were resistant to the growth-inhibitory effects of IFN-α and proliferated in the presence of 50 U/ml of IFN-α at a rate comparable to that of untreated controls (Fig. 3B). Cells possessing mutations in the vIL-6 promoter at either ISRE-1 or ISRE-2 had diminished IFN resistance and reduced proliferation at low concentrations of IFN-α.

Feedback inhibition of IFN signaling by vIL-6 provides a clear example of how virus subversion of host cell defenses can lead to cell proliferation. Why does cellular IL-6 not achieve the same effect? Both hIL-6 and vIL-6 can initiate IL-6 signaling in BCP-1 cells, as measured by electrophoretic mobility–shift assays in which the gamma-inteferon activation sequence (GAS) element from the interferon regulatory factor 1 (IRF-1) promoter is used as a probe, although vIL-6 signaling is more robust (*18*). The answer may lie in differences in receptor usage by the two cytokines. IFN-α treatment results in down-regulation of gp80 surface expression but has no effect on gp130 surface expression (Fig. 4A), an effect previously noted for other B cell lines, including the IL-6–dependent U266 multiple myeloma cell line (*26*). IFN-α also blocks hIL-6–induced but not vIL-6–induced gp130 tyrosine phosphorylation (Fig. 4B), demonstrating that the blockage occurs at the receptor level. gp80 mRNA expression is not markedly altered by IFN-α treatment, suggesting that gp80 blockade is largely due to posttranscriptional inhibition (fig. S4). This leads to a model (Fig. 4C) in which viral evolution has generated a modified cytokine that escapes regulatory control of IL-6 signaling by IFN-α. Infected cells that normally would either arrest or undergo apoptosis in response to IFN signaling continue to proliferate in the presence of vIL-6, resulting in a virus-human autocrine feedback circuit.

vIL-6 inhibits tumor-suppressor pathways activated during immune signaling, but it is important to emphasize that this is not solely responsible for PEL tumorigenesis, which results from multiple, combined viral and host cell genetic influences. Viruses have evolved a variety of ways to overcome host defenses against infection, including abrogating IFN signaling pathways (*27*, *28*). KSHV itself possesses another protein, vIRF-1, to inhibit IFN-mediated transcription. By sensing levels of IFN-α signaling, KSHV reacts to and modifies its cellular environment through vIL-6, exhibiting a fundamental property of biological systems called irritability that has been previously used to distinguish viruses from higher forms of life (*29*). In addition to immune evasion, it is possible that this mechanism plays a role in maintaining viral latency by preventing IFN induction of lytic replication. The autocrine loop established by vIL-6 illustrates mechanistically how interference with antiviral defenses can contribute to tumor cell proliferation and provides an attractive target for novel therapies directed against KSHV-related hematopoietic tumors.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S4
Table S1
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53BP1, a Mediator of the DNA Damage Checkpoint

Bin Wang,[1] Shuhei Matsuoka,[1] Phillip B. Carpenter,[4] Stephen J. Elledge[1,2,3]*

53BP1 binds to the tumor suppressor protein p53 and has a potential role in DNA damage responses. We used small interfering RNA (siRNA) directed against 53BP1 in mammalian cells to demonstrate that 53BP1 is a key transducer of the DNA damage checkpoint signal. 53BP1 was required for p53 accumulation, G_2-M checkpoint arrest, and the intra-S-phase checkpoint in response to ionizing radiation. 53BP1 played a partially redundant role in phosphorylation of the downstream checkpoint effector proteins Brca1 and Chk2 but was required for the formation of Brca1 foci in a hierarchical branched pathway for the recruitment of repair and signaling proteins to sites of DNA damage.

53BP1 was identified through its ability to bind to the tumor suppressor protein p53 through 53BP1's COOH-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 ionizing radiation (IR). These foci colocalize with those of the Mre11-Nbs1-Rad50 complex and phosphorylated γ-H2AX, which are thought to facilitate the recruitment of repair factors to damaged DNA (*9*–*11*). In response to IR, 53BP1 is phosphorylated in an ataxia telangiectasia mutated (ATM)–dependent manner (*10*–*12*), but its role in the DNA damage response is unclear.

To determine 53BP1's role, we used small interfering RNAs (siRNAs) in the form of two independent, nonoverlapping, 21–base pair RNA duplexes that target 53BP1 to inhibit its expression (*13*, *14*). U2OS cells were transfected with these siRNA oligonucleotides (oligos) and, within 3 days posttransfection, a portion of cells had undergone cell death (fig. S1). A similar phenotype was also observed in two other cell lines, Hct116 and Saos2 (*15*).

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

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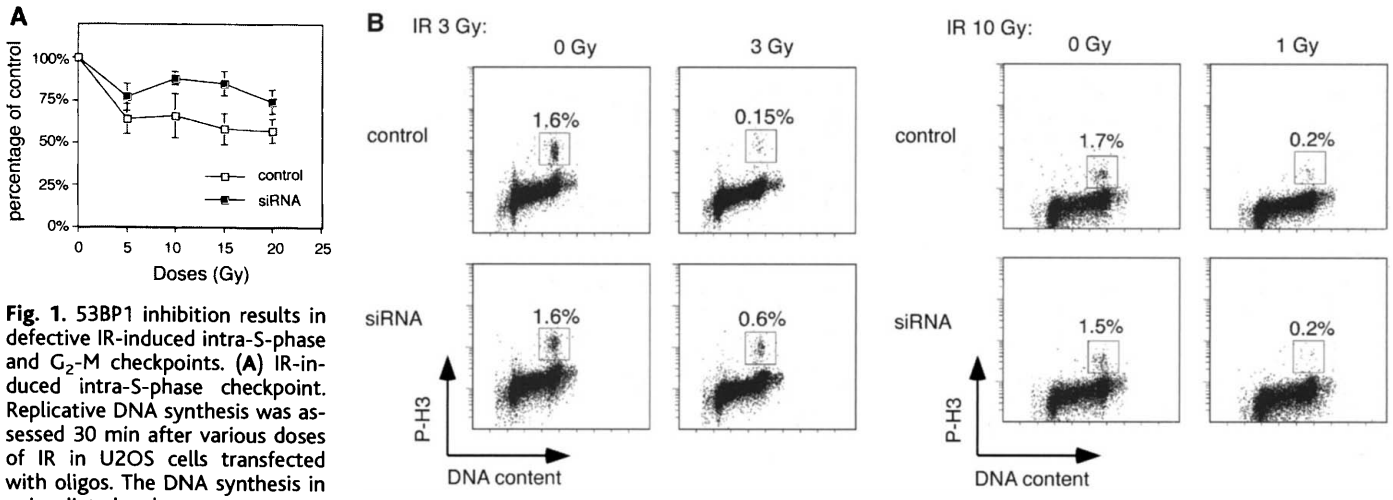

Fig. 1. 53BP1 inhibition results in defective IR-induced intra-S-phase and G_2-M checkpoints. (**A**) IR-induced intra-S-phase checkpoint. Replicative DNA synthesis was assessed 30 min after various doses of IR in U2OS cells transfected with oligos. The DNA synthesis in unirradiated cultures was set to 100% for cells transfected with control oligos or siRNA oligos targeting 53BP1 (*14*). Error bars represent the standard deviation of triplicate samples. (**B**) Analysis of the G_2-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 before fixation. Cells in mitosis were determined by staining with propidium iodide and antibody to phosphohistone H3 (P-H3) (Cell Signaling, Beverly, MA), followed by fluorescein isothiocyanate (FITC)–conjugated secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA), and the percentage of the M-phase cells was determined by flow cytometry.

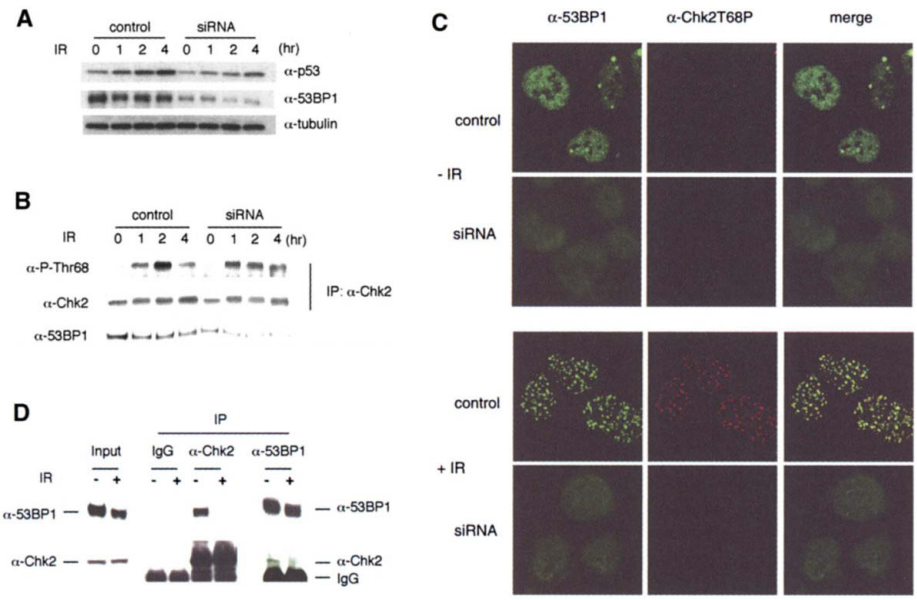

Fig. 2. 53BP1 regulates p53 and Chk2 in response to IR. (**A**) IR-induced p53 stabilization. U2OS cells were transfected with siRNA oligos targeting 53BP1 or control oligos for 2 days, then exposed to 10-Gy IR. Cell lysates were made from samples recovered from irradiation at the indicated times and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Western blots were performed with the use of antibodies to 53BP1, tubulin, and p53 (Oncogene, San Diego, CA). (**B**) Chk2 phosphorylation at Thr^{68} is reduced in 53BP1-inhibited cells. Chk2 immunoprecipitates were prepared from U2OS cells at the indicated hours after exposure to 10-Gy irradiation. Western blots were performed using antibodies to Chk2 (*14*) and to T68P-Chk2 (*14*). (**C**) IR-induced phospho-foci recognized by antibodies to P-T68 of Chk2 depend on 53BP1. SiRNA-transfected U2OS cells were irradiated with 10-Gy irradiation and 2 hours later were fixed with paraformaldehyde, permealized with Triton X-100, and then immunostained with antibodies to Chk2T68P (*23*) and 53BP1 (*23*) and the appropriate FITC- (Molecular Probes, Eugene, OR) and Cy3-conjugated secondary antibodies (Amersham). (**D**) 293T cells were untreated (–) or treated (+) with 20-Gy IR and harvested after 1 hour. Cell extracts were incubated with antibodies to immunoglobulin G (IgG, control), Chk2, or 53BP1 and protein A Sepharose. Immunoprecipitates were separated by SDS-PAGE and then immunoblotted with antibodies to 53BP1 and Chk2 as indicated.

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*) and indicates a role of 53BP1 in the intra-S-phase checkpoint.

To assess the G_2-M checkpoint, we irradiated 53BP1-inhibited and control cells with 3 or 10 gray (Gy) of IR. About threefold more 53BP1-inhibited cells than the control cells treated with 3 Gy entered into mitosis (Fig. 1B). However, inhibition of 53BP1 had no effect after 10-Gy IR. Therefore, 53BP1-inhibited cells also displayed an IR-induced G_2-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.

Because 53BP1 binds p53, we asked whether 53BP1 was required for p53 activation in response to IR. The induction of p53 in response to IR was substantially decreased in 53BP1-inhibited cells (Fig. 2A). We then examined Chk2, a checkpoint protein implicated in p53 regulation that is phosphorylated on Thr^{68} 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 twofold after 2 hours in response to IR in the 53BP1-inhibited cells (Fig. 2B). The reduction of Chk2 phosphorylation at Thr^{68} was reproducibly observed at 1 or 2 hours after IR in different experiments (*15*). A much stronger effect was observed in the formation of IR-induced foci recognized by antibodies to P-T68 of Chk2 (*17*), which were reduced 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 homolog of Chk2 (*16*). Like Rad9 and Rad53, we found that antibodies to Chk2 but not control antibodies could efficiently immunoprecipitate 53BP1 and that

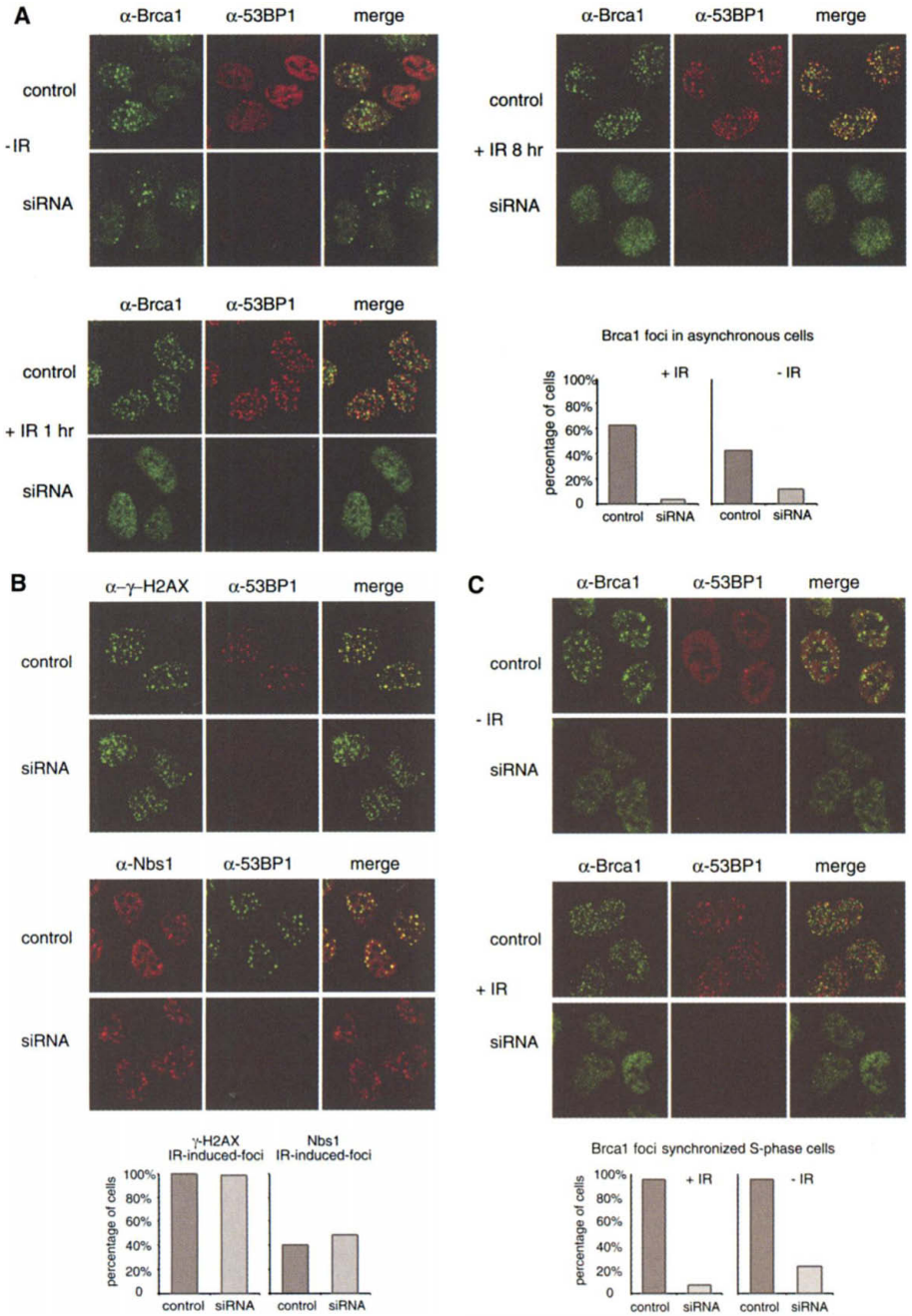

Fig. 3. Brca1 localization in S phase and relocalization in response to IR is dependent on 53BP1. (**A**) Brca1 localization in the presence and absence of 10-Gy IR. U2OS cells were transfected with siRNA targeting 53BP1 or control oligos and 2 days later exposed to 10-Gy IR. At the indicated times after IR, cells were permeablized with paraformaldehyde and fixed with Triton X-100. Immunostaining were performed with antibodies to 53BP1 and Brca1. Images were taken with a Zeiss confocal microscope. Quantitation of the BRCA1 foci are shown. These data were obtained with the use of siRNA oligo pair #1 targeting 53BP1. (**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 (*23*) staining were taken from cells recovered 2 hours after exposure to 10-Gy IR, and Nbs1 samples were cells recovered 6 hours after treatment with 10-Gy IR. Quantitation of foci are shown below. (**C**) Brca1 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 as 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.

Chk2 dissociates from 53BP1 in response to IR (Fig. 2D). This association was also detected in the reciprocal immunoprecipitate with the use of 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.

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 only a subpopulation of phospho-Chk2 were responsible for the foci. A second explanation would be if other proteins phosphorylated by the 53BP1 pathway besides Chk2 were recognized by these antibodies, because the immunofluorescence specificity of these antibodies for phospho-Chk2 has not been fully established (*17*). 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 hours post-IR, only 4% of the cells formed Brca1 nuclear foci when cells were treated with 53BP1siRNA, as 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*).

When asynchronous control cells were analyzed for Brca1 foci formation in the absence of IR, about 40% contained more than 20 Brca1 foci, reflecting the S-phase and G_2 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 with the use of 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 IR (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

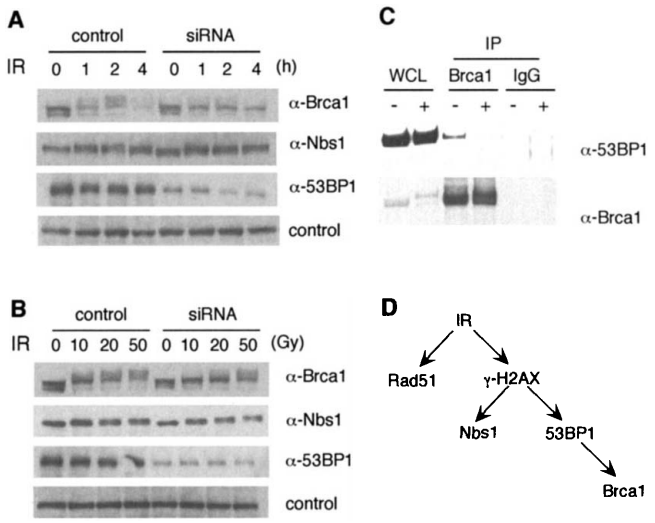

Fig. 4. 53BP1 regulation of Brca1. **(A)** Brca1 phosphorylation is reduced in the absence of 53BP1. U2OS cells were treated with siRNA oligos targeting 53BP1 or control oligos for 2 days. Cells were exposed to 10-Gy irradiation, and cell lysates were prepared at indicated times after irradiation. Immunoblots were performed with antibodies to Brca1 (Oncogene), Nbs1 (Norvus, Littleton, CO), and 53BP1. The control band is a nonspecific band from the same blot that was incubated with antibodies to Brca1. **(B)** Brca1 phosphorylation in response to different doses of irradiation. U2OS cells were transfected with siRNA oligos targeting 53BP1 or control oligos for 2 days, then treated with different doses of irradiation. Cell lysates were prepared at 2 hours after irradiation. **(C)** 53BP1 associates with Brca1. Cell lysates from untreated U2OS cells or 2 hours after exposure to 10-Gy IR were incubated with antibodies to Brca1 or rabbit IgG as a control. Western blots were performed with antibodies to 53BP1 and Brca1 (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.

siRNA oligos targeting 53BP1 relative to controls (Fig. 4A). As with the G_2-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*). The loss of 53BP1 did not have a general effect on the DNA damage-inducible phosphorylation of other proteins; for example, Nbs1 phosphorylation was not affected (Fig. 4, A and B). Furthermore, although BRCA1 phosphorylation showed less dependency on 53BP1 at 50-Gy 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.

An important 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 G_2-M checkpoints; similar results have been obtained by others (*19*). It is 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. Because it binds to p53, Chk2, and Brca1 and controls the phosphorylation of at least two of these proteins, 53BP1 has the property of a mammalian adaptor or mediator that might recruit a subset of substrates to the ATM and ATR (ataxia telangiectasia and rad3-related) 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 (*20*), 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 (*21*, *22*).

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