showed a variety of chromosome aberrations, including chromosomal breaks, dicentric chromosomes, and chromosomal telomeric association (Figure 4). Chromosomal abnormalities were detected in approximately 25% of BRIT1 knockdown cells, with no chromosomal aberrations being detected in mock- or control siRNA-treated cells (Figure 4). When pretreated with 3 Gy of ionizing radiation, the extent of chromosome aberration was even more severe (80% of BRIT1 knockdown cells showed aberrations). Similar sensitivity to chromosomal damage was also observed when normal human fibroblast BJ cells, MCF7 cells, and HeLa cells were evaluated (data not shown). These findings support the importance of BRIT1 in maintaining chromosomal integrity with or without exogenous genotoxic stress.

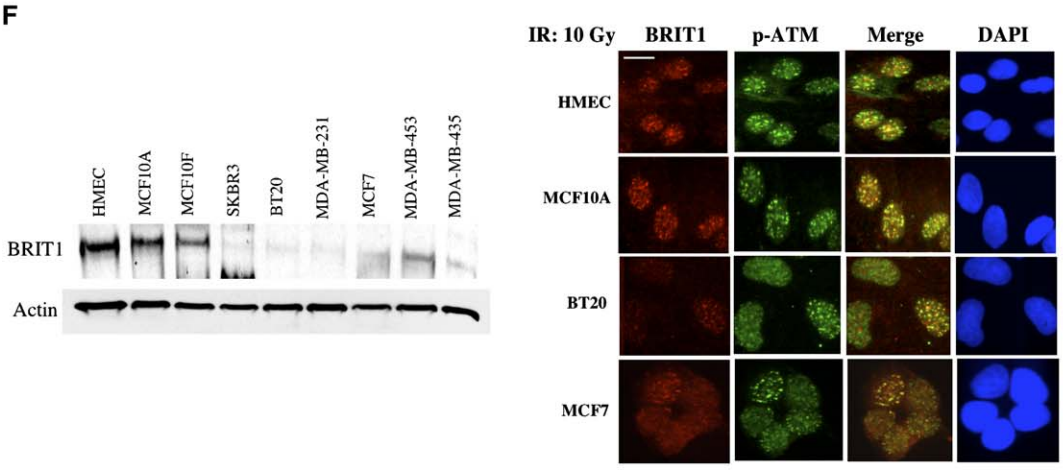
### BRIT1 is aberrant in human cancer

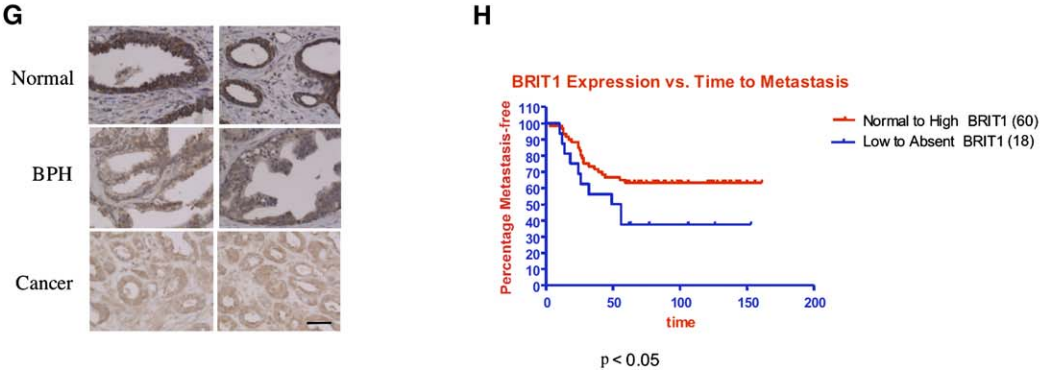
Failure of DNA damage checkpoint control leads to chromosomal aberrations (Gollin, 2005) and genomic instability (Eyfjord and Bodvarsdottir, 2005), both of which contribute to neoplastic transformation (Mills et al., 2003). We found that BRIT1, by its regulation of key checkpoint regulators, was required for the maintenance of chromosomal integrity in cells. Because BRIT1 is known to have a dual role in maintaining genomic stability and in repressing hTERT, we anticipated that defects in BRIT1 may contribute to human cancer development.

Using high-density array comparative genomic hybridization (CGH) (Snijders et al., 2001), we found substantial decreases in *BRIT1* DNA copy numbers in 35 of 87 cases (40%) of advanced epithelial ovarian cancer (Figure 5A). Interestingly, the loss of gene copy number of BRIT1 significantly correlated with overall genomic instability in these specimens. This result is consistent with the role of BRIT1 in maintaining genomic integrity (Figure 5B). BRIT1 mRNA levels were also found to be markedly decreased in 19 of 30 cases (63%) of ovarian cancer specimens relative to BRIT1 mRNA levels in benign ovarian tissue specimens (Figure 5C). Moreover, 72% of the 54 breast cell lines tested also showed decreases in *BRIT1* DNA copy number


A
BRIT1 Region DNA Copy Number Alterations in Ovarian Cancer Patient Samples
Patient Samples
B
BRIT1 vs Genomic Instability
Percent of Total Probes showing a change of at least one CN
p <0.001
BRIT1 Copy Number Change
C
Percentage Fold Change
Benign (n=7)
Tumor (n=30)
T-test: p = 0.0067


D
BRIT 1 Copy Number Alterations in Breast Cancer Cell Lines
Breast Cancer cell line
E
BRIT1 RNA Levels in Breast Cell Lines
p=0.0009
RNA-Fold Change
Normal Cell Lines
Cancer Cell Lines


F
HMEC
MCF10A
MCF10F
SKBR3
BT20
MDA-MB-231
MCF7
MDA-MB-453
MDA-MB-435
BRIT1
Actin
IR: 10 Gy
BRIT1
p-ATM
Merge
DAPI
HMEC
MCF10A
BT20
MCF7


G
Normal
BPH
Cancer
H
BRIT1 Expression vs. Time to Metastasis
Normal to High BRIT1 (60)
Low to Absent BRIT1 (18)
Percentage Metastasis-free
time
p < 0.05

(Figure 5D). In comparing BRIT1 expression between nontransformed breast epithelial cells (HMEC, MCF10A, and MCF10F) and established breast cancer cell lines, we also found significant decreases of BRIT1 RNA (Figure 5E) and protein (Figure 5F, left panel) expression in the breast cancer lines.

We further determined if the decrease of BRIT1 in cancer cells could contribute to defects in the activation of DNA damage response. As shown in Figure 5F (right panel), formation of irradiation-induced phospho-ATM containing foci were defective in cancer cell lines (BT20 and MCF7: 20%–25% of cells contained both BRIT1 and p-ATM foci) as compared to nontransformed breast cell lines (HMEC and MCF10A: more than 90% of cells contained both BRIT1 and p-ATM foci). The defect of p-ATM foci formation was readily restored when BRIT1 was ectopically expressed in MCF7 cells (Figure S4). These results, consistent with the BRIT1 knockdown experiments described above, indicate a crucial role of BRIT1 in early DNA damage responses.

We also examined the expression of BRIT1 in prostate cancer specimens, which offer the opportunity to study the transition of cells from normal to malignant in a single specimen. Immunohistochemical analysis revealed decreases of BRIT1 protein expression in benign prostate hypertrophy with further decreases in cancer cells, as compared with surrounding normal prostate tissue (Figure 5G).

Collectively, these findings suggest that changes in BRIT1 levels could contribute to tumor progression through increasing genomic instability. Indeed, BRIT1 expression was inversely correlated with the likelihood of breast cancer metastasis (van't Veer et al., 2002) (Figure 5H) and with the duration of relapse-free survival (Wang et al., 2005b) (data not shown). In addition to the reduced expression of BRIT1, we also sought to determine if genetic aberrations occurred within the BRIT1 coding region in cancer specimens. After sequencing an entire 2.7 kb of BRIT1 cDNA from ten breast cancer specimens, we identified a 38 base pair *BRIT1* deletion in exon 10 in one of the breast cancer specimens (BR7), which resulted in a premature stop codon in exon 11 (Figure 6A). The same deletion was detected in the plasmids of all cloned RT-PCR product analyzed from the same patient sample, indicating that the deletion was not due to the artifact from the RT-PCR process. Also, since no full-length BRIT1 cDNA could be detected in this patient sample, we suspect that there was an LOH in the other allele of BRIT1 gene. This deletion of BRIT1 led to a truncated protein with a predicted size of 72 kd that lacked both the two C terminus BRCT domains. Since BRCT domains are important for mediating DNA damage response (Yu et al., 2003), we suspected that the BR7 mutant was defective for its function in response to DNA damage. To address this question, we transfected U2OS cells with empty FLAG vector (FLAG-V) or FLAG-BRIT1 or FLAG-BR7 constructs with the endogenous BRIT1 knocked down by BRIT1 siRNA. As expected, only FLAG-BRIT1, not FLAG-V or FLAG-BR7, rescued the BRIT1-dependent MDC1 foci formation (Figure 6B). We also tested the function of FLAG-BR7 in the BRIT1-deficient MCF7 cells. As shown in Figure 6C, when ectopically expressed, FLAG-BRIT1 but not FLAG-BR7 rescued the irradiation-induced formation of p-RPA (left panel) or p-ATM foci (right panel).

Thus, we propose that BRIT1 functions as a tumor suppressor gene that contributes to both cancer initiation and cancer progression in a variety of cancer lineages.

## Discussion

The results presented here offer insight into a role of BRIT1 in mediating DNA damage signaling and in maintaining chromosomal integrity. Collectively, these functions, in combination with the previously demonstrated functions of BRIT1 in regulating BRCA1, Chk1, and hTERT, likely contribute to a tumor suppressor role for BRIT1 (Figure 7).

We previously reported that BRIT1 regulated the expression of two important checkpoint regulators, BRCA1 and Chk1, and was required for maintenance of the intra-S and $G_2$/M checkpoints (Lin et al., 2005). In the same study, we also demonstrated that BRIT1 was a chromatin binding protein that formed IRIF after ionizing irradiation. Because BRIT1 foci colocalized with $\gamma$-H2X foci, we hypothesized that, in addition to the transcriptional regulation of BRCA1 and Chk1, BRIT1 might participate directly in the transmission of DNA damage signaling. Our findings herein revealed that BRIT1 indeed has a crucial role in early DNA damage responses in both the ATM and ATR pathways. ATM is the major kinase involved in immediate responses to DSBs upon ionizing irradiation. In nonirradiated cells, ATM is not phosphorylated and exists as an inactive dimer. Following irradiation, DSBs trigger autophosphorylation of ATM and formation of phosphorylated monomers (Bakkenist and Kastan, 2003). The phosphorylated ATM monomers then relocate

**Figure 5.** BRIT1 deficiencies in human cancer

**A:** Array-based CGH was used to test 87 specimens of human serous ovarian cancer with bacterial artificial chromosome probes. Log base two logarithmic ratios of test to reference intensity were calculated for the probes across the region containing BRIT1. More than half of the samples arrayed showed a reduction in copy number, whereas approximately 30% showed a loss of at least one copy of the region across chromosome 8 encompassing *BRIT1*.

**B:** The losses of *BRIT1* gene copy in the ovarian cancer CGH database were compared with the gain or loss of at least one copy number change using different probes to other chromosome regions. The specimens that exhibited more BRIT1 gene loss correlated with the overall copy changes of various genes.

**C:** Comparing BRIT1 RNA transcript levels in benign ovarian tissue specimens and ovarian tumor specimens demonstrated a statistically significant reduction in BRIT1 RNA levels in the tumors as compared with the mean of RNA levels of the benign specimens.

**D:** CGH of 54 breast cancer cell lines showed that a striking majority showed large decreases in copy number, with loss of at least one copy, for the region on chromosome 8 containing BRIT1.

**E:** Expression of BRIT1 RNA in human normal (untransformed) breast cell lines or breast cancer cell lines were analyzed by quantitative real-time RT-PCR.

**F:** Left: total cell lysates were obtained from the cultured cell lines, subjected to Western blot analysis, and probed with antibodies against BRIT1 or actin. Right: the indicated cell lines were treated with 10 Gy of ionizing radiation. One hour after irradiation, cells were fixed and costained with antibodies to BRIT1 or phospho-ATM antibody. Scale bar, 20 μm. Note: the phospho-ATM foci appeared in a very small fraction of BT20 or MCF7 cells that showed detectable BRIT1 expression.

**G:** Prostate cancer specimens were analyzed by immunohistochemical staining and probed with BRIT1-specific antibody. The brownish color indicates BRIT1 expression in the regions of normal tissue, benign prostate hypertrophy (BPH), or prostate cancer from the same patient specimen. Scale bar, 50 μm.

**H:** BRIT1 RNA transcript levels were tested for correlation with time to metastasis of breast cancer in a database maintained by Stanford University. Normal to high BRIT1 levels were associated with longer time to develop metastasis and an overall reduction in the incidence of metastasis.

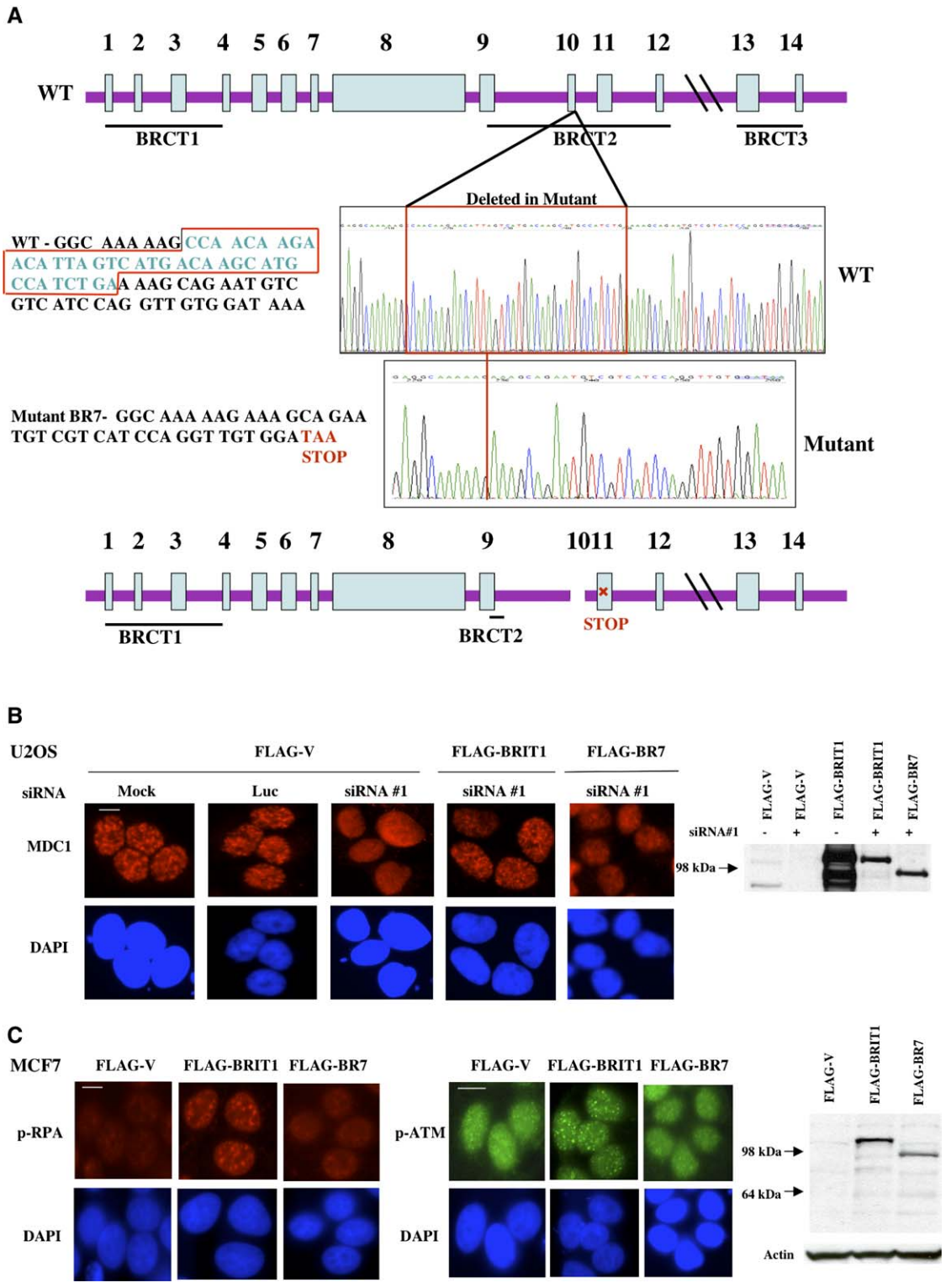



**Figure 6.** The deleted *BRIT1* gene identified from a breast cancer patient fails to activate foci formation for MDC1, p-RPA, and p-ATM

**A:** Exon 10 deletion of the *BRIT1* gene (BR7) was identified in a breast patient sample by sequence analysis (coding exons shown in gray). The premature termination (indicated by red x, bottom schematic) results in a protein lacking two C terminus BRCT domains.

**B:** U2OS cells were transfected with FLAG-V, FLAG-BRIT1, or FLAG-BR7 constructs. Four hours after the transfection, cells were mock transfected or transfected with control luciferase or BRIT1 siRNA#1. Forty-eight hours after the siRNA transfection, cells were treated with IR (10 Gy). One hour after irradiation, cells were fixed and stained with the MDC1 antibody. Cells were then washed and subsequently stained with rhodamine-antibody. Nuclei were visualized by DAPI staining (left). Scale bars, 20 μm. The lysates from the cells analyzed above were subjected to Western blot analysis and probed with BRIT1 antibody (right) to confirm the expression of the exogenous FLAG-BRIT1 and FLAG-BR7 protein.

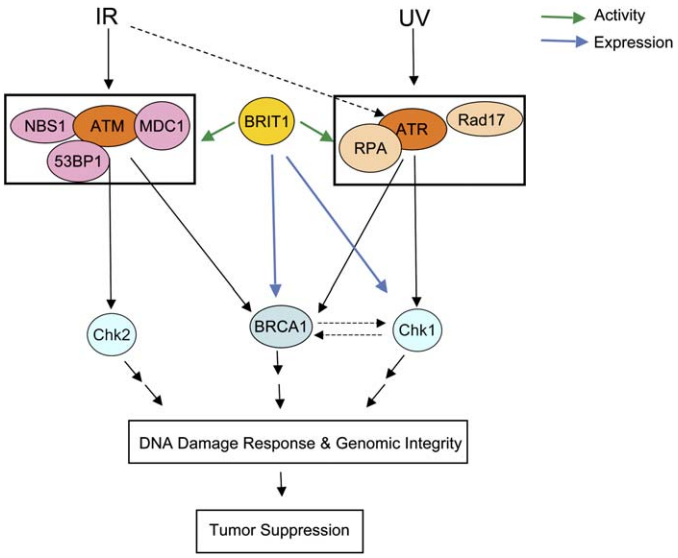



**Figure 7.** A model of BRIT1 functions in checkpoint signaling, chromosomal integrity, and cancer suppression

In addition to regulating BRCA1 and Chk1 expression, BRIT1 is also required for the function of DNA damage sensors or mediators in both the ATM and ATR pathways; BRIT1 is also required for the maintenance of chromosomal integrity and, potentially, for tumor suppression.

to the DSBs and form the IRIF. Recent studies have implicated MRN, possibly in a complex with MDC1 or 53BP1, in ionizing irradiation-induced ATM activation (Goldberg et al., 2003; Lee and Paull, 2005; Stewart et al., 2003). Because BRIT1 is required for the recruitment of NBS1 (a component of the MRN complex), MDC1, and 53BP1 to damaged DNA loci, it may function as a key regulator in initiating the ATM-dependent response. In fact, we found that knockdown of BRIT1 reduced IR-induced Chk2, NBS1, and SMC1, but not ATM phosphorylation (Figure S5; Lin et al., 2005), suggesting that BRIT1 was not required for autophosphorylation of ATM but the formation of p-ATM foci and the ATM downstream signaling.

Our findings also implicate BRIT1 in the ATR pathway after UV irradiation. Three complexes, RPA, ATR-ATRIP, and Rad17, are among the earliest response elements in this pathway (Unsal-Kacmaz et al., 2002; Zou and Elledge, 2003; Zou et al., 2003; Sancar et al., 2004). UV irradiation results in increases in ssDNA, which becomes coated by RPA (Zou and Elledge, 2003). The RPA-coated ssDNA then recruits the ATR-ATRIP complex and facilitates its recognition of substrates for phosphorylation and signal transduction (Zou and Elledge, 2003). In fact, RPA is important not only for the recruitment of the ATR complex but also for damage recognition by the Rad17 complex (Zou et al., 2003). Our findings raise the possibility that BRIT1 regulates the binding of the RPA to damaged DNA, which, in turn, recruits the Rad17 complex to initiate the ATR signaling. While our manuscript was under review, a study on MCPH1/BRIT1 was published in which the authors reported that MCPH1 had a function downstream of Chk1 in the ATR-signaling pathway (Alderton et al., 2006). In fact, we also found a direct association between BRIT1 and Chk1, suggesting that MCPH1/BRIT1 may regulate the ATR signaling at multiple levels (data not shown). We should note that the truncating mutations observed in MCPH syndrome patients do not impact on Chk1 or BRCA1 expression or early ATR-dependent damage-induced phosphorylation events (Alderton et al., 2006). As authors mentioned, the differential defects observed may have been due to the different tissue types or due to the incomplete loss of function of their mutants. Therefore, the functional defects observed from the MCPH1 mutants in microcephaly patients may be very different from the defects in BRIT1 knockdown or in the cancer cells.

We also show here that BRIT1 depletion did not interfere with the formation of radiation-induced γ-H2AX foci, indicating that BRIT1 functions in parallel with or downstream of γ-H2AX in the signal pathways. In fact, when we performed a kinetic study, we observed enhanced γ-H2AX levels and prolonged γ-H2AX responses in BRIT1 knockdown cells analyzed by Western blot analysis (Figure S6A) and fluorescent staining (Figure S6B). This observation may have been due to the impaired DNA repair in BRIT1 knockdown cells, a phenomenon observed in Rpa1 mutant mice (Wang et al., 2005a). Since both ATM and ATR signaling are defective when BRIT1 is knocked down, the IR-induced H2AX phosphorylation in BRIT1 knockdown cells is likely regulated by other kinases, such as DNA-PK (Stiff et al., 2004). Indeed, DNA-PK inhibitor dramatically reduced γ-H2AX foci formation in the BRIT1 knockdown cells (Figure S6C).

Disruption of the mechanisms that regulate DNA damage responses result in chromosomal aberrations and genomic instability. For example, haploinsufficiency for a variety of DNA damage genes, including ATM, ATR, γ-H2AX, and Chk1 (Barlow et al., 1999; Bassing et al., 2003; Celeste et al., 2003; Fang et al., 2004; Lam et al., 2004), or knockdown or knockout of NBS1 (Difilippantonio et al., 2005; Zhang et al., 2005), RPA (Balajee and Geard, 2004; Wang et al., 2005a), Rad17 (Budzowska et al., 2004), BRCA1 (Shen et al., 1998), and BRCA2 (Tutt and Ashworth, 2002), is associated with genomic instability. Similarly, cells lacking Rad17 were recently reported to exhibit acute chromosomal aberrations including chromosomal breakage, deletion, and endoduplication (Wang et al., 2003). siRNA knockdown of ATR in cells has also been shown to drastically increase chromosomal gaps and breaks (Casper et al., 2002). Consistent with a crucial role of BRIT1 in controlling DNA damage responses, we found that BRIT1 depletion led to significant increases in chromosomal aberrations in both nontransformed cells and cancer cell lines. The types of aberrations identified imply that BRIT1 knockdown probably leads to chromosomal instability through breakage-fusion-bridge cycles and subsequent missegregation and breakage during mitosis.

Loss of DNA damage checkpoint function and acquisition of the ability to proliferate indefinitely are two of the fundamental changes required for the development of cancer. BRIT1 was originally identified as an hTERT repressor that potentially prevents hTERT reactivation and cellular immortalization. Recent reports suggest that loss of the checkpoint responses is

**C:** MCF7 cells were transfected with FLAG-V, FLAG-BRIT1, or FLAG-BR7 constructs. Forty-eight hours after transfection, cells were treated with either UV (50 J/m$^2$) or IR (10 Gy). One hour after irradiation, cells were fixed and stained with the p-RPA and p-ATM antibodies, respectively. Cells were then washed and subsequently stained with rhodamine- or FITC-conjugated antibodies. Nuclei were visualized by DAPI staining. The lysates from the cells analyzed above were subjected to Western blot analysis and probed with BRIT1 antibody (right) to confirm the expression of the exogenous FLAG-BRIT1 and FLAG-BR7 protein.

a hallmark of cancer cells and is an early step in the development of cancer (Bartkova et al., 2005; Gorgoulis et al., 2005; Kastan and Bartek, 2004; Motoyama and Naka, 2004). Because BRIT1 has a dual role in controlling these two critical activities, we believe that BRIT1 deficiency may contribute to the development of cancer in humans. *BRIT1* is located on chromosome 8p23.1, a region implicated in the development of several malignancies, including breast, ovarian, and prostate cancer (Chan et al., 2002; DeMarzo et al., 2003; Lassus et al., 2001; Miller et al., 2003; Pribill et al., 2001; Shao et al., 2000; Thor et al., 2002; Veltman et al., 2003). Indeed, 8p23.1 is one of two most common sites of allelic loss or chromosomal deletions in prostate cancer (Bookstein, 2001; DeMarzo et al., 2003). Our findings with regard to *BRIT1* gene content and RNA and protein expression in different cancer specimens and the *BRIT1* gene aberration identified in a breast cancer patient are consistent with a potential role of BRIT1 as a tumor suppressor gene. Further, *BRIT1* status seems to correlate with the occurrence of metastasis and duration of the relapse-free interval in patients with breast cancer, suggesting that BRIT1 may contribute to tumor aggressiveness. Thus, BRIT1 defection seems to be a key pathological alteration in cancer initiation and progression, and as such, further understanding of its function may well contribute to novel, effective therapeutic approaches for cancer.

## Experimental procedures

### Cells

U2OS cells and breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). The U2OS cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum with glutamine, penicillin, and streptomycin; the breast cancer cell lines were maintained in the recommended media. Normal human mammary epithelial cells were purchased from Cambrex Corp. (East Rutherford, NJ) and maintained in Mammary Epithelial Basal Medium.

### siRNA

The siRNA duplexes were 19 base pairs with a 2 base deoxythymidine overhang (Dharmacon Research). The sequences of BRIT1 siRNA#1, #2, and #3 oligonucleotides are AGGAAGUUGGAAGGAUCCAdTdT, CUCUCUGUGU GAAGCACCUdTdT, and CUGGCGUAUACAAGAUGACdTdT, respectively. The control Luciferase siRNA has the following sequence: UAAGGCUAU GAAGAGAUACdTdT. Cells were transfected with siRNA duplexes using Oligofectamine (Invitrogen) following the manufacturer's instructions.

### Antibodies

BRIT1 antibody was generated by using a GST-BRIT1 fusion protein synthesized by Proteintech (Chicago, IL). Anti-ATR (N-19) and anti-Orc2 (C-18) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p-Rad17 (Ser645), p-Chk2, and anti-p-NBS1 antibodies were purchased from Cell Signaling Technology (Beverly, MA); anti-RPA70, anti-NBS1, anti-p-ATM, and anti-$\gamma$-H2AX antibodies were purchased from Upstate Biotechnology (Lake Placid, NY); anti-ATM antibody was purchased from Novus Biologicals (Littleton, CO); anti-RPA32 antibody was purchased from Neomarkers (Fremont, CA); p-RPA32-Ser4/Ser8 and p-SMC1 antibodies were purchased from Bethyl Laboratories (Montgomery, TX); and MDC1 antibody was purchased from Sigma (St. Louis, MO).

### Immunofluorescence staining

Cells cultured on coverslips were washed twice in phosphate-buffered saline (PBS), incubated in cytoskeleton buffer (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] [pH 6.8], 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM ethylene glycol bis-2-aminoethyl ether-N,N′,N″,n′-tetraacetic acid [EGTA], and 0.5% Triton X-100) for 3 min on ice. The cells were then washed with ice-cold PBS three times and incubated in stripping buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 1% Tween 20, and 0.25% sodium deoxycholate) for 3 min on ice. After another three washes with ice-cold PBS, cells were fixed with 4% paraformaldehyde at 4°C for 30 min, permeabilized in 1% Triton X-100 and 0.5% NP-40 for another 30 min, blocked with 1% bovine serum albumin, and incubated with primary antibody for 2 hr and secondary antibody (fluorescein isothiocyanate, or rhodamine) for 1 hr. Cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclear DNA. The coverslips were mounted onto glass slides with VectaShield antifade (Vector Laboratories, Burlingame, CA) and visualized by using a Leica DM LB fluorescence microscope. Images were captured with a Kodak digital imaging system.

### Chromatin isolation

A total of $4 \times 10^6$ cells were washed with PBS and resuspended in 200 $\mu$l of solution A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol, 10 mM NaF, 1 mM $Na_2Vo_3$, and protease inhibitors). Triton X-100 was added to a final concentration of 0.1%, and the cells were incubated for 5 min on ice. Cytoplasmic proteins were separated from nuclei by low-speed centrifugation (4 min at 1300 × g at 4°C). The isolated nuclei were washed once with solution A and then lysed in 200 $\mu$l of solution B (3 mM ethylenediamine tetraacetic acid, 0.2 mM EGTA, 1 mM dithiothreitol, and protease inhibitors). Insoluble chromatin was collected by centrifugation (4 min at 1700 × g at 4°C), washed once in solution B, and centrifuged again at high speed (10,000 × g) for 1 min. The final chromatin pellet was resuspended in 200 ml of Laemmli buffer and sonicated for 15 s. Chromatin was digested by resuspending nuclei in solution A containing 1 mM $CaCl_2$ and 50 units of micrococcal nuclease (Sigma) and incubating at 37°C for 1 min, after which the nuclease reaction was stopped by the addition of 1 mM EGTA.

### Chromosome preparation

For chromosomal aberration studies, cells were transfected twice with control or BRIT1-specific siRNA. Seventy-two hours after the second siRNA transfection, cytological preparations were made following standard procedures. Briefly, cells were exposed to Colcemid (0.04 $\mu$g/ml) for 1 hr, subjected to hypotonic treatment (0.075 M KCl for 20–25 min at room temperature), and fixation in a mixture of methanol and acetic acid (3:1 by volume). Slides were stained in Giemsa and examined blindly for structural and numerical abnormalities. These slides were decoded only after the entire scoring of aberrations was completed. From each sample, a minimum of 35 metaphase spreads were analyzed, and representative spreads were captured using a Genetiscan imaging system.

### Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was performed by using total RNA (isolated with Trizol [Invitrogen]) and the one-step RT-PCR TaqMan Fast Universal PCR Master Mix from Applied Biosystems (Foster City, CA). A fluorogenic TaqMan probe for BRIT1 was designed based on a sequence from Genbank, and a $\beta$-actin probe was used as the loading reference.

### Immunohistochemical staining

Ten formalin-fixed, paraffin-embedded sections of prostate cancer tissues obtained from the institutional prostate cancer bank were used for BRIT1 detection as follows. After slides were deparaffinized with xylene and rehydrated through ethanol series, microwave antigen retrieval was carried out by placing the slides in 0.1 M sodium citrate buffer (pH 6.0) in a microwave oven at 98°C for 8 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 12 min. Slides were washed three times with PBS and incubated for 1 hr in protein blocking buffer (5% normal horse serum plus 1% normal goat serum in PBS). Immunohistochemical staining was done by using the Vectastain ABC avidin biotin-peroxidase enzyme complex kit (Vector Laboratories) and BRIT1 antibody (1:100 dilution). Cells were visualized by adding 0.05% 3-3′ diaminobenzidine and counterstained with Gill's No. 3 hematoxylin. In parallel, cell pellets from BRIT1-siRNA treated or untreated HMEC cells were processed at the same time with the same staining procedure to serve as positive and negative staining controls. The studies were performed according to protocols approved by the University of Texas M.D. Anderson Cancer Center Institutional Review Board, and all subjects provided written informed consent.

### Mutation analysis

For mutation analysis, full-length BRIT1 cDNAs were amplified from ten breast cancer samples using SuperScript III One Step RT-PCR Platinum Taq HiFi (Invitrogen). RT-PCR products were then cloned into Topo-XL PCR vector (Invitrogen) for sequencing.

### Array CGH

Array-based CGH was used to test 87 specimens of human serous ovarian cancer with bacterial artificial chromosome probes as described by Pinkel et al. (1998). Log base two logarithmic ratios of test to reference intensity were calculated for the probes across the region containing BRIT1. The studies were performed according to protocols approved by the University of Texas M.D. Anderson Cancer Center Institutional Review Board, and all subjects provided written informed consent.

### Supplemental data

The Supplemental Data include six supplemental figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/2/145/DC1/.

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