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Women with germline mutations in the breast and ovarian cancer gene 1 (Brcal) have an approximately 50% lifetime risk of developing ovarian cancer and almost 90% chance of breast cancer. Brcal mutations account for a significant percentage of all breast cancer cases. It appears that the main role for the Brcal protein in cells is to prevent the accumulation of mutations in key growth regulatory genes in response to DNA damage. BRCA1 is phosphorylated in response to DNA damage by an elaborate surveillance mechanism, called a checkpoint that detects DNA damage and prevents the accumulation of mutation. We are investigating the role these phosphorylation events play in the regulation of BRCA1. We have mapped phosphorylation sites and will mutate them to determine their function. We are also planning to investigate the mechanism through which the BRCA1 protein localizes to sites of DNA damage within cells.

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## Introduction

Maintenance of genomic integrity is crucial for the development and health of organisms. Cell cycle checkpoints and DNA repair mechanisms help ensure the distribution of an intact genome to all cells and progeny. The inactivation of many of the genes involved in these activities have been linked to syndromes that cause a predisposition to cancer in humans. The *ATM*, *Brcal*, and *Brca2* genes are three such tumor suppressors involved in preventing genetic damage (1). Mutations in *ATM* cause ataxia telangiectasia (A-T), a disorder characterized by atrophy of the cerebellum and thymus, immunodeficiency, premature aging, predisposition to cancer, and sensitivity to ionizing radiation (2). Furthermore, heterozygous carriers of a dysfunctional *ATM* gene are predisposed to breast cancer (3). Mutations in *Brcal* and *Brca2* are linked to inherited, early-onset breast cancer (4). Mutations in *Brcal*, *Brca2*, or *ATM* cause defects in cellular proliferation, genomic instability, and sensitivity to DNA damage (5-7).

*ATM* is a member of a protein family related to phosphoinositide kinases that includes *ATR*, *MEC1*, *TEL1* and *RAD3*. These proteins are essential for signaling the presence of DNA damage and activating cell cycle checkpoints (8). *ATM* is activated in response to DNA damage and is required for efficient DNA double strand break repair and optimal phosphorylation and activation of the p53, c-Abl, and Chk2 proteins that promote apoptosis or cell cycle arrest (9-14).

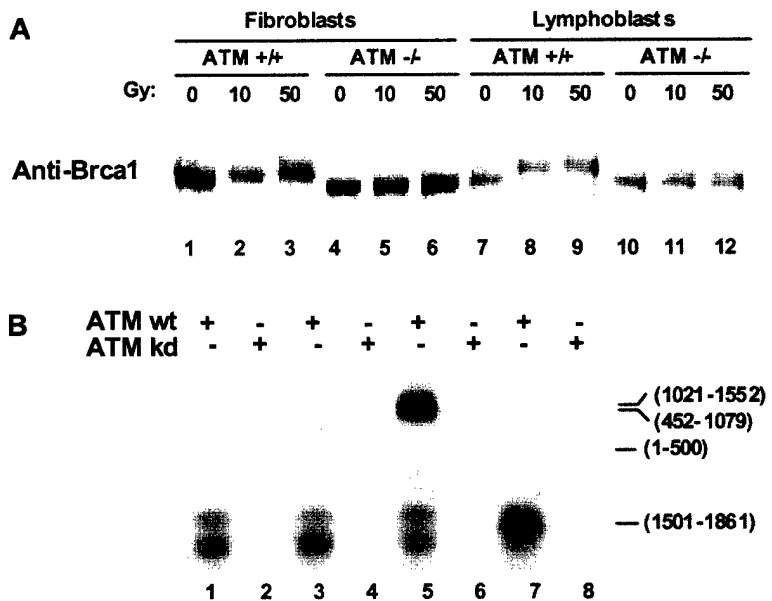
The *Brcal* and *Brca2* proteins form a complex with Rad51, a RecA homologue required for homologous recombinational repair of DNA double stranded breaks (6,15-17). These three proteins localize to discrete nuclear foci during S phase of the cell cycle, share developmental expression patterns, and are maximally expressed at the G1-S transition (16-19). *Brcal* mutations in mice result in genetic instability, defective G2/M checkpoint control and reduced homologous recombination (7). Exposure of cells to ionizing radiation or hydroxyurea causes dispersal of *Brcal* foci and relocalization to sites

of DNA-synthesis where DNA repair may occur (18). Brca1 is phosphorylated during S-phase and is also phosphorylated in response to DNA damage (18,20).

**(6) Body**

In the course of identifying BRCA1-associated proteins by mass spectrometry, we identified ATM and confirmed this association by reciprocal co-immunoprecipitation. Given this physical association, we tested whether ATM was required for phosphorylation of Brca1 in response to DNA damage. Brca1 from  $\gamma$ -irradiated wild-type cells migrated more slowly than the Brca1 from untreated cells on SDS-PAGE gels indicating phosphorylation (18, 20, and Fig. 1B below). Brca1 in ATM-deficient fibroblast and lymphoblast cells derived from A-T patients was not hyper-phosphorylated after exposure to  $\gamma$ -irradiation (Fig. 1A). These studies were initiated prior to this grant and have been continued since then via this grant.

In Technical objective 1 we mapped phosphorylation sites in BRCA1 isolated after DNA damage treatment. These



**Figure 1 A and B ATM-Dependent Brca1 Phosphorylation.** (A) Wild type or A-T fibroblasts or lymphoblasts were treated with 10 or 50 Gy of  $\gamma$ -irradiation. Cell lysates were harvested one hour after irradiation, fractionated on SDS-PAGE, and immunoblotted with anti-Brca1 (Ab-1, Oncogene Research) antibody. (B) GST-fusion proteins containing Brca1 amino acids 1-500 (lanes 1 and 2), or 452-1079 (lanes 3 and 4), were used as substrates in an *in vitro* kinase assay with wild-type or kinase-defective ATM. The kinase reactions were separated by SDS-PAGE, stained with Coomassie blue and exposed to film. The two phosphorylated proteins observed in all of the odd numbered lanes are unidentified proteins co-immunoprecipitated with ATM from 293T cells.

are shown below in Table 1 and were described in detail in the first progress report. We were able to mutate several of these sites and ask for the ability of the mutant BRCA1 to function. We observed that BRCA1 mutants with serine 1423 and serine 1524 mutated were less able to complement the BRCA1 mutant cell line HCC1937. One potential criticism of these results was the fact that we got very low levels of the BRCA1 expressed although the wild-type protein was also poorly expressed at the same levels. Thus although the mutant BRCA1 protein failed to properly function, it was not clear whether it would still be defective if it were expressed at the levels normally found in cells. We have had very little success getting high levels of BRCA1 expressed from retroviruses. This problem hampered our efforts in technical objective 3 as described below. Still, the key findings are that BRCA1 is a phospho-protein and is phosphorylated on multiple sites in vivo, some of which are inducibly phosphorylated in response to DNA damage by ATM and ATR. Thus we accomplished Technical Objective 1.

**Table 1. Summary of in vitro and in vivo phosphorylated sites on Brca1 as detected by mass spectrometry.**

<b>Site</b>	<b>Peptide</b>
<b><u>In vitro</u></b>	
S1330	<sup>326</sup> HQSE <u>S</u> QGVGLSDKELVSDDEER <sup>1347</sup>
S1423	<sup>1407</sup> LQQEMAELEAVLEQHGS <u>S</u> QPSNSYPSIISDSSALEDLRNPEQSTSEK <sup>145</sup>
S1466	<sup>1460</sup> SSEYPIS <u>S</u> QNPEGLSADKFEVSADSSTSK <sup>1487</sup>
S1524	<sup>1521</sup> NYP <u>S</u> QEELIKVVDVEEQLEESGPHDLTETSY <sup>1552</sup>
S1542	<sup>1531</sup> VVDVEEQLEES <u>S</u> GPHDLTETSY <sup>1552</sup>
?	<sup>1279</sup> ASQEHHLSEETKCSASLFSSQCSELEDLTANTNTQDPFLIGSSK <sup>1322</sup>
<b><u>In vivo</u></b>	
S1189	<sup>1189</sup> SPSPFTHTHLAQGYR <sup>1203</sup>
S1457	<sup>1453</sup> AVLT <u>S</u> QKSSEYPISQNPEGLSADKFEVSADSSTSK <sup>1487</sup>
S1542	<sup>1531</sup> VVDVEEQLEES <u>S</u> GPHDLTETSYLPR <sup>1555</sup>
S1524, S1542	<sup>1521</sup> NYP <u>S</u> QEELIKVVDVEEQLEES <u>S</u> GPHDLTETSYLPR <sup>1555</sup>

# Amino acids in underlined bold faced type were unambiguously determined to be sites of phosphorylation by LC/MS/MS. \* At least one phosphorylated amino acid within these

peptides could not be determined unambiguously by LC/MS/MS. †Peptides with both one and two moles of phosphate were observed.

In Technical Objective 2, we proposed to identify novel phosphorylation sites on BRCA1 in response to DNA replication stress by hydroxyurea and UV light. These experiments were performed but no new phosphorylation sites were identified beyond what was already seen for IR-induced DNA damage. We feel that this is because the ATR kinase is primarily responsible for BRCA1 phosphorylation in response to hydroxyurea and UV. However, ATR is activated in response to IR in addition to ATM and ATM and ATR share the vast majority of substrate sites. Thus, the initial study identifying phospho-sites in response to ionizing radiation also identified the same sites phosphorylated by ATR in response to UV and HU. For these reasons Technical Objective 2 was accomplished but did not yield the anticipated insights into BRCA1 function we had initially hoped for.

In our final Technical Objective, 3, the identification of the nuclear foci localization domain in BRCA1, we were unable to get consistent results because we could not see the BRCA1 protein reproducibly when expressed in retroviruses. This was especially true for deletion derivatives. During our studies, a paper was published showing that the BRCT-domain of BRCA1 was required for foci formation and mutations in this domain were required for proper localization. Therefore we did not further pursue this aim and instead focused our interests in more fruitful areas.

However, the negative results from Technical Objectives 2 and the inconclusive results of Technical Objective 3 (and being scooped on Objective 3) did allow us to pursue additional findings that came from BRCA1 analysis. In the course of identifying BRCA1-associated proteins by mass spectrometry, we identified ATM and confirmed this

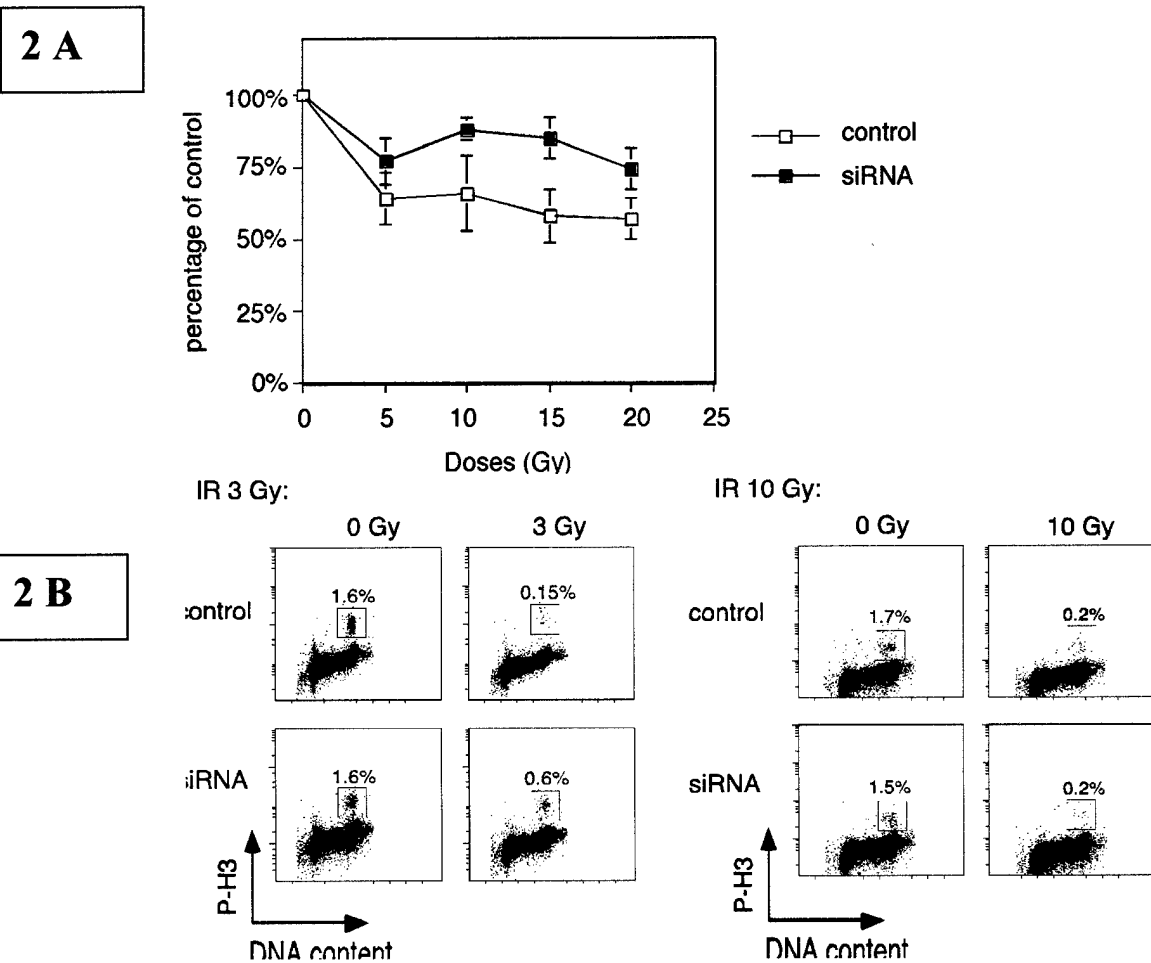
association by reciprocal co-immunoprecipitation. Given this physical association, we tested whether ATM was required for phosphorylation of Brca1 in response to DNA damage. We found several phosphorylation sites and showed they were ATM targets in vivo. This was all detailed in the previous project report. Since then we have gone on to determine what other molecules might be required to help ATM phosphorylate BRCA1 since these proteins might also be tumor suppressors in the breast. We focussed on a BRCA1-related protein 53BP1. 53BP1 was originally identified through its ability to bind to the tumor suppressor protein p53 through 53BP1's C-terminal BRCT (Brca1 carboxyl terminus) repeats (21,22) which are found in many DNA damage response proteins (3-8). 53BP1 responds to DNA double strand breaks (29-32), quickly relocalizing to discrete nuclear foci upon exposure to IR. These foci colocalize with those of the Mre11/Nbs1/Rad50 complex, BRCA1 and phosphorylated  $\gamma$ -H2AX which are thought to facilitate recruitment of repair factors to damaged DNA (29-3). In response to IR, 53BP1 is phosphorylated in an ATM (ataxia telangiectasia mutated) dependent manner (30-32), but its role in the DNA damage response is unclear.

To determine 53BP1's role, small interfering RNAs (siRNA) in the form of two



independent, non-overlapping 21-base pair RNA duplexes targeting 53BP1, were used to inhibit its expression (33). U2OS cells were transfected with these siRNA oligos and, within three days post transfection, a portion of cells had undergone cell death (data not shown). A similar phenotype was also observed in two other cell lines, Hct116 and Saos2 (data not shown).

To determine whether 53BP1 plays a role in DNA damage cell cycle checkpoints, we examined the response of 53BP1-inhibited cells to IR. IR induces the intra-S-phase checkpoint which reduces DNA synthesis. Unlike the control cells, 53BP1-inhibited

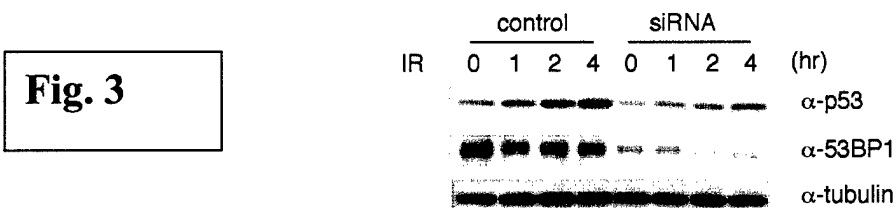


**Fig. 2** 53BP1 inhibition results in defective IR-induced intra-S-phase and G2/M checkpoints. **(A)** IR-induced intra-S-phase checkpoint. Replicative DNA synthesis was assessed 30 min after various doses of ionizing irradiation in U2OS cells transfected with oligos. The DNA synthesis in unirradiated cultures was set to 100% for cells transfected with control oligos or siRNA oligos against 53BP1. **(B)** Analysis of the G2/M DNA damage checkpoint. Cells were either untreated or irradiated with either 3 Gy or 10 Gy as indicated, then incubated for 1 hour at 37 °C prior to fixation. Cells in mitosis were determined by staining with propidium iodide and antibody to phospho-histone H3 and percentage of the M-phase cells was determined by flow cytometry.

cells showed radio-resistant DNA synthesis (Fig. 2A). This was also seen in Saos2 and Hela cells with both siRNAs (data not shown) and indicates a role of 53BP1 in the intra-S phase checkpoint.

To assess the G2/M checkpoint, 53BP1-inhibited and control cells were irradiated with 3 or 10 Gy of ionizing radiation. Approximately three-fold more 53BP1-inhibited cells entered into mitosis than the control cells treated with 3 Gy (Fig. 2B). However, inhibition of 53BP1 had no effect following 10 Gy IR. Therefore, 53BP1-inhibited cells also displayed an IR-induced G2/M checkpoint defect. The fact that 53BP1-inhibited cells were only defective in response to lower doses of irradiation indicates the existence of an alternative signaling pathway that operates at higher doses of IR.

As 53BP1 binds p53, we asked whether 53BP1 was required for p53 activation in response to IR. P53 induction in response to IR was significantly decreased in 53BP1-inhibited cells (Fig. 3).



**Fig. 3**

**Fig. 3.** 53BP1 regulates p53 in response to IR. IR-induced p53 stabilization. U2OS cells were transfected with siRNA oligos against 53BP1 or control oligos for two days, then exposed to 10Gy ionizing irradiation. Cell lysates were made from samples at indicated times recovered from irradiation and separated on SDS-PAGE gel. Western blots were performed using anti-53BP1, anti-tubulin and anti-p53 antibodies.

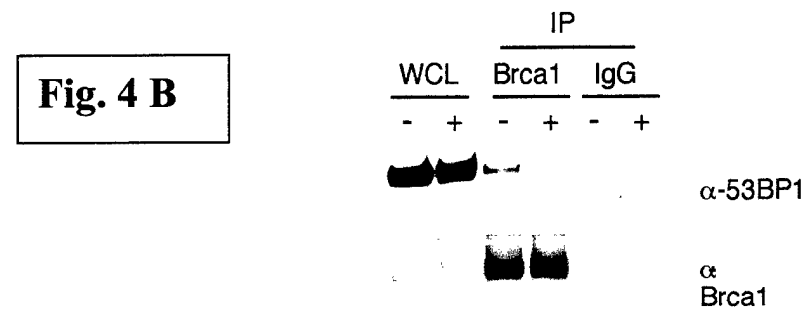
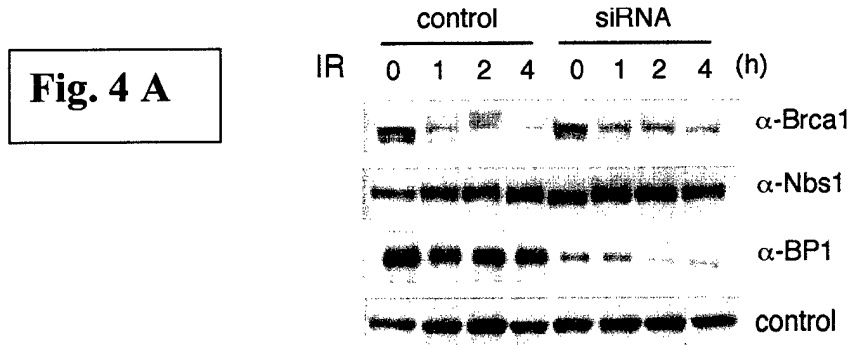
53BP1 forms foci that overlap with BRCA1 foci in response to DNA damage. Generally there are more 53BP1 foci than BRCA1 foci and they appear to form faster than BRCA1. To test whether 53BP1 might be required for BRCA1 foci, we examined

the ability of proteins to form foci in the absence of 53BP1. Brca1, Nbs1, and  $\gamma$ -H2AX all form foci in response to IR (36). IR-induced Brca1 foci formation was largely abolished in 53BP1-inhibited cells. Brca1 showed diffuse staining and rarely formed distinctive foci in response to IR at different time points. In an asynchronous cell population, at 2 hr post-IR, only 4% of the cells formed Brca1 nuclear foci when cells were treated with 53BP1siRNA, compared to 60% of the control cells. Similar results were obtained in Hct116 and HeLa cells with both oligo pairs. In contrast, formation of  $\gamma$ -H2AX foci or Nbs1 foci after IR remained unchanged in cells treated with control oligos or siRNA oligos. Rad51 foci were also unchanged.

When asynchronous control cells were analyzed for Brca1 foci formation in the absence of IR, approximately 40% contained more than 20 Brca1 foci, reflecting the S phase and G2 population. In 53BP1-inhibited cells, both the number of foci and the percentage of cells containing foci were reduced. Only 12% of 53BP1-inhibited cells contained more than 20 Brca1 foci. To control for cell cycle differences, we synchronized cells using a double-thymidine block, and S-phase cells (4 hours after release from the block) were used for immunostaining. BRCA1 foci were also dependent on 53BP1 in S-phase cells in the presence or absence of ionizing irradiation.

Although the IR-induced foci formation of Brca1 is dependent on the presence of 53BP1, Brca1 foci did not show complete colocalization with 53BP1 foci at early times. The strong effect on BRCA1 foci formation, coupled with the fact that the 53BP1 and BRCA1 foci do not initially fully overlap suggests that 53BP1 may regulate BRCA1 through a mechanism other than direct recruitment to foci. One means by which this might be achieved is through regulation of BRCA1 phosphorylation. In IR-treated cells,

Brca1 phosphorylation was reduced in the samples prepared from cells treated with siRNA oligos against 53BP1 relative to controls (Fig. 4A).



**Fig. 4.** 53BP1 regulation of Brca1. **(A)** Brca1 phosphorylation is reduced in the absence of 53BP1. U2OS cells were treated with siRNA oligos against 53BP1 or control oligos for two days. Cells were exposed to 10 Gy irradiation and cell lysates were prepared at indicated times after irradiation. Immunoblots were performed with antibodies against Brca1 (Oncogene), Nbs1 (Norvus) and 53BP1. The control band is a non-specific band from the same blot that was incubated with antibodies against Brca1. **(B)** 53BP1 associates with Brca1. Cell lysates from untreated U2OS cells or 2 hour after exposure to 10 Gy IR were incubated with antibodies against Brca1 or rabbit IgG as a control. Western blots were performed using anti-53BP1 and anti-Brca1 antibodies (Oncogene). Ten percent of the cell lysate used for immunoprecipitation were loaded in the control lanes (WCL).

As with the G2/M checkpoint, the strongest dependency of Brca1 phosphorylation appeared to be at lower doses of IR (not shown). High levels of IR have been shown to obscure BRCA1 regulation by other proteins such as ATM (38). Loss of 53BP1 did not have a general effect on the DNA damage-inducible phosphorylation of other proteins. Nbs1 phosphorylation was not affected (Fig. 4A). Furthermore, while BRCA1

phosphorylation showed less dependency on 53BP1 at 50Gy IR, these cells still failed to form foci (data not shown).

Next we examined whether 53BP1 associated with BRCA1. Brca1 interacts with 53BP1 in vivo, and this interaction was abolished in response to IR (Fig. 4B). Thus, this dynamic association is likely to be important for regulation of 53BP1's ability to regulate BRCA1 function in response to DNA damage.

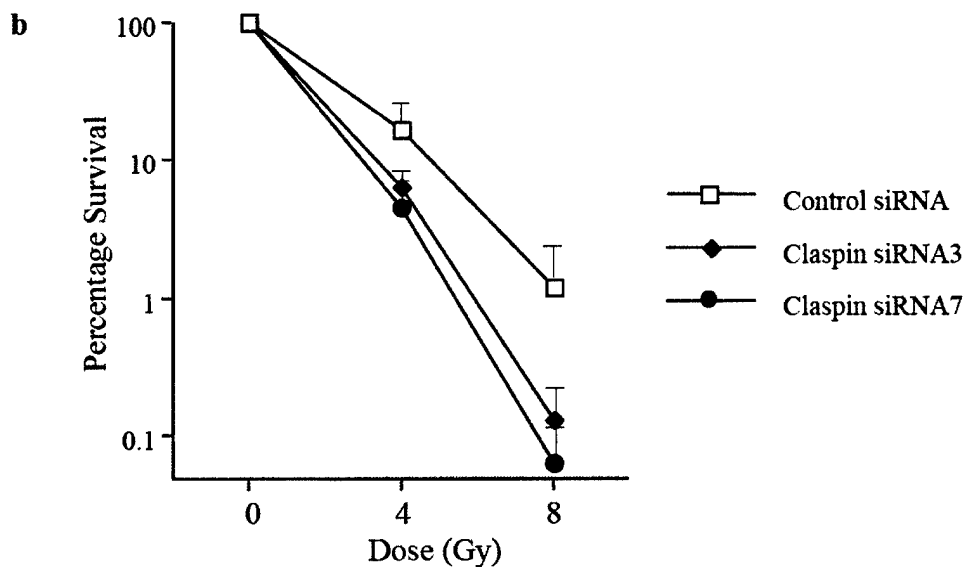
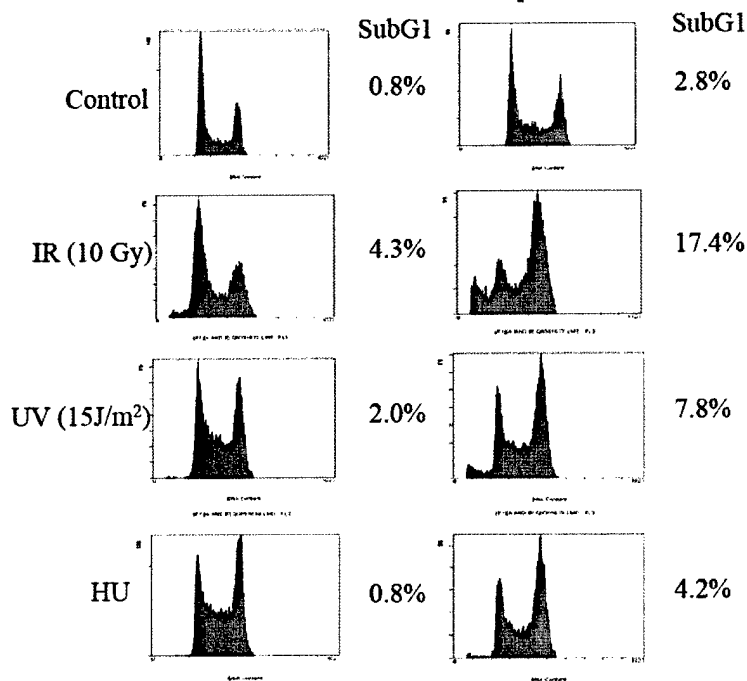
The major finding of these additional studies is that 53BP1 is a critical transducer of the DNA damage signal and is required for both the intra-S phase and G2/M checkpoints. It is clearly part of a partially redundant branch of the signaling apparatus and its loss results in a partial decrease in phosphorylation of key checkpoint target proteins. As it binds to p53, and Brca1 and controls BRCA1 phosphorylation, it has the property of a mammalian adaptor or mediator that might recruit a subset of substrates to the ATM/ATR-ATRIP checkpoint kinases.

A second key finding of this study is that the pathway leading to the assembly of repair/signaling foci in response to damage is branched and shows a regulatory hierarchy in which H2AX is required for Nbs1 and 53BP1 foci (39) and 53BP1 controls the ability of at least BRCA1 but not Nbs1 to form foci. The nature of this disruption in foci formation is unknown but may be related to the role of 53BP1 in control of phosphorylation of these or other proteins. Regardless of the mechanism, it is clear that 53BP1 is a central transducer of the DNA damage signal to BRCA1 and other tumor suppressor proteins and is likely to play an important role in the maintenance of genomic stability and prevention of cancer.

In addition to the p53 studies, we were able to follow up on another interaction with BRCA1. In this case we studied Claspin. Both BRCA1 and Claspin are required for activation of the Chk1 kinase in response to DNA damage. We therefore investigated whether there was a connection between the two in vivo.

To determine if claspin plays a role in the DNA damage and replication checkpoints.

U2OS cells were transfected twice with two independent small interfering RNAs (siRNAs) against two non-overlapping sequences on Claspin. Forty-eight hours after the second transfection, cells were challenged with different genotoxic insults; ionizing radiation (10 Gy), UV (50 J/m<sup>2</sup>) or hydroxyurea (2 mM). As judged by the increase in the sub G1, apoptotic population, Claspin-depleted cells were more sensitive to all three stresses (Fig. 5a). The increase in sensitivity to ionizing radiation was further confirmed by a colony forming assay. Compared to control siRNA treatment, the Claspin-siRNA treated cells were significantly more sensitive to ionizing irradiation (Fig. 5b). These results indicate that Claspin plays an important role in the cellular responses to both DNA damage and replication blocks.



**Fig. 5.** Claspin deficiency increases the sensitivity to genotoxic stress. (a) U2OS cells were transfected twice with control or Claspin siRNAs. 48 hours after the second transfection, cells were treated with different genotoxic agents as indicated. 72 hours after treatment with the indicated agents, DNA contents were analyzed by FACS. (b) U2OS cells were transfected with siRNAs as described above. The transfected cells then were plated at low density, irradiated and the colonies were counted after two weeks.

### **Claspin regulates DNA damage checkpoints**

Before massive apoptosis occurred (72 hours after IR), we observed a prolonged G2 phase accumulation for Claspin depleted U2OS cells (data not shown). A prolonged G2 accumulation after IR has previously been linked to a defective S-phase checkpoint in cells lacking BRCA1 or Nbs1 (40), suggesting a role of Claspin in intra-S phase checkpoint. Thus we examined DNA synthesis inhibition in response to IR (41) in cells lacking Claspin. As shown in Fig. 6a, Claspin deficient cells showed a significant radio-resistant DNA synthesis (RDS) phenotype, indicating a role for Claspin in the intra-S-phase checkpoint. We also examined if Claspin played a role in the G2/M checkpoint. Cells treated with Claspin-specific siRNA were irradiated and labeled with anti-phospho-histone H3 antibody as the marker for M phase cells (42). In contrast to the control cells which were arrested in G2, a significantly higher population of Claspin-depleted cells entered mitosis, indicating the requirement of Claspin for the G2/M checkpoint (Fig. 6b). Together, our results indicate that human Claspin plays an important role in DNA damage checkpoints, contributing to resistance to the toxic effect of DNA damage.

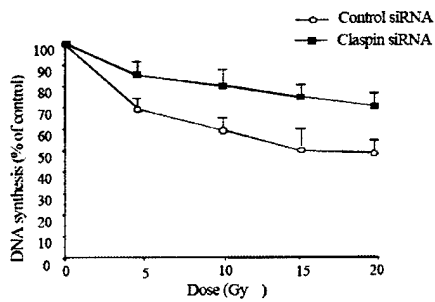
### **Claspin is required for Chk1 activation upon DNA damage.**

It has been shown that disruption of Chk1 abrogates the IR-induced intra-S phase and G2 checkpoints (46). Furthermore, Claspin has been implicated in Chk1 regulation in *Xenopus*. Therefore, we sought to determine if Claspin depletion blocked IR-induced Chk1 activation. As shown in Fig. 6c (upper panel), cells treated with Claspin siRNA showed significantly reduced Chk1 S345 phosphorylation either in response to ionizing

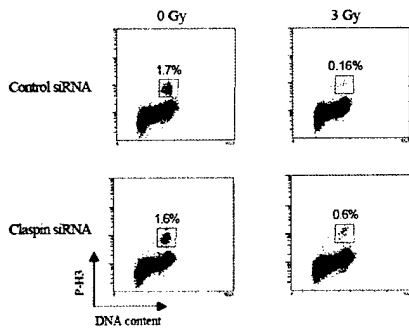


radiation or UV. This effect was Chk1-specific because another effector kinase, Chk2,

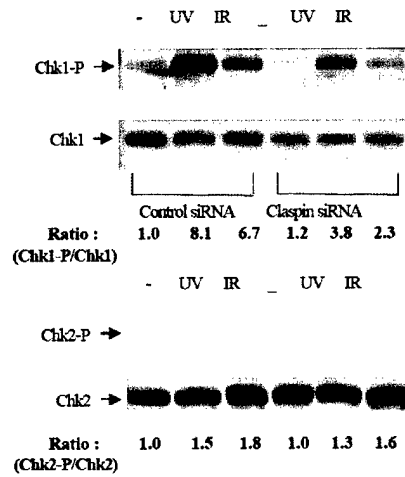
**Fig. 6**



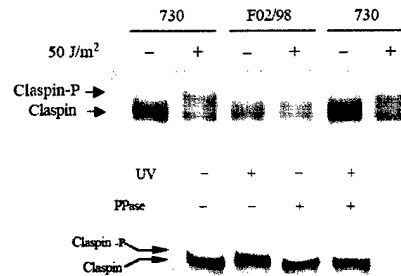
**b**



**c**



**d**



**Fig. 6.** Claspin is required for the IR-induced intra-S and G2/M checkpoints. (a) IR-induced intra-S phase checkpoint. DNA synthesis was assessed 30 min after various doses of IR in U2OS cells twice-transfected with Claspin siRNA or the control siRNA. (b) IR-induced G2/M checkpoint analysis. U2OS cells were either untreated or irradiated with 3 Gy then incubated for 1 hr before fixation. Cells in mitosis were determined by staining with propidium iodide and phospho-histone H3 antibody followed by FITC16 conjugated secondary antibody. The percentage of M-phase cells was determined by FACS for p-H3. (c) U2OS cells were transfected with control or Claspin siRNA twice. 48 hours after the second transfection, cells were either unirradiated or irradiated with UV (50 J/m<sup>2</sup>) or IR (10 Gy). Two hours after irradiation, cells were harvested for Western blotting and probed with antibodies against S345P-Chk1 or Chk1 (top panel) or antibodies against T68P-Chk2 or Chk2 (bottom panel). (d) Top panel: 730 cells, a normal human primary fibroblast line and F02/98 cells, a primary fibroblast line from a Seckel Syndrome patient, were unirradiated or irradiated with UV. Two hours after the treatment, cells were harvested for Western blotting and probed with anti-Claspin antibodies. Bottom panel: 730 cells were either unirradiated or irradiated with UV (50 J/m<sup>2</sup>). Two hours after the treatment, cells were harvested and the lysates were treated with or without  $\lambda$  protein phosphatase for Western blotting and probed with anti-Claspin

antibodies.

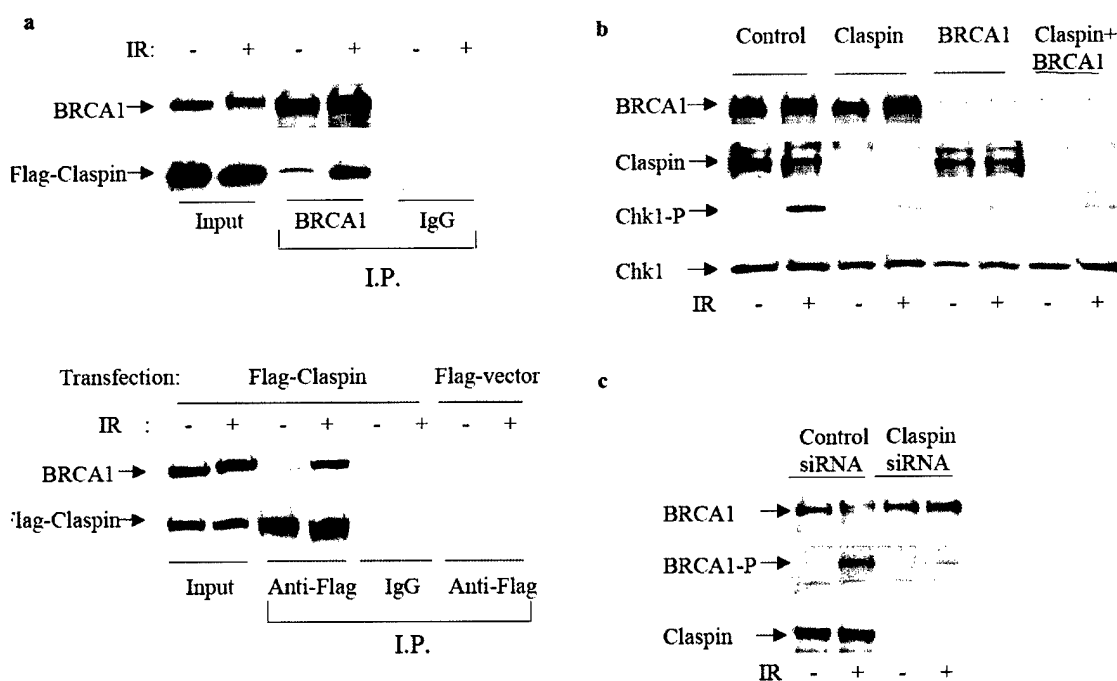
was not affected (Fig. 6c, bottom panel). Our results, therefore, indicated that Claspin may be a general regulator for Chk1 activation in the responses to both DNA damage and replication stress. Since Chk1 activation depends on both ATR and Claspin, we sought to determine if there was functional interactions between ATR and Claspin. Claspin is phosphorylated after replication block or UV irradiation, which causes the slower migration of the protein on SDS PAGE. Recently, primary fibroblasts derived from a Seckel Syndrome patient were found to contain a splicing mutation in the ATR gene that resulted in reduced expression of the wild type ATR product (44). As shown in Fig. 6d, UV induced Claspin phosphorylation in normal fibroblasts but not in fibroblasts harboring the ATR mutation, indicating a dependency for Claspin phosphorylation on the ATR pathway. Claspin phosphorylation has been previously shown to be required for binding and activation of xChk1 in *Xenopus* and we suspect that Claspin may be a direct target of ATR to facilitate Chk1 binding just as Mrc1 is a target of the ATR homolog Mec1 in yeast.

#### **Claspin binds to BRCA1 and regulates BRCA1 phosphorylation.**

The functions of Claspin in checkpoint regulation and Chk1 activation are very similar to the functions of another important checkpoint protein, BRCA1. BRCA1 has been shown to be involved in both intra-S phase and G2-phase checkpoints after ionizing radiation (42). In addition, its role in the G2/M checkpoint is believed to be through the activation of Chk1 activity (45). Because of those functional similarities, we suspected that Claspin

and BRCA1 may interact in response to DNA damage. To determine if these two proteins physically associate, we expressed Flag-tagged Claspin in HeLa cells and performed immunoprecipitation assays to assess their binding. Using an anti-BRCA1 antibody, we detected a small amount of Flag-Claspin associated with the endogenous BRCA1 protein (Fig. 7a, top panel).

Fig. 7



**Fig. 7.** Claspin interacts with BRCA1 and regulates IR-dependent Chk1 phosphorylation. HeLa cells were transfected with Flag-tagged Claspin and either unirradiated or irradiated with IR (10 Gy). 1 hr after treatment, cells were harvested for immunoprecipitations. (a) Top panel: cell extracts were incubated with antibodies against BRCA1 or immunoglobulin G (IgG, control) and protein A Sepharose. Immunoprecipitates were separated by SDS-PAGE and immunoblotted with antibodies against BRCA1 or the Flag epitope. Bottom panel: The same as the top panel except anti-Flag antibodies were used instead of anti-BRCA1 antibodies for immunoprecipitation. (b) U2OS cells were either transfected twice with control siRNA, Claspin siRNA, BRCA1 siRNA alone or in combination. 48 hours after the second transfection, cells were either unirradiated or irradiated with 10 Gy ionizing radiation. 1 hour after the treatments, cells were harvested for Western blotting and probed with the indicated antibodies. (c) Cell lysates from the control or Claspin depleted cells were prepared for Western blotting and probed with antibodies against BRCA1, p-S1524-BRCA1 or Claspin.

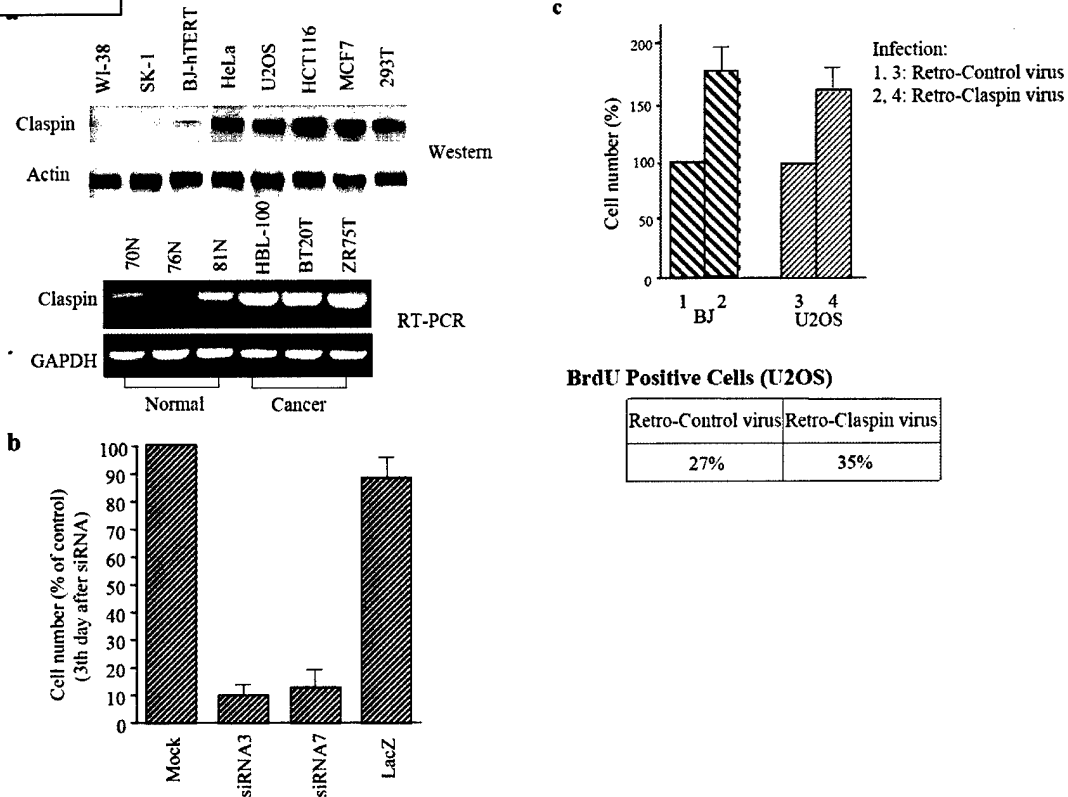
Their co-immunoprecipitation significantly increased after cells were exposed to 10 Gy ionizing radiation. Reciprocally, when Flag-Claspin was immunoprecipitated, BRCA1 protein was co-immunoprecipitated and the binding increased significantly after IR (Fig. 7a, bottom panel).

Next, we sought to determine if Claspin and BRCA1 belonged to the same pathway or two distinct pathways in Chk1 regulation. We either depleted individual proteins or double-depleted both proteins and analyzed their effects on Chk1 activation after IR. As shown in Fig. 7b, the depletion of either Claspin or BRCA1 repressed IR-dependent Chk1 S345 phosphorylation to the same extent. The combination of both siRNAs did not further inhibit Chk1 activation, suggesting that Claspin and BRCA1 functioned in the same signaling pathway. A recent study indicated that Claspin bound to chromatin around the time of initial unwinding step of replication and suggested it functions to monitor the replication process like Mrc1. Therefore Claspin may help to recruit signal mediators, such as BRCA1 to transduce signals to downstream effectors. BRCA1 is phosphorylated upon DNA damage at various sites, including S1524. If Claspin functions upstream of BRCA1, depletion of Claspin should interfere with BRCA1 phosphorylation. Using antibodies against phospho-S1524-BRCA1, we demonstrated a requirement for Claspin on IR-dependent BRCA1 phosphorylation. BRCA1 was strongly phosphorylated at S1524 after IR and this phosphorylation was significantly reduced when Claspin was depleted in cells (Fig. 7c). Therefore, Claspin acts upstream of BRCA1 or together with BRCA1 to regulate Chk1 activation.

## Claspin regulates cell proliferation

Since Claspin is involved in maintenance of intact checkpoints and probably genomic stability, we were interested in determining if Claspin expression was reduced in some cancer cells. Surprisingly, when we analyzed the expression levels of Claspin in different cell lines, we found that Claspin was expressed at much higher levels in cancer cells compared with the normal primary cells (Fig. 8a, top panel). The same expression patterns were also seen when we compared Claspin RNA expression between cells derived from normal mammary glands and three different breast cancer cell lines (Fig. 8a, bottom panel).

Fig. 8



**Fig. 8.** Claspin functions in DNA replication and proliferation. (a) Top panel: Claspin protein levels were analyzed in untransformed cells (WI-38, SK-1, BJ-hTERT) or cancer cell lines (all other five cell lines). Bottom panel: Claspin RNA levels were determined by RT-PCR in the cells derived from normal mammary glands (70N, 76N, 81N) or breast

cancer cell lines (HBL-100, BT20T, ZR75T). (b) U2OS cells were mock-transfected or transfected with siRNAs against Claspin (siRNA3 and siRNA7) or LacZ (control) twice. Equal cell numbers were plated 48 hours after the second transfection and the total cell numbers were counted again three days after. (c) Top panel: BJ cells or U2OS cells were infected with retrovirus expressing Claspin (2, 4) or the vector control (1,3). After puromycin selection, stable clones were first pooled together and then equal cell numbers from the pooled population were plated and the total numbers of cells were counted again three days after. Bottom panel: U2OS cells described above were stained and analyzed for the percentage of BrdU positive cells by immunohistochemical staining and counted under microscope.

These results suggested that Claspin may have a cell growth promoting function distinct from its role in DNA damage responses. In fact, in our previous studies we have already discovered a dual role of Mrc1 in yeast. Like Mrc1 mutants which show a slower S phase progression, we observed a significant growth defect and reduced replication in Claspin deficient cells. When U2OS cells were transfected with either control siRNA or two different Claspin, we found that the Claspin depleted populations had many fewer cells three days later than the control (Fig. 8b), reflecting lower growth rates in Claspin depleted cells. Also, when we performed thymidine incorporation to assess the DNA synthesis, we found that Claspin-depleted cells had a 30-40% reduction of thymidine incorporation, indicating a reduction of replication (data not shown). Since Mrc1 and *Xenopus* Claspin were previously reported to either move along with the replication forks or bind to replicating chromatin, human Claspin may promote DNA replication. Consistent with this supposition, when we infected either BJ cells or U2OS cells with the retroviral vector encoding wild type Claspin, we observed significant increase of proliferation rates in both cell lines (Fig 8c, top panel), which was consistent with an increase in the number of cells staining positive for BrdU in cells ectopically expressing Claspin (Fig. 8c, bottom panel).

In this report, we investigated the role of human Claspin in both DNA damage checkpoints and cellular proliferation. Previous studies on *Xenopus* Claspin were mainly focused on its role in response to replication stress during S phase. Here, we showed that Claspin status affected sensitivity to both replication stress and DNA damage induced by either ionizing radiation or UV. In addition, we demonstrated that Claspin was required for the IR-induced intra-S and G2/M checkpoints in U2OS cells. Therefore, in addition to associating with the replicating chromatin to ensure proper replication process, Claspin may regulate the checkpoints through replication-independent mechanisms, probably in part through its regulation on Chk1 activity. While this report was in preparation, a study by Chini and Chen (46) showed that Claspin was required for Chk1 activation in response to HU, consistent with our studies.

Chk1 is one of the key effector kinases which inhibits CDC25s to prevent S phase progression and the G2/M transition when cells countered replication stress, UV or ionizing radiation (43, 47, 48). Chk1 is believed to be activated by the ATR-ATRIP kinase (48, 49). How ATR-ATRIP controls phosphorylation and activation of Chk1, however, is still unclear. In *Xenopus* egg extracts, Claspin was shown to be required for ATR-dependent Chk1 activation. In this study, we demonstrated that Claspin itself was a downstream target of ATR and functioned with BRCA1 in the same signal transduction pathway to activate Chk1. Double depletion of Claspin and BRCA1 has no additional effects on Chk1 phosphorylation compared to depletion of individual proteins. Furthermore, Claspin depletion impaired BRCA1 phosphorylation at Ser-1524, a site mainly phosphorylated by the ATR/ATRIP complex. Therefore, our results placed

Claspin upstream of BRCA1 in ATR-Chk1 pathway and its function may be to recruit BRCA1 and Chk1 to ATR at the damaged sites on chromatin.

In addition to its roles in DNA damage responses, Claspin has a separate function involved in cell proliferation. We found that overproduction of Claspin stimulated cell proliferation. Conversely Claspin depletion slowed down proliferation, consistent with the role of its budding yeast homolog Mrc1 which is thought to act to tether DNA polymerases to sites of replication. However, a function for Claspin in control of a rate limiting step in cell proliferation is unexpected based on any of its known functions and may indicate that it positively regulated a critical cell cycle transition such as G1/S. If it has such an oncogenic role it might prove to be an antiproliferation target for cancer cells. Thus, both these studies on Claspin and the previous studies on 53BP1 forged new connections between BRCA1 and DNA damage signaling.

## **Key Research Accomplishments**

1. Identification of DNA damage induced phosphorylation sites on BRCA1.
2. Demonstration that some of these sites were required for the function of BRCA1.
3. Discovery that 53BP1 controls p53 activation
4. Discovery that 53BP1 binds to BRCA1 and releases it after DNA damage
5. Discovery that 53BP1 controls BRCA1 phosphorylation.
6. Discovery that Claspin is required for checkpoint arrest.
7. Discovery that Claspin and BRCA1 work together to control Chk1 activation.
8. Discovery that Claspin and BRCA1 form a complex after DNA damage
9. Discovery that overexpression of Claspin causes mammalian cells to proliferate more rapidly.
10. Discovery that Claspin controls the phosphorylation of BRCA1 on S1524.



## Reportable Outcomes

Two papers were published from work shown here.

Wang, B., Matsuoka, S. , Carpenter, P. B., and Elledge, S.J. (2002) 53BP1, a Mediator of the DNA Damage Checkpoint. *Science* 298:1435-1438.

Lin SY, Li K, Stewart GS, Elledge SJ (2004) Human Claspin works with BRCA1 to both positively and negatively regulate cell proliferation. *Proc. Natl. Acad. Sci. U. S. A.* 101:6484-9.

## Conclusions

The conclusions from these studies are that BRCA1 is a phospho-protein that becomes hyper-phosphorylated in response to DNA damage by the Checkpoint kinases ATM and ATR. In addition to these kinases, two other mediator proteins, 53BP1 and Claspin both work together with BRCA1 to carryout downstream function. 53BP1 controls some phosphorylation of BRCA1 and is involved in activation of the Checkpoint kinase Chk2, a known breast cancer gene. Claspin, a regulator of activation of Chk1 works together with BRCA1. In response to DNA damage, Claspin binds BRCA1 and controls its phosphorylation through the ATR pathway. Together BRCA1 and Claspin regulate Chk1 kinase activity and cell cycle arrest. These complexes act upstream of and together with BRCA1 to control its function and are therefore implicated in breast cancer. This will serve as a fertile area of future investigation into the causes of Breast Cancer.

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