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Introduction

In the last progress report, I described our progress on the isolation of Chk2 mutant cells and their characterization. That aim is essentially completed. In the last year we have concentrated on Aim 2, which includes finding Chk2 associated proteins and potential Chk2 substrates. To identify Chk2 associated proteins, we took the FHA domain (Forked-Head Associated) of Chk2 which is known to be involved in protein-Protein interaction and produced it in E. coli as a GST fusion. We then went on to use this GST-FHA domain protein as an affinity column to purify associated proteins. In this way we identified a known DNA damage regulated protein 53BP1. We provide the data for this experiment below in the Body section. 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 (1,2) which are found in many DNA damage response proteins (3-8). 53BP1 responds to DNA double strand breaks (9-12), quickly relocalizing to discrete nuclear foci upon exposure to IR. These foci colocalize with those of the Mre11/Nbs1/Rad50 complex and phosphorylated γ-H2AX which are thought to facilitate recruitment of repair factors to damaged DNA (9-11). In response to IR, 53BP1 is phosphorylated in an ATM (ataxia telangiectasia mutated) dependent manner (10-12), but its role in the DNA damage response is unclear.

Body

Identification of 53BP1 as a Chk2 FHA-domain interacting protein. To search for Chk2 associated proteins we attempted to immunoprecipitate large quantities of Chk2 from human extracts. While we could isolate large amounts of Chk2, we could not identify large quantities of associated proteins in sufficient levels to identify them by

mass spectroscopy. In an alternative approach we used a GST-Chk2 fusion construct that contain the FHA domain of Chk2. We made an affinity column of this Gst-FHA fusion protein and poured extracts from hela cells over the column. Proteins specifically associated withthis matrix were eluted and analyzed by mass spectroscopy. In this analysis we isolate 53BP1 (data not shown). We then set out to understand the function of 53BP1 in this pathway.

Analysis of the genetic role of 53BP1.

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 (13, 14). U2OS cells were transfected with these siRNA oligos and, within three days post transfection, a portion of cells had undergone cell death. 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 cells showed radio-resistant DNA synthesis (Fig. 1A). This was also seen in Saos2 and Hela cells with both siRNAs (15, 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. 1B). 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.

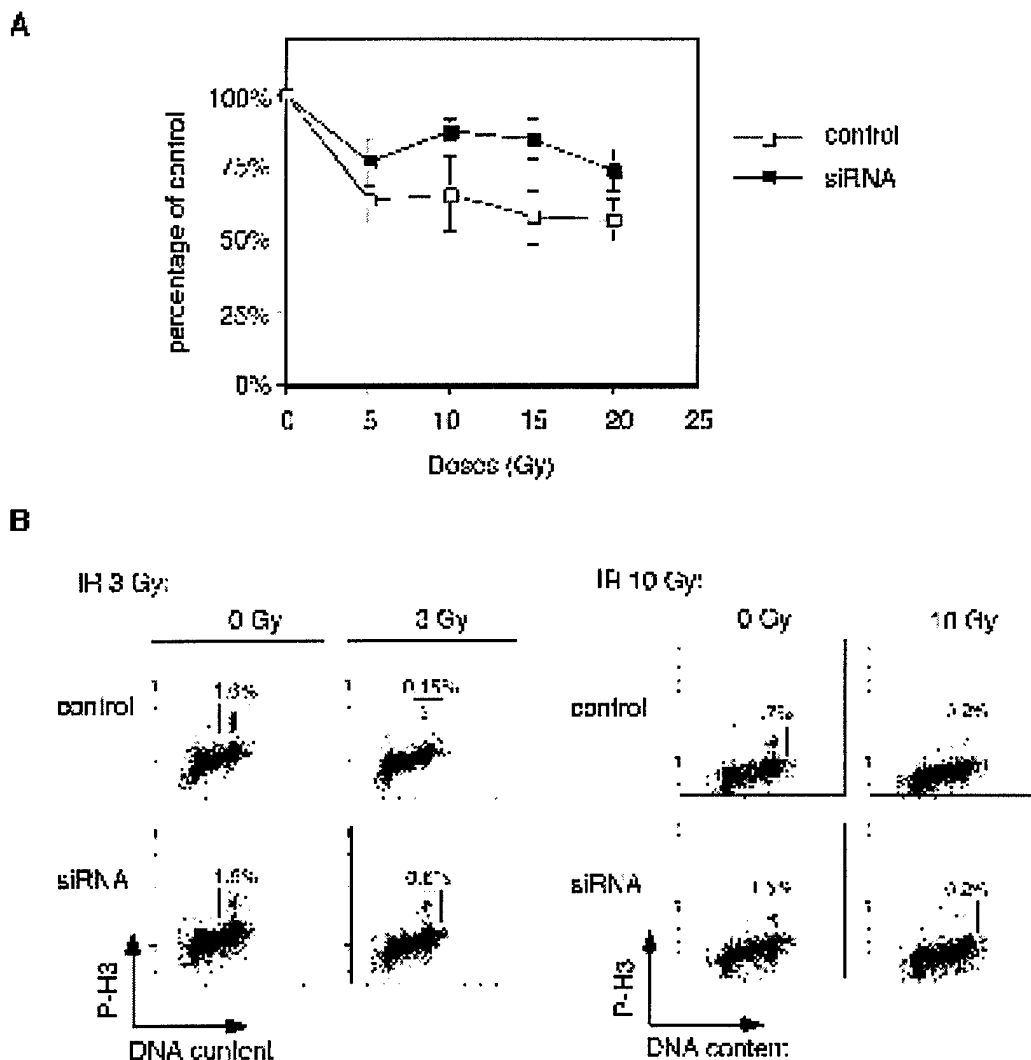

Fig. 1. 53BP1 inhibition results in defective IR-induced intra-S-phase and G2/M checkpoints. (**A**) IR-induced intra-S-phase checkpoint. Replicative DNA synthesis was assessed 30 min after various doses of ionizing irradiation in U2OS cells transfected with oligos. The DNA synthesis in unirradiated cultures was set to 100% for cells transfected with control oligos or siRNA oligos against 53BP1 (14). Error bars represent the standard deviation of triplicate samples. (**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 (Cell Signaling), followed by FITC-conjugated secondary antibody (Jackson Immunoresearch Laboratories), and percentage of the M-phase cells was determined by flow cytometry.

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. 2A). We then examined Chk2, a checkpoint protein implicated in p53 regulation that is phosphorylated on Thr68 and forms foci in response to IR (16,17). Quantification of the ratio of Chk2 phosphorylated on Thr 68 to the total amount of Chk2 revealed that Chk2 phosphorylation at Thr 68 was reduced 2-fold at 2h in response to IR in the 53BP1-inhibited cells (Fig. 2B). The reduction of Chk2 phosphorylation at Thr 68 was reproducibly observed at 1 h or 2h after IR in different experiments (15). A much stronger effect was observed in the formation of IR-induced foci recognized by antibodies raised against P-T68 of Chk2 (17), which were nearly completely abolished in 53BP1 siRNA-treated cells, but were unaffected in control cells (Fig. 2C).

53BP1 resembles the Rad9 BRCT-repeat protein of budding yeast, which binds to and is required for the DNA-damage induced activation of Rad53, a homologue of Chk2 (16). Like Rad9 and Rad53, we found that antibodies to Chk2, but not control antibodies, could efficiently immuno-precipitate 53BP1 and that Chk2 dissociates from 53BP1 in response to IR (Fig. 2D). This association was also detected in the reciprocal IP using 53BP1 antibodies. These data suggest that 53BP1 may act as an adaptor that facilitates Chk2 phosphorylation. It is likely that 53BP1 facilitates Chk2 activation in a transient complex, and upon activation, Chk2 dissociates from the 53BP1 complex.

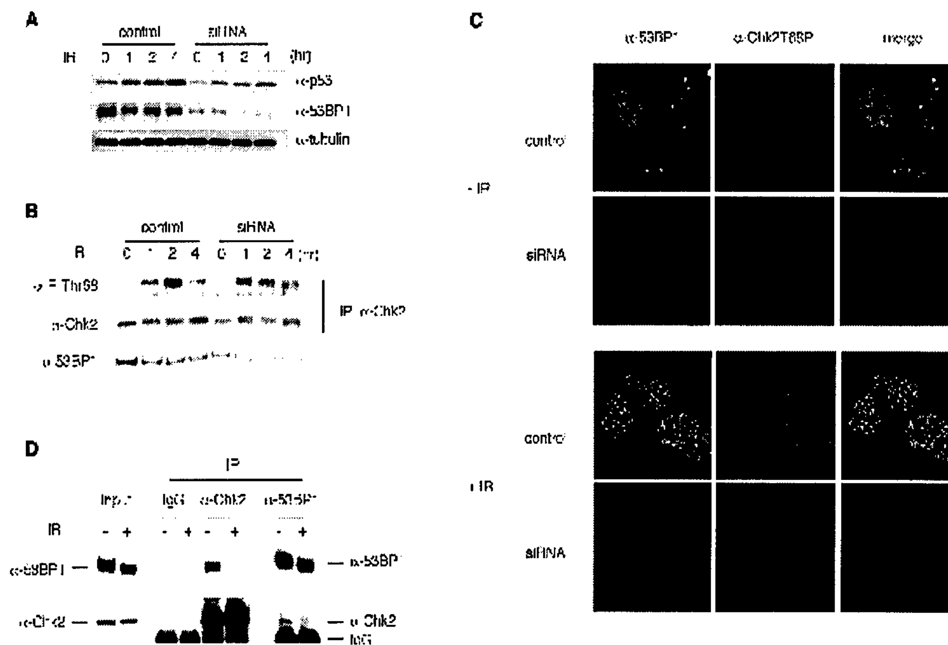

Fig. 2. 53BP1 regulates p53 and Chk2 in response to IR. **(A)** IR-induced p53 stabilization. U2OS cells were transfected with siRNA oligos against 53BP1 or control oligos for two days, then exposed to 10Gy ionizing irradiation. Cell lysates were made from samples at indicated times recovered from irradiation and separated on SDS-PAGE gel. Western blots were performed using anti-53BP1, anti-tubulin and anti-p53 (Oncogene) antibodies. **(B)** Chk2 phosphorylation at Thr 68 is reduced in 53BP1-inhibited cells. Chk2 immunoprecipitates were prepared from U2OS cells at indicated hours after exposure to 10 Gy irradiation. Western blots were performed using anti-Chk2 (14) and anti- T68P - Chk2 antibodies (14). **(C)** IR-induced phospho-foci recognized by antibodies against P-T68 of Chk2 depend on 53BP1. SiRNA transfected U2OS cells were irradiated with 10 Gy irradiation, and 2 hour later were fixed with paraformaldehyde, permealized with Triton X-100, and then immunostained with antibodies against Chk2T68P (provided by J. Chen) and 53BP1 (provided by T. D. Halazonetis) and the appropriate FITC- (Molecular Probes) and Cy3-conjugated secondary antibodies (Amersham). **(D)** 293T cells were untreated (-) or treated (+) with 20 Gy IR and harvested after 1h. Cell extracts were incubated with control IgG, anti-Chk2 or anti-53BP1 antibodies and protein A Sepharose. Immunoprecipitates were separated by SDS-PAGE, and then immunoblotted with anti-53BP1 and anti-Chk2 antibodies as indicated.

The discrepancy between the partial dependency of 53BP1 for Chk2 phosphorylation and its major role in the formation of phospho-foci could be explained if Chk2 or other proteins phosphorylated via the 53BP1 pathway were solely responsible for the foci recognized by these antibodies. Alternatively, 53BP1 might function as a general regulator of foci formation. To test this, we examined the ability of other proteins to form foci, in the absence of 53BP1. Brca1, Nbs1, and γ-H2AX all form foci in

response to IR (16). 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 (Fig. 3A). 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 (Fig. 3A). Similar results were obtained in Hct116 and Hela cells with both oligo pairs (15). In contrast, formation of γ-H2AX foci or Nbs1 foci after IR remained unchanged in cells treated with control oligos or siRNA oligos (Fig. 3B). Rad51 foci were also unchanged (15).

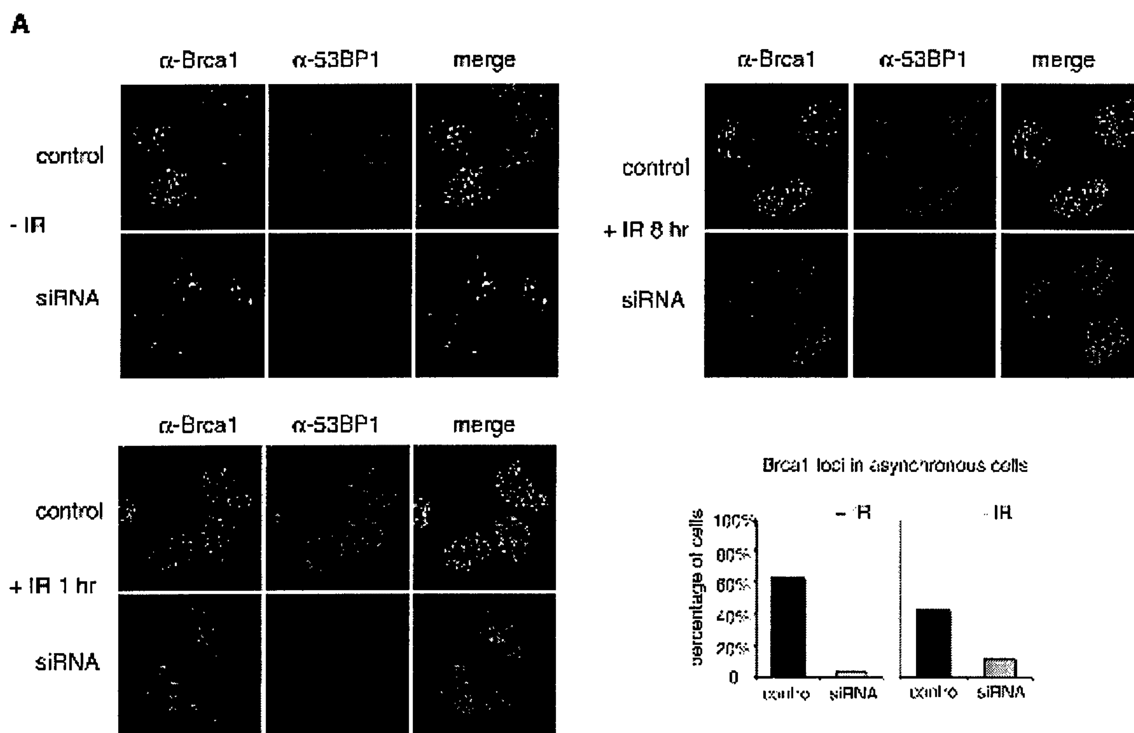

Fig. 3. Brca1 localization in S-phase and relocalization in response to IR is dependent on 53BP1. (A) Brca1 localization in the presence and absence of 10 Gy IR. U2OS cells were transfected with siRNA against 53BP1 or control oligos and 2 days later exposed to 10 Gy IR. At the indicated times after IR, cells were permeablized with paraformaldehyde and fixed with Triton X-100. Immunostaining were performed using antibodies against 53BP1 and Brca1. Images were taken using a Zeiss confocal microscope. Quantitation of the BRCA1 foci are shown. These data were obtained using siRNA oligo pair #1 against 53BP1.

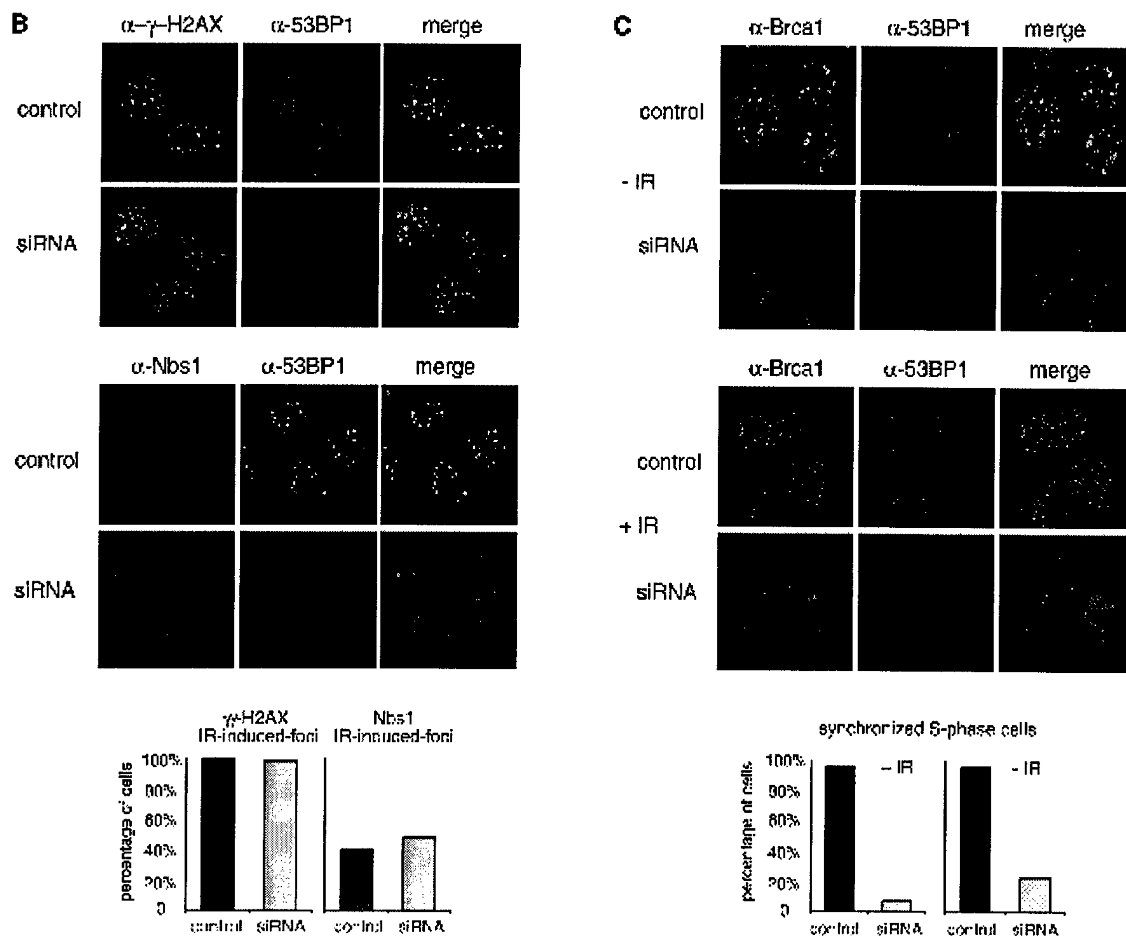

Fig. 3. (**B**) IR-induced Nbs1 and γ-H2AX nuclear foci are independent of 53BP1. U2OS cells were treated and fixed as described in (A). Samples for γ-H2AX (antibodies provided by W.M. Bonner) staining were taken from cells recovered 2 hour after exposure to 10 Gy IR, and Nbs1 samples were cells recovered 6 hour after treatment with 10 Gy IR. Quantitation of foci are shown below. (**C**) Brcal nuclear foci in synchronized S-phase cells in the presence and absence of 10 Gy IR are dependent on 53BP1. U2OS cells were sychronized using a double-thymidine block and released as described (14). At 4 hours after release, >80% of the cells were in S-phase indicated by flow cytometry. Cells at this stage were treated with 10 Gy irradiation, and recovered for 1 hour at 37 °C. Cells were fixed and immunostained as described. Quantitation of foci are shown below.

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 (Fig. 3A). To control for cell cycle differences, we

synchronized cells using a double-thymidine block (14), 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 (Fig. 3C).

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 (Fig. 3A). 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). As with the G2/M checkpoint, the strongest dependency of Brca1 phosphorylation appeared to be at lower doses of IR (Fig. 4B).

High levels of IR have been shown to obscure BRCA1 regulation by other proteins such as ATM (18). 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 and 4B). Furthermore, while BRCA1 phosphorylation showed less dependency on 53BP1 at 50Gy IR, these cells still failed to form foci (15).

Next we examined whether 53BP1 associated with BRCA1. Brca1 interacts with 53BP1 in vivo, and like Chk2, this interaction was abolished in response to IR (Fig. 4C). Thus, this dynamic association is likely to be important for regulation of 53BP1's ability to regulate both Chk2 and BRCA1 function in response to DNA damage.

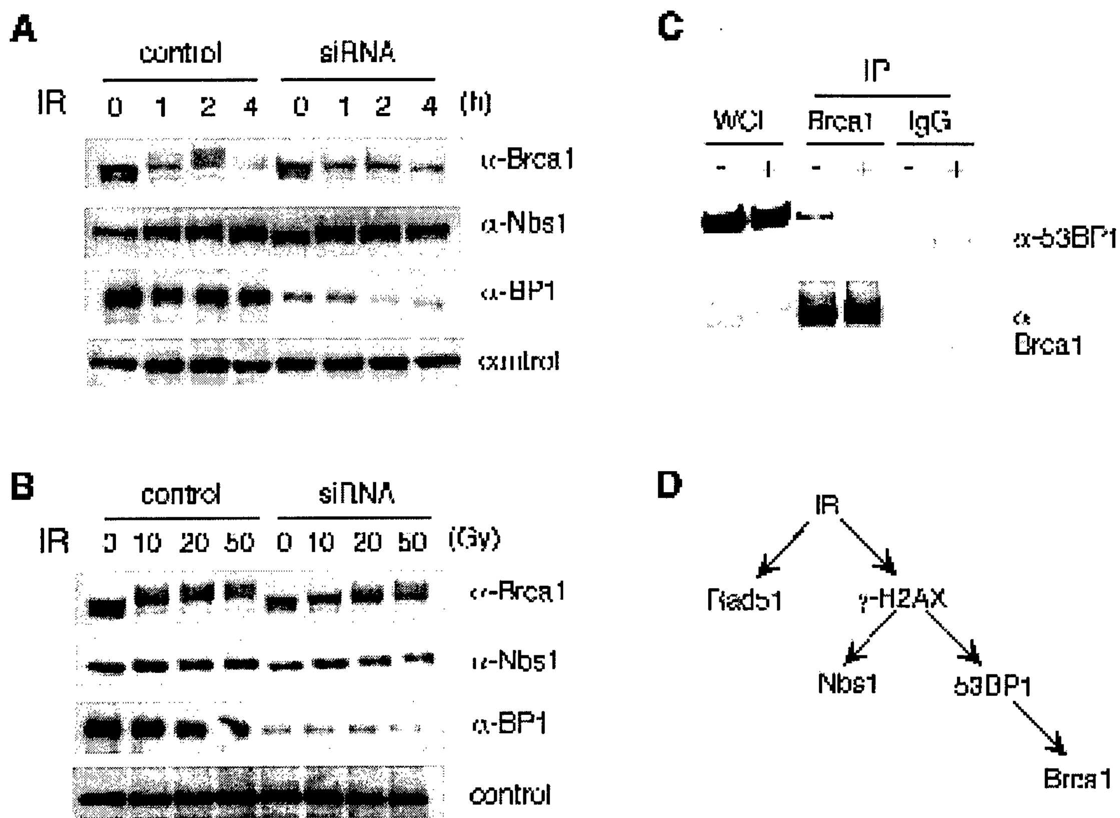

Fig. 4. 53BP1 regulation of Brca1. **(A)** Brca1 phosphorylation is reduced in the absence of 53BP1. U2OS cells were treated with siRNA oligos against 53BP1 or control oligos for two days. Cells were exposed to 10 Gy irradiation and cell lysates were prepared at indicated times after irradiation. Immunoblots 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)** Brca1 phosphorylation in response to different doses of irradiation. U2OS cells were transfected with siRNA oligos against 53BP1 or control oligos for two days, then treated with different doses of irradiation. Cell lysates were prepared at 2h after irradiation. **(C)** 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). **(D)** A schematic showing the genetic dependence for formation of nuclear foci for different proteins in response to IR.

We will continue these efforts to identify additional Chk2 associated proteins. In addition, we plan to start to look for Chk2 substrates by new phospho-proteomic methods we are currently collaborating on these new methods with other investigators here at Harvard. We are trying to identify all DNA Damage induced phospho-peptides in cells and hope to be able to sort out which belong to Chk1, Chk2, Atm and Atr in the future. This is a large undertaking and we do not yet know the boundaries of the effort.

Key research Accomplishments
A) Discovery that Chk2 interacts with 53BP1.

Reportable Outcomes
Wang, B., Matsuoka, S. , Carpenter, P. B., and Elledge, S.J. *Science* 298:1435-1438.

Morales, J.C., Xia, Z., Lu, T., Aldrich, M.B., Wang, B., Rosales, C., Kellems, R.E., Hittelman, W.N., Elledge, S.J. and Carpenter, P.B. *J. Biol. Chem.* 278:14971-7.

Conclusions

The major finding of these 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 and similar results have been obtained by others (R. DiTullio and T. Halazonetis, personal communication). 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, Chk2 and Brca1 and controls the phosphorylation of at least two of these proteins, 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 (19) and 53BP1 controls the ability of at least BRCA1 but not Nbs1 to form foci as depicted in the pathway model shown in Fig. 4D. 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 p53 and other tumor suppressor proteins and is likely to play an important role in the

maintenance of genomic stability and prevention of cancer (20, 21). We have also recently helped make 53BP1 mutant cells, which have many of the properties we observed for the siRNA treated cells. These cells were derived from a 53BP1 mutant mouse generated by Phil Carpenter. We did not contribute to making the mouse, we worked with only the mouse embryonic fibroblasts from these mice. That work is described in Moraleset al. (2003) *J. Biol. Chem.* 278:14971-7. We played only a small role in that project.

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