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PRINCIPAL INVESTIGATOR: Lei Zheng, Ph.D.
Nicholas Ting, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
Health Science Center at San Antonio
San Antonio, Texas 78229-3900

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6. AUTHOR(S)
Lei Zheng, Ph.D.
Nicholas Ting, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas
Health Science Center at San Antonio
San Antonio, Texas 78229-3900
E-Mail: ting@uthscsa.edu

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13. ABSTRACT ***(Maximum 200 Words)***

Mutations in the breast cancer susceptibility gene *BRCA1* account for 50% of familial cases, and about 2 to 5% of all cases of breast cancer. Most of the *BRCA1* linked tumors have undergone loss of heterozygozity at this locus, classifying *BRCA1* as a tumor suppressor. Substantial evidence implicates BRCA1 to be involved in cellular DNA damage response and DNA repair pathways. Mouse cells deficient for *Brca1* show genetic instability, defective G2/M checkpoint control and reduced homologous recombination. BRCA1 also interacts with proteins of the DNA repair machinery and regulates expression of *p21* and *GADD45* genes. However, it remains unclear how DNA damage signals are transmitted to modulate the repair function of BRCA1. Previous work from our laboratory have shown that BRCA1 becomes hyperphosphorylated in a cell cycle dependent manner and in response to genotoxic insults. Here, we further investigated how this phosphorylation event contributes to the cellular function of BRCA1. Our results revealed a novel DNA damage response pathway that involves the protein kinase mutated in Ataxia telangiectasia (ATM), a BRCA1 associated protein, CtIP, and BRCA1, thus providing a potential link between ATM deficiency and breast cancer.

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INTRODUCTION:

Mutations to the *BRCA1* gene are responsible for nearly 50% of inherited cases of breast cancer. However, the precise mechanism by which BRCA1 contributes to the progression of tumor development remains to be elucidated. Recent evidence have suggested that BRCA1 is important for cellular DNA damage repair and/or DNA damage response pathway [reviewed in 1, 2]. First, mechanistic studies have shown that BRCA1 associates with the DNA repair protein triplex, Mre11/Rad50/NBS1 to form distinct nuclear foci that correlates to sites of DNA damage in cells treated with genotoxic agents [3]. Second, overexpression of BRCA1 induces the expression of GADD45 and p21, two DNA-damage responsive genes [4,5]. Based on these reports, it has been proposed that absence of functional BRCA1 leads to aberrant repair of DNA, which in turn, can lead to genomic instability and ultimately, tumorigenesis.

Previous work in our laboratory has shown that BRCA1 is a 220 kDa nuclear protein that becomes hyperphosphorylated in a cell-cycle dependent manner [6]. BRCA1 also becomes hyperphosphorylated when cells are treated with genotoxic agents [4,7]. We have also noted that a BRCA1 associated protein, called CtIP (for C-terminal interacting protein), is also phosphorylated upon exposure of cells to ionizing radiation (IR). CtIP was originally isolated by its association with the adenovirus E1A co-repressor protein CtBP [4]. CtIP binds to the C-terminal region of BRCA1, and this association is disrupted upon IR *in vivo* [4]. Co-expression of BRCA1 with CtIP and co-repressor CtBP, is able to repress BRCA1-mediated transcriptional activity from the p21 promoter (4). Therefore, we were interested in identifying the protein kinase that is responsible for this DNA damage induced phosphorylation of CtIP and BRCA1, and study how this event contributes to the function of BRCA1 in the DNA damage repair and response pathway.

BODY:

To this end we initially tested the DNA-damage induced phosphorylation status of CtIP and BRCA1, in cell lines deficient for two protein kinases involved in DNA damage signaling: DNA-dependent protein kinase (DNA-PK) and ATM (for mutated in Ataxia Telangiectasia) [reviewed in 8]. In DNA-PK deficient cells, the IR induced phosphorylation of BRCA1 and CtIP was observed. On the other hand, the IR induced phosphorylation of CtIP was absent, while IR induced phosphorylation of BRCA1 was slightly compromised in ATM deficient cells. Because the reduction of IR induced phosphorylation of BRCA1 was not as prominent compared to CtIP in the ATM deficient cells, we concentrated our studies on CtIP. Transfection of wild type ATM into ATM deficient cells restored IR-induced phosphorylation of CtIP, suggesting that CtIP is a target for ATM kinase following DNA damage.

Ataxia Telangiectasia is a rare autosomal recessive genetic disorder characterized by immune system deficiencies, growth retardation, neuronal degeneration, and a 100 fold increase in the incidence of some cancers such as leukemia and lymphoma [reviewed in 9]. The gene product encodes for a 220 kDa serine/threonine protein kinase that is required for p53 activation following DNA damage [8]. Inspection of the amino acid sequence of CtIP revealed that it has several ATM kinase consensus phosphorylation sites, but only two sites are conserved in mouse and human, Ser 664 and Ser 745. We substituted these two serine residues to alanine, and expressed recombinant wild type CtIP or mutant CtIP (S664/745A) and asked whether they are substrates of ATM. Immunoprecipitated ATM was able to phosphorylate CtIP(wt), but not CtIP(S664/745A) *in vitro*. We then performed two-dimensional phosphopeptide maps to show that Ser 664 and Ser 745 on CtIP are phosphorylated *in vivo* following IR.

The next set of experiments were performed to determine the consequence of IR induced ATM-dependent phosphorylation of CtIP. Immunoprecipitation studies indicated that ATM exists in the previously determined CtIP-BRCA1 complex *in vivo* [4]. Following DNA damage, CtIP dissociates from BRCA1; however, in ATM deficient cells, the association between BRCA1 and CtIP persisted after IR. We then transfected flag-tagged CtIP(wt) or CtIP(S664/745A) into ATM proficient cells and performed immunoprecipitation studies with anti-flag antibodies. Following IR, the CtIP(wt)/BRCA1 complex, but not the CtIP(S664/745A)/BRCA1 complex was disrupted, suggesting that ATM dependent phosphorylation of Ser 664 and Ser 745 on CtIP is required for dissociation of CtIP from BRCA1 following DNA damage.

We have shown that BRCA1 can promote transcriptional activity from the p21 promoter [4]. It has been recently shown that BRCA1 induced the expression of *GADD45*, and this induction requires the intron 3 region of *GADD45* [5]. To further investigate the significance of IR-induced ATM-dependent phosphorylation of CtIP, we assayed transcriptional activity using a luciferase reporter construct containing the intron 3 sequence of *GADD45* (pI3). As expected, expression of BRCA1 alone induced pI3 reporter activity by about 5 fold in human osteosarcoma U20S cells (ATM proficient). Co-expression of BRCA1 with CtIP/CtBP led to repression of pI3 reporter activity, which was relieved following exposure of cells to IR. This derepression was not seen with cells co-expressing BRCA1 with the mutant CtIP(S664/745A) and CtBP, suggesting that phosphorylation on these serine sites is critical for relief of repression. Consistently, the derepression of transcriptional activity from the pI3 reporter was not observed in ATM deficient cells following IR treatment. Taken together, these data suggest that IR induced phosphorylation of CtIP on Ser 664 and Ser 745 by ATM is required to dissociate CtIP/CtBP from BRCA1, enabling BRCA1 to participate in the expression of *GADD45*.

To further explore the biological significance of the phosphorylation of CtIP by ATM, we established MCF7 cell lines that constitutively expressed wild type CtIP

(CtIP-wt) or CtIP harboring serine to alanine mutations at amino acid positions 664 and 745 (CtIP-S664/745A). We observed that cells expressing CtIP-S664/745A were approximately 3 fold more sensitive to IR when compared to cells expressing CtIP-WT. Mammalian cells will arrest at different phases of the cell cycle in response to genomic insults to allow for repair, thus preventing propagation of damaged DNA. Since GADD45 has been implicated in the checkpoint control of G2 to M phase of the cell cycle in response to DNA damage [14], we tested the status of the G2/M checkpoint in cells expressing CtIP-S664/745A. Following exposure to IR, analysis of the mitotic index in these cells revealed that cells expressing CtIPS664/745A showed a defect in G2/M checkpoint. As expected, the level of GADD45 in these cells were decreased approximately 2.5 folds, compared to cells expressing WT-CtIP. In short, these results suggest that phosphorylation of CtIP by ATM at Ser664 and Ser745 is important to arrest cells at the G2/M boundary, mediated in part, through the expression of GADD45, following DNA damage.

KEY RESEARCH ACCOMPLISHMENTS:

- Our results have provided evidence for a functional connection between the gene mutated in Ataxia Telangiectasia, *ATM*, and the breast cancer susceptibility gene *BRCA1* in cellular response to DNA damage.
- We have identified a novel DNA damage response pathway, involving BRCA1, CtIP and ATM that could explain how the absence of BRCA1 and ATM may be involved in the genesis of cancer.
- More specifically, we have determined that phosphorylation of CtIP by ATM following DNA damage is required to release BRCA1 to participate in the expression of GADD45, which is crucial to establish a checkpoint control at the G2/M cell cycle boundry.

REPORTABLE OUTCOMES:

Meeting presentation:

- Li, S., Ting N.S.Y., Zheng, L., Ziv, Y., Chen, P-L., Shiloh, Y., Lee, E.Y-H., Lee, W-H. *Functional link of ATM and BRCA1 in DNA damage response.* Era of Hope, Dept. of Defense Breast Cancer Research Program Meeting, Atlanta, GA. June 2000

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- Li, S., Ting N.S.Y., Zheng, L., Ziv, Y., Chen, P-L., Shiloh, Y., Lee, E.Y-H., Lee, W-H. *Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response.* Nature **406**: 210-215 (2000).

CONCLUSIONS:

These results are reported in a manuscript entitled, Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response, published in *Nature* [10]. Recent work have also shown that Ser 1423 and Ser 1524 on BRCA1 is phosphorylated by ATM in response to IR [11]. Mutation of these serines to alanine, resulted in aberrant BRCA1 mediated cellular response to DNA damage [11]. Combined with our data, a link between ATM and BRCA1 has been established, which may explain the increased risk of breast cancer in certain Ataxia Telangiectasia heterozygotes [12, 13]. It is likely that in one DNA damage response pathway ATM transduces the DNA damage signal by phosphorylating CtIP and BRCA1. Phosphorylation of CtIP by ATM dissociates BRCA1 from the CtIP/CtBP repressor complex, subsequently enabling BRCA1 to partake in the induction of p21 and GADD45. We have further shown that the phosphorylation event on CtIP is important for the expression of GADD45 which is crucial to establish a G2/M cell cycle checkpoint. In the absence of functional ATM, the activity of BRCA1 and CtIP may become disregulated leading, in turn, to a defect in the cellular response to DNA damage, which may lead genomic instability and tumorigenesis.

In sum, these results have provided insights into the function of BRCA1 and ATM, which subsequently provides a basis for developing treatment for BRCA1 and ATM associated cancers.

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Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response

Shang Li*, Nicholas S. Y. Ting*, Lei Zheng*, Phang-Lang Chen*, Yael Ziv†, Yosef Shiloh†, Eva Y.-H. P. Lee* & Wen-Hwa Lee*

* *Department of Molecular Medicine/Institute of Biotechnology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78245, USA*
† *Department of Human Genetics and Molecular Medicine, Sackler School of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel*

BRCA1 **encodes a familial breast cancer suppressor that has a critical role in cellular responses to DNA damage[1,2]. Mouse cells deficient for *Brca1* show genetic instability, defective G2–M checkpoint control and reduced homologous recombination[3,4]. BRCA1 also directly interacts with proteins of the DNA repair**

machinery[5] and regulates expression of both the *p21* and *GADD45* genes[6–8]. However, it remains unclear how DNA damage signals are transmitted to modulate the repair function of BRCA1. Here we show that the BRCA1-associated protein CtIP[9–12] becomes hyperphosphorylated and dissociated from BRCA1 upon ionizing radiation. This phosphorylation event requires the protein kinase (ATM) that is mutated in the disease ataxia telangiectasia[13]. ATM phosphorylates CtIP at serine residues 664 and 745, and mutation of these sites to alanine abrogates the dissociation of BRCA1 from CtIP, resulting in persistent repression of BRCA1-dependent induction of GADD45 upon ionizing radiation. We conclude that ATM, by phosphorylating CtIP upon ionizing radiation, may modulate BRCA1-mediated regulation of the DNA damage-response *GADD45* gene, thus providing a potential link between ATM deficiency and breast cancer.

BRCA1 interacts with the transcriptional co-repressor complex of CtIP and CtBP through its BRCT domains, and DNA damage-induced dissociation of BRCA1 from this complex may be important for BRCA1 function[9]. To explore the mechanism regulating this interaction, we examined the phosphorylation of CtIP in T24 cells treated with ionizing radiation (IR), ultraviolet or methylmethane sulphonate (MMS). CtIP immunoprecipitated from extracts of treated cells showed slower migrating forms (Fig. 1a, compare lanes 3–5 with lane 2), which were especially prominent in cells γ-irradiated at the dose of 20–40 Gy (Fig. 1a, lane 3; and Fig. 1b). The alteration in electrophoretic mobility was attributed to phosphorylation, as it was sensitive to λ-phosphatase but inhibited by NaF and Na_3VO_4 (Fig. 1c). λ-Phosphatase-treated CtIP migrated faster than the CtIP immunoprecipitated from control cells (Fig. 1c, compare lanes 4 and 2), suggesting that CtIP is a phosphoprotein that becomes hyperphosphorylated after exposure of cells to DNA-damaging agents.

The phosphatidylinositol3-OH kinase [PI(3)K] related family of serine/threonine protein kinases, which includes ATM and DNA-PK (DNA-dependent protein kinase), have been implicated in cell signalling events in response to DNA double-strand breaks[13,14]. It appears that a PI(3)related kinase is involved in the phosphorylation of CtIP, as the IR-induced hyperphosphorylation of CtIP was inhibited in cells treated with the PI(3)K inhibitor, wortmannin (20 μM) (Fig. 1d, lane 4). In two isogenic cell lines, M059J (with deficient DNA-PK activity) and M059K (with normal levels of DNA-PK activity)[15], the IR-induced hyperphosphorylation of CtIP remains unchanged (data not shown); however, the hyperphosphorylated forms of CtIP appeared only in ATM wild-type GM00637G cells, but not in ATM-deficient GM09607A cells after IR (Fig. 1e). Two other ATM-deficient cell lines, GM05849B and GM02052C, showed identical results (Fig. 1f). Similarly, in two isogenic stable clones derived from ATM-deficient fibroblasts (AT22IJE-T) carrying either vector alone (plasmid pEBS7) or Flag-tagged wild-type ATM (plasmid pEBS7-YZ5)[16], CtIP hyperphosphorylation was restored only in cells expressing wild-type ATM (Fig. 1g, bottom panel, compare lanes 2 and 4). The presence of wild-type ATM in these cells was confirmed by western blot analysis (Fig. 1g, top panel). These observations suggest that ATM is responsible for the phosphorylation of CtIP following IR.

ATM has been shown to phosphorylate Ser 15 in the highly conserved amino terminus of p53 with the sequence $_{10}$VEPPLSQE$_{17}$ in response to IR[17,18]. The amino-acid sequence of CtIP has six SQ sites bearing similar sequences surrounding Ser 15 of p53. Two of these serines, 664 and 745, are conserved between human and

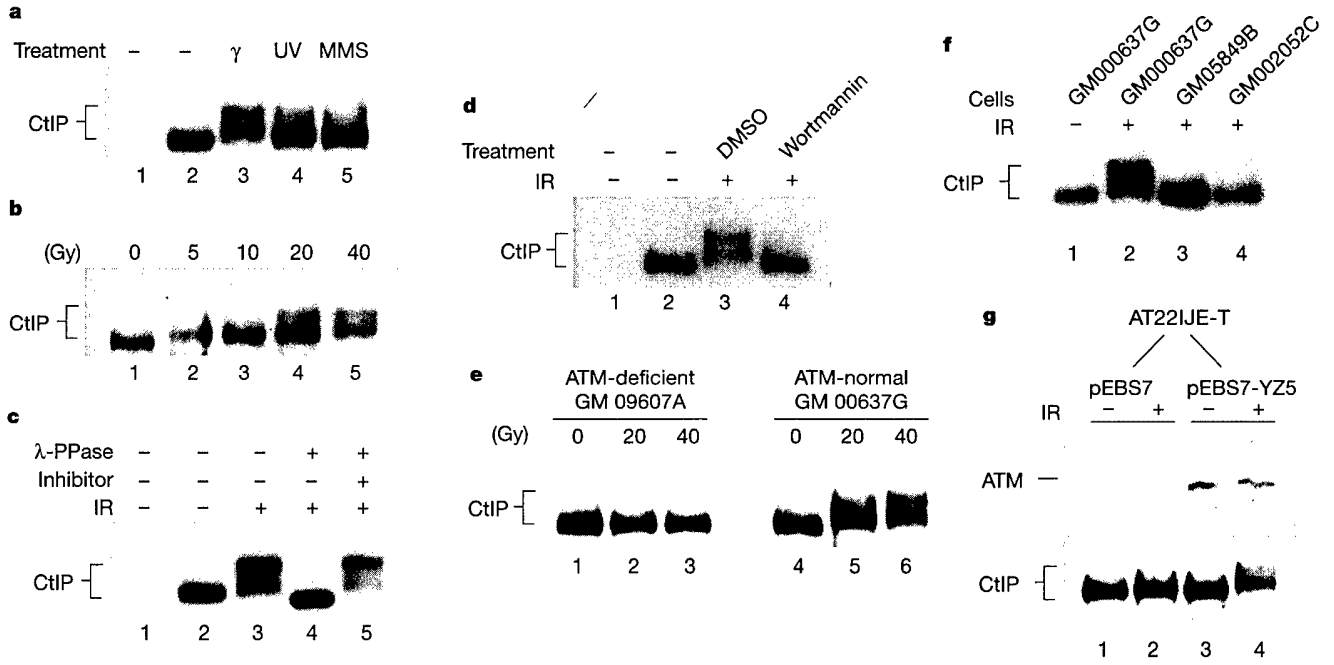

Figure 1 IR-induced phosphorylation of CtIP is ATM dependent. **a**, Change in CtIP gel mobility upon DNA damage. T24 cells were untreated (–) or treated with IR (γ), ultraviolet (UV) or MMS; extracts were immunoprecipitated using anti-CtIP antibody (lanes 2–5) and immunoblotted for CtIP. **b**, Dose-dependent IR phosphorylation of CtIP. T24 cells treated with IR (5–40 Gy) were immunoblotted for CtIP. **c**, Changes in mobility are due to the phosphorylation of CtIP. CtIP immune complexes from untreated (–) or γ-irradiated (+) T24 cells were untreated or treated with λ-phosphatase ± phosphatase inhibitors, as indicated (lanes 2–5), and immunoblotted with anti-CtIP antibodies. **d**, Inhibition of CtIP phosphorylation by wortmannin. T24 cells were untreated (lanes 1 and 2), or pre-incubated with either dimethyl-sulphoxide (DMSO; lane 3) or 20 μM wortmannin (lane 4) before IR (40 Gy); the extracts were then immunoprecipitated and blotted for CtIP (lanes 2–4). Extracts were immunoprecipitated using pre-immune serum in lane 1 (**a**,**c**,**d**). **e**, Phosphorylation of CtIP upon IR is compromised in ATM-deficient cells. Cell extracts from ATM-deficient (GM09607A, lanes 1–3) or normal human fibroblasts (GM00637G, lanes 4–6) treated with IR for the indicated dosages were immunoblotted for CtIP. **f**, Absence of CtIP hyperphosphorylation in additional ATM-deficient cell lines (GM05849B, lane 3; GM02052C, lane 4) upon IR (40 Gy). **g**, Ectopic expression of wild-type ATM in A-T cells restored the phosphorylation of CtIP upon IR. ATM-deficient AT22IJE-T cells were stably transfected with pEBS7 (vector) or pEBS7-YZ5 (carrying Flag-tagged wild-type ATM cDNA). Extracts from IR-treated cells (40 Gy) were immunoblotted with monoclonal antibodies: 2C1, for ATM (top); C11 for CtIP (bottom).

mouse (Fig. 2a), and we mutated these to alanines. Recombinant glutathione *S*-transferase (GST)–CtIP proteins carrying either the double mutations [GST–CtIP(C-S664/745A)] or no mutation [GST–CtIP(C)] were expressed and isolated for *in vitro* ATM kinase assays[17,18]. Immunoprecipitated Flag-tagged wild-type ATM was capable of phosphorylating recombinant GST–CtIP(C), but not GST–CtIP(C-S664/745A) (Fig. 2b, bottom panel, compare lanes 2 and 5), whereas inactive mutant ATM(D2870A) did not phosphorylate either GST–CtIP(C) or GST–CtIP(C-S664/745A) (Fig. 2b, bottom panel, lanes 3 and 6,). The presence of Flag-tagged ATM and ATM(D2870A) was confirmed by immunoblot analysis (Fig. 2b, top panel). These data indicate that ATM may directly phosphorylate CtIP on Ser 664 and Ser 745 *in vitro*.

To determine whether these residues on CtIP are phosphorylated *in vivo*, we transfected Flag-tagged CtIP(WT), CtIP(S664A) or CtIP(S664/745A) into human osteosarcoma U2OS cells and metabolically labelled these cells with inorganic ^{32}P-phosphate. After IR, CtIP(WT), CtIP(S664A) or CtIP(S664/745A) were immunopreci-

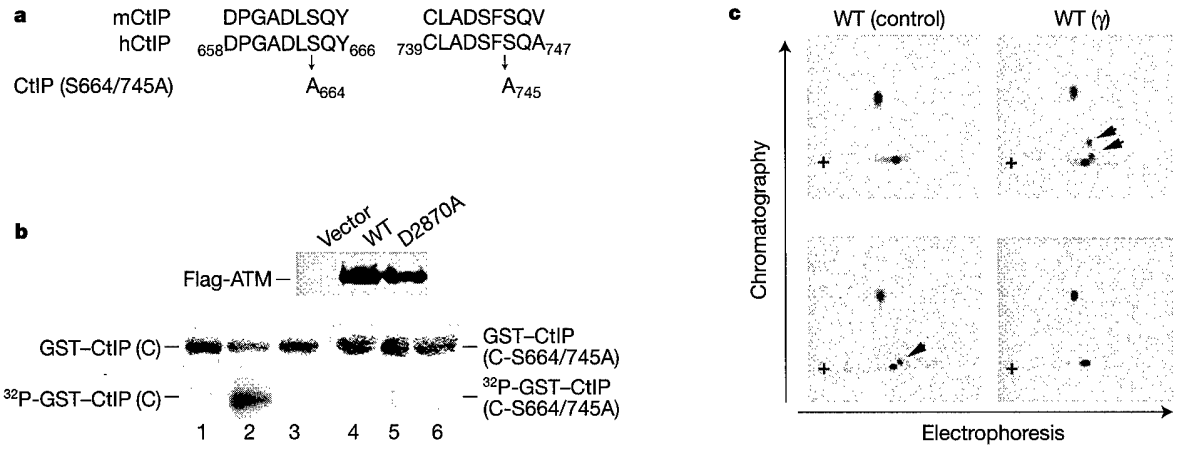

Figure 2 Identification of ATM phosphorylation sites on CtIP. **a**, Sequences of two potential ATM-phosphorylation sites in CtIP and the alignment between human (hCtIP) and mouse (mCtIP) CtIP. For the CtIP phosphorylation site mutant, CtIP(S664/745A), Ser 664 and Ser 745 were mutated to alanine. **b**, Phosphorylation of CtIP by ATM-associated kinase *in vitro*. 293 cells were transiently transfected with vector alone (lanes 1 and 4), or vectors expressing Flag-tagged wild-type ATM (WT) (lanes 2 and 5) or mutated ATM(D2870A) (lanes 3 and 6). Transfected cell extracts were immunoprecipitated with anti-Flag mAb for *in vitro* kinase assay using GST–CtIP(C) (lanes 1–3) and GST–CtIP(C-S664/745A) (lanes 4–6) as substrates. Top, western blot of ectopically expressed ATM using anti-Flag mAb. Middle, Coomassie blue gel of GST–CtIP(C) and GST–CtIP(C-S664/745A). Bottom, autoradiogram of phosphorylated GST–CtIP(C) and CtIP(C-S664/745A). **c**, Phosphorylation of Ser 664 and Ser 745 of CtIP *in vivo* upon IR. U2OS cells transiently transfected with Flag-tagged CtIP(WT), CtIP(S664A) or CtIP(S664/745A) expression vectors, were metabolically labelled with ^{32}P, then either untreated or treated with IR (20 Gy). CtIP was isolated for tryptic phosphopeptide mapping analysis[19]. Two new spots appeared for CtIP(WT) upon IR (arrowheads). Mutation of Ser 664 to alanine resulted in the disappearance of one IR-induced spot, whereas mutation of both Ser 664 and Ser 745 to alanine resulted in the loss of both spots on the tryptic phosphopeptide map.

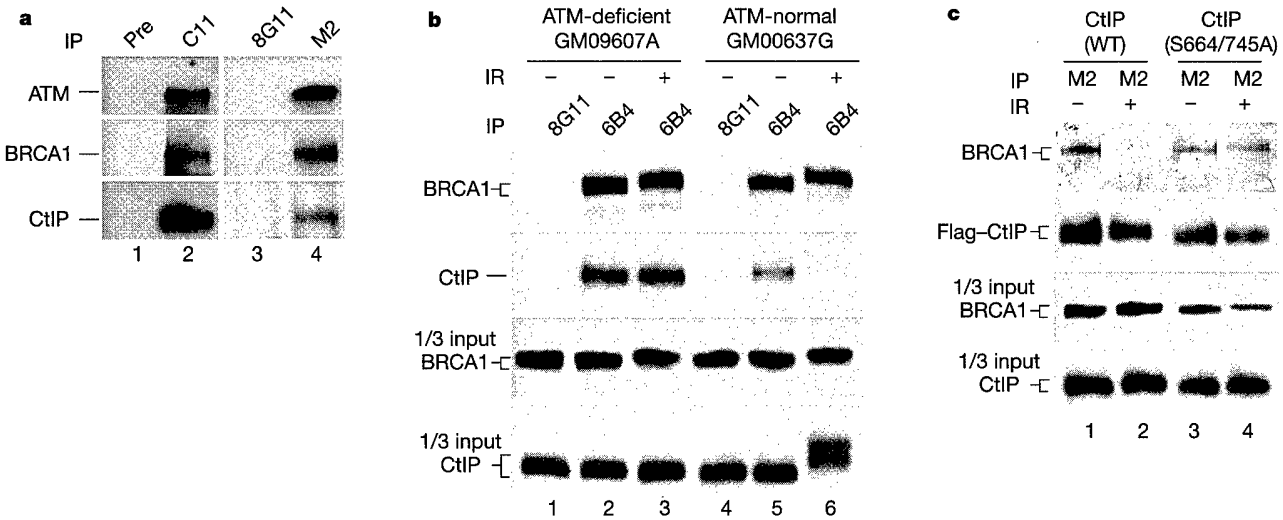

Figure 3 Phosphorylation of CtIP by ATM is essential for dissociation of CtIP from BRCA1 upon IR. **a**, Formation of ATM–BRCA1–CtIP complex *in vivo*. HCT116 cell lysates were immunoprecipitated using pre-immune serum (lane 1) or anti-CtIP polyclonal antibody, C11 (lane 2), and immunoblotted for ATM, BRCA1 or CtIP. In lanes 3 and 4, HCT116 cells were transiently transfected with Flag-tagged ATM expression construct. The lysates were immunoprecipitated using anti-GST (8G11) monoclonal antibody (mAb) as a control or anti-Flag mAb, and immunoblotted for ATM, BRCA1 or CtIP. **b**, Dissociation of BRCA1 and CtIP is abrogated in ATM-deficient cells upon IR. ATM deficient (GM09607A, lanes 1–3) or normal human fibroblasts (GM00637G, lanes 4–6) were untreated (–) or treated with IR (+, 40 Gy). Extracts were immunoprecipitated using control (8G11) mAb or anti-BRCA1 (6B4) mAb, and immunoblotted for BRCA1 (top) or CtIP (bottom). Total input of BRCA1 and CtIP is also shown. **c**, CtIP phosphorylation mutant fails to dissociate from BRCA1 upon IR. Flag-tagged wild-type (lanes 1 and 2) or mutated (lanes 3 and 4) CtIP were transiently transfected into U2OS cells, and the cells were either untreated (–) or treated with IR (40 Gy). The extracts were immunoprecipitated using anti-Flag mAb (M2). Top, immunoblot for BRCA1; bottom, immunoblot for Flag-tagged CtIP. Total input of BRCA1 and Flag-tagged CtIP is also shown.

pitated with anti-Flag antibody (M2), and subjected to two-dimensional tryptic peptide analysis[19]. Two additional tryptic ^{32}P-phosphopeptide spots arose from CtIP(WT) immunoprecipitated from γ-irradiated cells compared with non-irradiated cells (Fig. 2c, top panel, arrows); however, both of these two ^{32}P-phosphopeptide spots were absent in CtIP(S664/745A), and only one of these ^{32}P-phosphopeptides spots was present in CtIP(S664A) (Fig. 2c, bottom panel). Together, these data indicate that both Ser 664 and Ser 745 on CtIP may be phosphorylated *in vivo* in response to IR.

To explore the biological significance of ATM-dependent phosphorylation of CtIP upon IR, we initially tested whether ATM associates with the CtIP and BRCA1 complex *in vivo* by co-immunoprecipitation. BRCA1, CtIP and ATM were co-immunoprecipitated from the human colon carcinoma cell line HCT116 using an anti-CtIP polyclonal antibody (Fig. 3a, compare lanes 1 and 2). As the ATM-specific antibody, 2C1, was not efficient for immunoprecipitation, HCT116 cells were first transfected with a Flag-tagged wild-type ATM expression vector, and subsequently immunoprecipitated with anti-Flag M2 antibody. BRCA1 and CtIP were both co-immunoprecipitated with Flag-tagged ATM (Fig. 3a, lane 4). These data suggest that ATM is present in the previously identified BRCA1–CtIP complex *in vivo*.

We then proceeded to evaluate the consequence of IR-induced ATM-dependent phosphorylation of CtIP on CtIP–BRCA1 complex formation. In the absence of IR, CtIP associated with BRCA1 in both ATM-deficient (GM09607A) and ATM-normal (GM00637G) cells (Fig. 3b, lanes 2 and 5). Upon IR, CtIP dissociated from BRCA1 in ATM-normal cells, but not in ATM-deficient cells (Fig. 3b, compare lanes 3 and 6). Western blot analysis of the cell lysates clearly indicated that IR-induced hyperphosphorylation of CtIP is absent in ATM-deficient cells (Fig. 3b, '1/3 input' panel, compare lanes 3 and 6). Because ATM also phosphorylates BRCA1 (ref. 20), the persistent association of CtIP and BRCA1 in ATM-deficient cells after IR could be attributed to a deficiency in the phosphorylation of BRCA1, of CtIP, or of both. To discriminate between these alternatives, we performed similar experiments with U2OS cells transiently transfected with Flag-tagged CtIP(WT) or CtIP(S664/745A). BRCA1 associated with both CtIP(WT) or CtIP(S664/745A) in the absence of IR (Fig. 3c, lanes 1 and 3), whereas BRCA1 dissociated from CtIP(WT), but remained associated with CtIP(S664/745A) upon IR (Fig. 3c, compare lanes 2–4). Because U2OS cells contain wild-type ATM, IR-induced ATM-dependent phosphorylation of BRCA1 has no apparent effect on the status of the CtIP–BRCA1 complex (Fig. 3c). It should be noted that the change in electrophoretic mobility of Flag-tagged wild-type CtIP was less prominent after IR, perhaps due to the addition of the Flag epitope. These

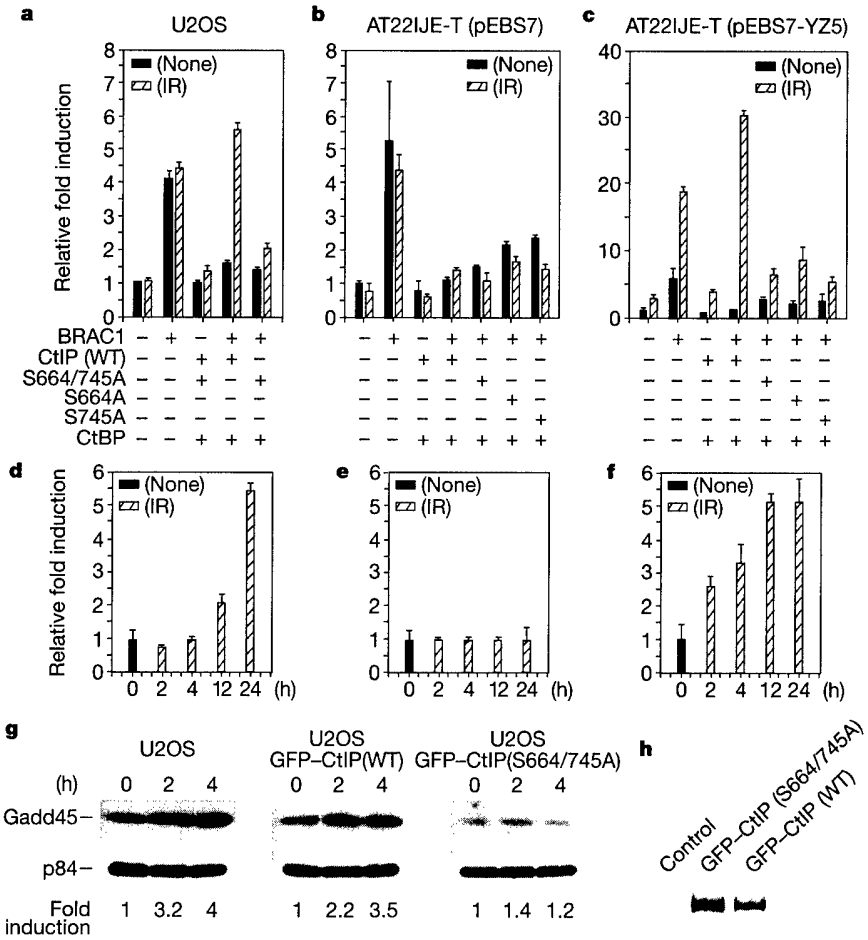

Figure 4 Regulation of GADD45 expression by BRCA1, CtIP and CtBP. **a–c**, U2OS, AT22IJE-T (pEBS7) or AT22IJE-T (pEBS7-YZ5) cells were co-transfected with pI-3 (containing *GADD45* intron 3 driving expression of a luciferase reporter gene), pSV40–β-gal (control plasmid) and the plasmids indicated. The luciferase activity was measured and normalized with β-galactosidase activity. Scale in **c** is different from in **a** and **b**. Results were derived from three independent transfection experiments. **d–f**, pI-3 reporter activity in U2OS, AT22IJE-T (pEBS7), or AT22IJE-T (pEBS7-YZ5) cells treated with IR. Cells were co-transfected with pI-3 and pSV40–β-gal plasmid, and untreated (solid bar) or treated (hatched bar) with IR (30 Gy) at 36 h after transfection. Transfected cells were then assayed for luciferase activity at the time points indicated. **g**, IR-induced expression of cellular GADD45 protein. U2OS cells stably expressing either GFP–CtIP (WT) or GFP–CtIP(S664/745A) were collected 2 and 4 h after IR and immunblotted for GADD45 and a nuclear matrix protein p84 as an internal control[9]. Blots were developed using an ECL kit and quantified by an SI Densitometer (Molecular Dynamics). The protein ratio of GADD45:p84 is shown. **h**, Immunoblot analysis of ectopically expressed GFP–CtIP(WT) and GFP–CtIP(S664/745A) in the stable cell clones using anti-GFP antibody.

results recapitulate the behaviour of the CtIP–BRCA1 complex in γ-irradiated ATM-deficient cells (Fig. 3b), and suggest that ATM-dependent phosphorylation of CtIP on Ser 664 and Ser 745 is essential for its dissociation from BRCA1 upon IR.

Dissociation of the CtIP–CtBP co-repressor complex from BRCA1, leading to relief of transcription repression, could represent one of the mechanisms regulating the transcriptional activity of BRCA1 upon DNA damage. To test this hypothesis, we examined the effect of CtIP–CtBP on the transactivation activity of BRCA1 on the *GADD45* regulatory element (intron 3 of *GADD45*) which was induced by BRCA1 (ref. 8). Consistently, expression of BRCA1 activated transcription from the reporter plasmid (pI-3) containing intron 3 (+1,553 to +1,695) of *GADD45* by about fourfold compared with the pcDNA3.1 vector alone in U2OS cells (Fig. 4a). Co-expression of CtIP(WT) or CtIP(S664/745A) and CtBP with BRCA1 repressed this BRCA1-induced activity by about 70–80%. After IR, however, CtIP(WT) no longer repressed the activity of BRCA1, whereas CtIP(S664/745A) retained persistent repression (Fig. 4a). The same persistent repression was observed with both CtIP(WT) and CtIP(S664/745A) in ATM-deficient cells [AT22IJE-T(pEBS7)] after IR (Fig. 4b). Re-introduction of wild-type ATM into these cells [AT22IJE-T(pEBS7-YZ5)] eliminated repression mediated by the CtIP(WT)–CtBP complex after IR, whereas CtIP(S664/745A)–CtBP maintained its repression (Fig. 4c). These results suggest that a defect in the ATM-dependent phosphorylation of CtIP(S664/745A) inhibited the dissociation of CtIP(S664/745A) from BRCA1 upon DNA damage, leading to the continuous repression of transcriptional activity from the intron 3 of *GADD45*. Furthermore, it appears that phosphorylation of both Ser 664 and Ser 745 is required for dissociation, as a single mutant form of CtIP(S664A or S745A) maintained its repressive effect after IR treatment (Fig. 4b, c). It was noted that the overall fold induction of the reporter (pI-3) was higher in the AT22IJE-T (pEBS7-YZ5) cells compared with in the U2OS cells in these experiments. This is because the reporter activity 4 h after IR was induced 2–3-fold in AT22IJE-T (pEBS7-YZ5) cells but very little in U2OS cells (Fig. 4d, f). The transcriptional activity of the reporter construct alone in ATM-deficient cells [AT22IJE-T(pEBS7)] remained constant after IR, which is consistent with previous results showing that induction of *GADD45* in response to IR requires ATM (compare Fig. 4e and f)[21]. Together, these data suggest that phophorylation of CtIP by ATM is critical for releasing BRCA1 from its repressive state. Consistently, overexpression of BRCA1 could titrate the endogenous CtIP–CtBP repressor complexes and thereby liberate BRCA1 from repression (Fig. 4a–c).

The persistent repression exerted by CtIP(S664/745A) suggests that it may act as a dominant-negative inhibitor of BRCA1-mediated transcriptional activity following IR. To test this possibility, we created U2OS cells stably expressing green fluorescent protein (GFP) tagged CtIP(WT) or CtIP(S664/745A), challenged these cells with IR, and assessed endogenous expression of GADD45 by immunoblot analysis. Overexpression of CtIP(S664/745A) but not CtIP(WT) inhibited the induction of GADD45 after IR (Fig. 4g). The expression of GFP-tagged CtIP proteins was confirmed by immunoblot analysis (Fig. 4h). These data further support the notion that phosphorylation of CtIP at Ser 664 and Ser 745 is important for BRCA1-mediated induction of GADD45 in response to DNA damage.

In response to genomic insult, ATM probably transduces the DNA damage signal by phosphorylating downstream effector molecules involved in regulating cell-cycle progression or DNA damage repair. For example, IR-induced ATM-dependent phosphorylation of p53 and hMDM2 contributes to the activation of p53, leading to the induction of p21 and GADD45 (refs 17, 18, 22). BRCA1 has also been demonstrated to induce expression of p21 and GADD45 (refs 6–8). Our study suggests another DNA damage-response pathway in which the signal is transmitted through phosphorylation of CtIP by ATM, leading to dissociation of the CtIP–CtBP repressor complex from BRCA1, which in turn, activate transcription of *GADD45* (Fig. 5). This regulatory mechanism may also explain our previous results concerning repression by CtIP/CtBP on the expression of p21 mediated by BRCA1 (ref. 9). Apparently, BRCA1 and p53 are important in p21 and GADD45 expression in response to IR. It is likely that both p53 and BRCA1 mediate synergistic and parallel pathways to ensure a proper cellular response to DNA damage.

These results provide a link between ATM and BRCA1 through CtIP, which may explain the increased risk for breast cancer in certain populations of ataxia telangiectasia heterozygotes[23,24]. In the absence of functional ATM, the activity of BRCA1 may become disregulated leading, in turn, to a defect in the cellular response to DNA damage, concomitant genomic instability and, ultimately, tumorigenesis. □

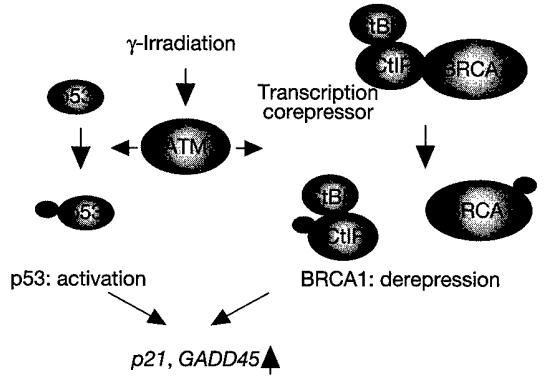

Figure 5 Model showing how ATM modulates the BRCA1 transcriptional regulation of DNA damage-response genes following IR. In response to IR, ATM kinase becomes activated and phosphorylates CtIP to disrupt the CtIP–CtBP–BRCA1 complex. Consequently, BRCA1 is released and participates in the activation of DNA damage-response genes *p21* and *GADD45*.

Methods

Plasmid constructs

The pCNF–CtIP(WT) plasmid that expresses the Flag-tagged CtIP was generated by subcloning Flag-tagged CtIP cDNA into pcDNA3.1 vector (Invitrogen). The pCNF–CtIP(S664/745A) with mutations at Ser 664 and Ser 745 was engineered by site-directed mutagenesis. pCMV–ATM expresses Flag-tagged wild-type ATM, whereas pCMV–ATM(D2870A) expresses mutant ATM with Asp 2,870 changed to alanine by site-directed mutagenesis. GST–CtIP(C) was constructed by inserting the carboxy-terminal fragment of CtIP (amino acids 324–897) into the *Sma*I site of pGEPK3 vector. GFP–CtIP contains the full-length CtIP cDNA downstream of GFP-tagged expression vector[25]. pcDNA–BRCA1, pRcCMV–CtBP and pSV40–β-gal plasmids have been described[9]. The pI-3 plasmid was constructed as described[8].

Cell treatment, immunoprecipitation and western blot analysis

Cells were incubated in fresh medium for 3 h and treated with different dosages of γ-ray (5–40 Gy), ultraviolet (1 mJ cm^{-2}) or 0.01% MMS. The cells were collected 1 h after treatment and lysed in Lysis 250 buffer. Immunoprecipitation and western blots were carried out as described[9]. We used the following antibodies: anti-CtIP mouse polyclonal antibody C11, anti-BRCA1 monoclonal antibody 6B4, anti-GST monoclonal antibody 8G11 (ref. 9), ATM monoclonal antibody 2C1 (GeneTex), anti-Flag monoclonal antibody M2 (Sigma), anti-GADD45 rabbit polyclonal Ab H165 (Santa Cruz Biotechnology) and anti-GFP monoclonal antibody (Clontech).

λ-Phosphatase treatment

Immune complexes containing CtIP were washed in Lysis 250 buffer in the absence of phosphatase inhibitors. Parallel samples were resuspended in λ-phosphatase buffer (New England Biolabs) either in the presence or absence of phosphatase inhibitors, NaF (50 mM final concentration) and Na_3VO_4 (2 mM final concentration). λ-Phosphatase (400 U) was added to each sample followed by incubation at 30 °C for 1 h.

Transfection and luciferase assay

Plasmid DNA including 10 μg of pCMV (vector), pCMV-ATM or pCMV-ATM(D2870A) was transfected into 293 cells (2×10^6 in 10-cm dish) by the calcium phosphate/DNA

co-precipitation method. Transfection into HCT116 cells was performed using lipofectin. For measuring the transactivation activity by luciferase assay, U2OS cells were transfected by calcium phosphate/DNA co-precipitation with 0.5 μg of pI-3 reporter construct, along with 5 μg of pcDNA–BRCA1, 2.5 μg of pRcCMV–CtBP, 2.5 μg of pCNF–CtIP(WT), pCNF–CtIP(S664/745A), pCNF–CtIP(S664A), pCNF–CtIP(S745A), or pcDNA3.1 as the control vector to equalize the amount of the transfected DNA. One microgram of pSV40–β-gal was co-transfected for standardization of transfection efficiency by measurement of β-galactosidase activity. The cells were irradiated with 30 Gy 36 h after transfection, and assayed for luciferase and β-galactosidase activities 4 h after treatment using standard procedures[9].

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Correspondence and requests for materials should be addressed to W.-H.L. (e-mail: leew@uthscsa.edu).