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TITLE: Involvement of 53BP1, a p53 Binding Protein, in Chk2 Phosphorylation of p53 and DNA Damage Cell Cycle Checkpoints

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Involvement of 53BP1, a p53 Binding Protein, in Chk2 Phosphorylation of p53 and DNA Damage Cell Cycle Checkpoints

53BP1, a p53 binding protein, is involved in DNA damage response. It is phosphorylated in response to DNA damage and rapidly relocalizes to sites of damage, forming nuclear foci that colocalize with those formed by phosphorylated histone H2AX (-H2AX), Mre11-Rad50-Nbs1 complex, MDC1, Brc1 and other DNA damage signaling proteins. Our studies aimed to determine the role of 53BP1 in DNA damage response and tumor suppression. We studied the function of 53BP1 in mammalian cells by knocking down expression of 53BP1 using small interfering RNA (siRNA) against 53BP1. We also generated 53BP1-defective (53BP1tr/tr) mouse model with expression of a defective C-terminal truncation of the m53BP1 protein. We have shown that 53BP1 is a key transducer in the DNA damage response signaling. Inhibition of 53BP1 by siRNA in human cancer cell lines resulted in defective S-phase and G2/M checkpoints in response to ionizing irradiation (IR). 53BP1 interacts with tumor suppressors p53, Chk2 and Brc1, and is involved in regulation of these proteins in response to IR. 53BP1tr/tr mice were growth retarded and IR-sensitive. Mouse embryonic fibroblast (MEF) cells generated from 53BP1tr/t mice were hypersensitive to IR, and exhibited higher level of chromosomal abnormalities when treated with genotoxic stress, indicating a role of 53BP1 in maintaining genomic stability. Thus 53BP1 is likely to play an important role in maintenance of genomic stability and cancer prevention. These studies allowed us gain further insights into the DNA damage response pathway and the tumor suppression pathway in mammalian cells.

53 BP1, Chk2, Checkpoint, DNA damage repair, tumor suppressor, mice knockout

14. ABSTRACT

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Introduction

53BP1 binds to the tumor suppressor protein p53 and has a potential role in DNA damage responses. We have shown that 53BP1 is a key transducer in the DNA damage response signaling. Inhibition of 53BP1 by siRNA in human cancer cell lines resulted in defective S-phase and G2/M checkpoints in response to ionizing irradiation (IR). Mouse embryonic fibroblast (MEF) cells generated from 53BP1-/- mice were hypersensitive to IR, and displayed a slight G2/M checkpoint deficiency in response to lower dose of IR. We also showed that 53BP1 binds to p53, Chk2 and Brcal. 53BP1 plays an important role in p53 stabilization, Chk2 phosphorylation in response to IR.

Body

Task 1. To determine the role of 53BP1 in Chk2 activation and phosphorylation of p53 (months 1-12)
   a. To identify the association of Chk2 with 53BP1 in response to DNA damage (months 1-6)
   b. To determine the involvement of 53BP1 in Chk2 phosphorylation and/or stabilization of p53 (months 6-12)

In the previous reports, we have shown that 53BP1 associated with Chk2, and the association is a dynamic process. Chk2 binds to 53BP1 in the absence of IR, and dissociates in the presence of IR. 53BP1 is also partially responsible for Chk2 T-68 phosphorylation and p53 stabilization (attached paper Wang et al., 2002). To further understand the role of 53BP1 in Chk2 phosphorylation and p53 stabilization, we seek to find 53BP1 associated proteins, we found that DNA damage repair protein Ku80 interacted with 53BP1 (Figure 1). This indicates a role of 53BP1 in non-homologous end joining. Meanwhile, by deletion mutagenesis, we found that 53BP1 could oligomerize in the presence and absence of DNA damage (Figure 2). We also confirmed that p53 binds to the BRCT region of 53BP1. Although BRCT motifs are proposed to be phosphopeptide binding modules, the interaction of p53 with 53BP1 is phosphorylation independent.

Task 2. To determine the role of 53BP1 in cell cycle checkpoints in response to DNA damage (months 1-24)
   a. Generation of the 53BP1 somatic knockout cells derived from hTERT-immortalized human epithelia cells (months 1-8)
   b. Characterization of the sensitivity to DNA, chemotherapeutic agents and transformation potential for 53BP1 null cells (months 9-12)
   c. To determine if 53BP1 deficiency will affect the G1, S, G2/M checkpoints and affect the known key players (i.e. ATM, ATR, Chk2, Chk1) (months 12-24)

We have used siRNA against 53BP1 in human cancer cell lines to generate 53BP1-deficient cells. By analyzing such cells, we were able to show that 53BP1 is involved in S-phase checkpoint and G2/M phase checkpoint. We also found that immortalized 53BP1-/− MEFs were IR-sensitive, but only displayed checkpoint
deficiency in response to lower doses of IR. We showed that Chk2 phosphorylation was partially compromised in the 53BP1-siRNA treated cells. We then tested whether other key players of the DNA damage response, such as ATM, ATR, Chk1, NBS1, SMC1, etc. were affected with inhibition of 53BP1 in response to different types of DNA damage. No difference in activation of ATM, Chk1, or phosphorylation of NBS1 and SMC1 could be detected in the 53BP1 siRNA-treated cells and the control cells. Therefore, 53BP1 seems a specific mediator in regulating Chk2 and p53 in response to IR.

**Task 3.** To determine the role of 53BP1 in development and tumor suppression (1-36)

- **a.** Generation of 53BP1-deficient mice (months 1-12)
- **b.** Characterization of 53BP1 in development (months 12-24)
- **c.** Determination of 53BP1 in tumor suppression (months 24-36)

In collaboration with Dr. Phillip Carpenter group in UT Medical School, we have been able to genotype and analyze the 53BP1-/- mice that were generated by Lexicon Inc. (Woodland, TX). We have shown that 53BP1-/- mice were hypersensitive to IR and cells from these animals exhibited chromosomal abnormalities (attached paper Morales et al., 2003). This suggests a role of 53BP1 in maintaining genomic stability and tumor suppression.

**Figure 1. 53BP1 interacts with Ku80.** 293T cells were unirradiated or irradiated with 10-Gy IR. Cells were lysed 2 hours after irradiation, in the presence or absence of Benzonase (Novagen) or lambda phophatase (NEB). Immunoprecipitation was performed with anti-53BP1 antibody or IgG (control). Western blot was carried out using anti Ku80 antibody or anti 53BP1 antibody.

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![Western blot image]
Figure 2. Flag-tagged 53BP1 interacts with HA-tagged 53BP1. (A) A diagram showing the HA-tagged fragments of 53BP1 protein interacting with Flag-tagged full-length 53BP1 protein. Black rectangle represents one copy of the SV40 nuclear localization signal (NLS). (B) Flag-tagged full-length 53BP1 was co-expressed with HA-tagged different fragments of 53BP1 in 293T cells. Lysates were made from cells treated with or without 10 Gy IR for 2 hours. Immunoprecipitates were made from anti-flag antibody, western blots was performed with anti-HA antibody. (C) Flag tagged full-length 53BP1 was coexpressed with HA-tagged different fragments of 53BP1 in 293T cells. Cell lysates were immunoprecipitated with anti-Flag or anti-HA antibody.
Key Research Accomplishments

- 53BP1 is a checkpoint protein. It is involved in S-phase and G2/M checkpoints
- 53BP1 associates with Chk2 and is involved in its phosphorylation in response to IR
- 53BP1 is involved in p53 stabilization in response to IR
- 53BP1 associates with Brca1 and is involved in its phosphorylation in response to IR
- 53BP1 interacts with DNA damage repair protein Ku80 that is involved in non-homologous end joining

Reportable Outcomes


Conclusions

We have shown that 53BP1 is one of the functional homologs of yeast Rad9, functioning as a mediator of DNA damage response signaling. We also showed that 53BP1 is involved in regulation of three tumor suppressors, p53, Chk2 and Brca1. These evidences supports that 53BP1 may be a new tumor suppressor protein. Currently we are seeking to understand how 53BP1 regulates Chk2 and p53 in response to IR. These studies will allow us gain further insights into the DNA damage response pathway and the tumor suppression pathway in mammalian cells.
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-interferon regulatory factor 1 (IRF-1) promoter is used as a probe, although vIL-6 signaling is more robust (16). The answer may lie in differences in receptor usage by the two cytokines. IFN-α treatment results in down-regulation of gpl30 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 gpl30 tyrosine phosphorylation (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 gpl30 tyrosine phosphorylation (Fig. 4A), an effect previously noted for other B cell lines, including the IL-6–dependent U266 multiple myeloma cell line (26).

In response to IR, 53BP1 is phospho-
lysed through its ability to bind to the tumor suppressor protein p53 through 53BP1's COOH-terminal BRCT (Brca1 carboxyl-terminal) repeats (1, 2), which are found in many DNA damage response proteins (3–5). 53BP1 responds to DNA double-strand breaks (9–11), quickly relocating 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 repair 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-terminal) repeats (1, 2), which are found in many DNA damage response proteins (3–5).

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53BP1, a Mediator of the DNA Damage Checkpoint

Bin Wang,1 Shuhei Matsuoka,1 Phillip B. Carpenter,4 Stephen J. Elledge1,2,9

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, G2–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.

R E P O R T S
Fig. 1. 53BP1 inhibition results in defective IR-induced intra-S-phase and G<sub>2</sub>-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 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 phospho-histone 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<sup>68</sup> 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, or to T68P-Chk2 (14). (C) IR-induced phospho-foci were transfected with siRNA oligos targeting 53BP1 or control oligos for 2 days, then exposed to 10-Gy IR. Cell lysates were incubated with antibodies to Chk2 (14) and to T68P-Chk2 (14). The reduction of Chk2 phosphorylation at Thr<sup>68</sup> 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 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<sub>2</sub>-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 mitosis (Fig. 1B). However, inhibition of 53BP1 had no effect after 10-Gy IR. Therefore, 53BP1-inhibited cells also displayed an IR-induced G<sub>2</sub>-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.
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 53BP1 siRNA, as compared to 60% of the control cells (Fig. 3A). Similar results were obtained in HeLa and HeLa cells with both oligo pairs (Fig. 3B). 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 (Fig. 3B).

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 G2 populations. 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 (Norvis, 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.

References and Notes
14. Material and methods are available as supporting material on Science Online.
23. Antibodies to Chk2 and p53 were provided by D. Chen, 53BP1, T. D. Halazonetis, and γ-H2AX, W. M. Bonner.
24. We thank D. Cortez for helpful discussions W. M. Bonner, T. D. Halazonetis, J. Qin, and J. Chen for providing antibodies and T. Halazonetis for sharing unpublished checkpoint information and suggesting the use of STAG4. B.W. is a fellow of the U.S. Army Breast Cancer Postdoctoral Trainee Award and S.J.E. is an Investigator with the Howard Hughes Medical Institute.

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MDC1 is a mediator of the mammalian DNA damage checkpoint

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To counteract the continuous exposure of cells to agents that damage DNA, cells have evolved complex regulatory networks called checkpoints to sense DNA damage and coordinate DNA replication, cell-cycle arrest and DNA repair. It has recently been shown that the histone H2A variant H2AX specifically controls the recruitment of DNA repair proteins to the sites of DNA damage. Here we identify a novel BRCA1 carboxy-terminal (BRCT) and forkhead-associated (FHA) domain-containing protein, MDC1 (mediator of damage checkpoint protein 1), which works with H2AX to promote recruitment of repair proteins to the sites of DNA breaks and which, in addition, controls damage-induced cell-cycle arrest checkpoints. MDC1 forms foci that co-localize extensively with γ-H2AX foci within minutes after exposure to ionizing radiation. H2AX is required for MDC1 foci formation, and MDC1 forms complexes with phosphorylated H2AX. Furthermore, this interaction is phosphorylation dependent as peptides containing the phosphorylated site on H2AX bind MDC1 in a phosphorylation-dependent manner. We have shown by using small interfering RNA (siRNA) that cells lacking MDC1 are sensitive to ionizing radiation, and that MDC1 controls the formation of damage-induced 53BP1, BRCA1 and MRN foci, in part by promoting efficient H2AX phosphorylation. In addition, cells lacking MDC1 also fail to activate the intra-S phase and G2/M phase cell-cycle checkpoints properly after exposure to ionizing radiation, which was associated with an inability to regulate Chk1 properly. These results highlight a crucial role for MDC1 in mediating transduction of the DNA damage signal.

Mediators are an emerging class of checkpoint proteins involved in transducing the DNA damage signal. The prototypical mediator

Figure 1 MDC1 is phosphorylated in response to DNA damage and DNA replication stress. a, A diagrammatic representation of the MDC1 protein. The amino acids encompassing each domain are indicated. PST indicates proline-serine/threonine repeat domain. The red arrows indicate potential proline/threonine/serine kinase-like kinase phosphorylation motifs (SO/TQ). The fragments of the MDC1 protein used to make anti-MDC1 antibodies (Ab-1, Ab-2, Ab-3) are indicated. b, Alignment of the 41-amino-acid repeat sequence that composes the PST domain. Black boxed shaded boxes indicate conserved amino acids and grey boxes indicate similar amino acids. c, Recognition of three isoforms (I, II and III) of human MDC1. PI refers to pre-immune serum. d, DNA damage-induced phosphorylation of MDC1. Cells were treated with 20 Gy IR, 50 J m⁻² UV or 2 mM HU and harvested at the times indicated. IR-treated cell extracts were also incubated with and without λ protein phosphatase (PPase). e, MDC1 is phosphorylated in response to IR in an ATM- and Nbs1-dependent manner. Normal, NBS and A-T lymphoblasts were irradiated with 20 Gy of IR and harvested at the times indicated.

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is the Rad9 protein in Saccharomyces cerevisiae. Rad9 is phosphorylated by the ATR (ataxia-telangiectasia and Rad3 related) homologue, Mec1, in response to DNA damage and controls the activation of the Chk1 and Chk2 (scRad53) homologues. Rad9 contains two BRCT motifs that are required for its checkpoint functions. No clear mammalian homologue of S. cerevisiae Rad9 has been identified. However, several large BRCT-repeat-containing proteins, such as TopBP1, 53BP1 and BRCA1, which have been implicated in the cellular response to DNA damage, may compose this class of mediator protein in higher eukaryotic cells.

To investigate the role of MDC1 in the DNA damage response, we investigated proteins with motifs common to DNA damage response proteins and found a protein (Kazusa DNA Research Institute clone KIAA0170) that possesses the characteristics of a DNA damage mediator and named it MDC1. MDC1 contained two carboxy-terminal BRCT-repeats and an amino-terminal phospho-amino-acid-binding motif called a FHA domain, which is also found in several DNA damage response proteins such as Chk2, Rad53, Cds1 and Nbs1 (refs 12–15). A large S/TQ cluster domain can be found encompassing the N-terminal half of the protein (Fig. 1a). Both BRCA1 and Chk2 have S/TQ cluster domains and are phosphorylated within these domains by ATM (ataxia-telangiectasia mutated) and ATR after DNA damage. MDC1 also contains a large central proline/serine/threonine-rich repeat (PST) domain that has no significant homology to any other protein in the database (Fig. 1b).

To address whether the expression or mobility of MDC1 was affected by DNA damage, cells were exposed to a variety of genotoxic agents and blotted for MDC1. All isoforms demonstrated the altered mobility after exposure of cells to ionizing radiation (IR), ultraviolet radiation (UV) and hydroxyurea (HU) (Fig. 1d). The phosphorylation of MDC1 after IR is dependent on the presence of functional ATM and Nbs1 (Fig. 1e). Both ATM and ATR could be shown to phosphorylate a fragment of the MDC1 protein encompassing the S/TQ cluster domain in vitro (data not shown).

**Figure 2** MDC1 regulates BRCA1, 53BP1 and Nbs1 foci formation. a, IR-induced MDC1 foci formation. Cells were untreated or irradiated with 10 Gy, fixed and stained with indicated antibodies. Cells were fixed at 6 h post-irradiation to visualize Nbs1 foci. The percentages of cells with the respective foci are indicated. When cells were treated with MDC1 siRNA, the percentage of cells listed refers to the percentage of cells that lack detectable MDC1 but contain the indicated foci. Images were taken with a Zeiss confocal microscope.

- **a** IR 10 Gy
  - Un-irradiated
  - 15 min
  - 30 min

- **b** 10 Gy
  - Anti-γ-H2AX
  - Anti-MDC1
  - Merge
  - 15 min
  - 30 min

- **c** Anti-Brc1
  - Anti-MDC1
  - Merge

  - Control
  - siRNA

  - Anti-53BP1
  - Anti-MDC1
  - Merge

  - Control
  - siRNA

- **d** IR-induced 53BP1 foci
  - IR-induced Brca1 foci
  - IR-induced Nbs1 foci

  - Percentage of cells

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Many DNA damage-signalling proteins are recruited to sites of damage. It has been suggested that the order and timing of these events are critical for normal DNA repair. To assess whether MDC1 could also localize to sites of damage, cells were treated with IR and stained with anti-MDC1 antiserum. MDC1 rapidly formed foci over 95% of cells within 15 min after exposure to IR (Fig. 2a). A proportion of un-irradiated cells also contained MDC1 foci, indicating that MDC1 may be responding to endogenous damage or replication stress. No foci were observed in the cells stained with the pre-immune serum (Supplementary Information Fig. 1). MDC1 also formed foci after UV-irradiation (data not shown) indicating that MDC1 can respond to multiple types of DNA aberrations.

A time-dependent, sequential assembly of repair factors at the sites of damage has been demonstrated: γ-H2AX (the phosphorylated form of H2AX) foci appear within minutes after irradiation. Cells lacking MDC1 sites of damage has been shown to contain over 53BP1, BRCA1 and Nbs1 foci. Cells lacking MDC1 may be responding to endogenous damage or replication stress. No foci were observed in the cells stained with the pre-immune serum (Supplementary Information Fig. 1). MDC1 assembly of damage-responsive foci. H2AX is required for the replication stress. No foci were observed in the cells stained with the There appears to be a hierarchy of proteins involved in the repair of DNA damage and to a lesser extent with ATM and the FANCD2 protein similar kinetics of foci formation after IR, we examined the ability of MDC1 to form foci in the absence of H2AX and other checkpoint proteins. Mouse MDC1 showed a punctate nuclear staining pattern in wild-type mouse embryo fibroblasts (MEFs), which re-organized into large foci in response to DNA damage. MDC1 foci failed to form in H2AX null MEFs (Fig. 4a) but did form foci in Chk2 null MEFs (data not shown). Thus, H2AX acts upstream of MDC1. Surprisingly, the phosphorylation of MDC1 is also partly dependent on the presence of H2AX (Fig. 4b) but Chk2 loss has little or no effect on phosphorylation. Given that one of the functions of H2AX is to specifically recruit DNA repair proteins to sites of damage, this suggests that the modification of MDC1 by ATM and other kinases might be enhanced by recruitment to sites of DNA damage.

In addition, the damage-induced phosphorylation of 53BP1 was also found to be partly dependent on H2AX (Fig. 4b), consistent with its interaction with MDC1. This contrasts with the ATM-dependent phosphorylation of Nbs1, which occurs normally in the absence of H2AX (ref. 2).

The fact that H2AX and MDC1 form completely overlapping foci with identical kinetics suggests that they might have a more intimate relationship. To examine this we immunoprecipitated MDC1 and tested for the presence of phospho-S139 H2AX. Phosphorylated H2AX co-immunoprecipitated with MDC1 and the association was increased in the presence of DNA damage (Fig. 4c). The presence of γ-H2AX in undamaged cells may represent intrinsic cellular damage. It is thought that the phosphorylation of H2AX on S139 triggers its ability to recruit factors such as MDC1 to sites of DNA damage.

To examine this we used an S139-phosphorylated and unphosphorylated peptide from the C-terminal tail of H2AX coupled to beads to assess whether MDC1 could be pulled down from cellular extracts. MDC1 bound specifically to the phosphorylated H2AX peptide and not to the unphosphorylated H2AX peptide or a control phosphopeptide from cyclin E (Fig. 4d). In addition to MDC1, 53BP1 was also pulled down by the phospho-H2AX peptide, confirming the previously published association of H2AX and 53BP1. Not all DNA repair/checkpoint proteins were found to...
were done with the antibody indicated or a non-specific, species-matched c, 53BP1 phosphorylation is dependent on H2AX. Wild type, H2AX-/- MEFs, CHK2-/- and as specificity controls. e, fixed and stained with anti-MDC1 antibodies at 30 min post-irradiation. b, dependent. H2AX-/- MEFs and the corresponding wild-type MEFs were exposed to IR, peptide from the C-terminal tail of H2AX. (P) indicates phosphorylation of the peptide Figure 4 Interactions between MRN and ATM cannot bind in the context of the bead for steric synthesis. In contrast, cells treated with MDC1 siRNA oligonucleotides that already have γ-H2AX in it or that the complex containing exhibited a decrease of approximately 50% in the rate of DNA incorporation of tritiated thymidine was determined. Control cells MDC1 IPs, we had expected to recover these proteins with MDC1; tides. After 48 h, cells were exposed to 15 Gy of IR and the rate of H2AX p-S139 peptide. As we previously detected Nbs1 and ATM in fected with control siRNA or MDC1-specific siRNA oligonucleo- and hChk2 (data not shown) were not significantly enriched by the in regulating the intra-S-phase checkpoint, cells were either trans- associate with H2AX as components of the MRN complex: ATM and hChk2 (data not shown) were not significantly enriched by the H2AX p-S139 peptide. As we previously detected Nbs1 and ATM in MDC1 IPs, we had expected to recover these proteins with MDC1; however, it is possible that these proteins exist in a complex with MDC1 that already has γ-H2AX in it or that the complex containing MRN and ATM cannot bind in the context of the bead for steric reasons.

To further assess the interaction of MDC1 and the H2AX phospho-peptide, MDC1 was translated in vitro by using a rabbit reticulocyte lysate system and incubated with the H2AX-peptide-coupled beads. The MDC1 translated in vitro was pulled down specifically by the H2AX phospho-peptide, again demonstrating that the interaction is phospho dependent (Fig. 4e). However, it is unclear whether this interaction is direct as the reticulocyte lysates may contain bridging proteins.

To examine the genetic role of MDC1 in the DNA damage response, siRNA was used to deplete MDC1. Cells were transiently transfected with either a control siRNA or one of three MDC1-specific siRNAs, harvested 48–72 h after transfection, and their protein expression determined. All three MDC1-specific siRNA oligonucleotides decreased by more than 80% in the overall MDC1 protein expression, compared with the mock or control siRNA-transfected cells (Fig. 5a). To examine the ability of MDC1-depleted cells to respond to damage, siRNA-transfected cells were plated at low density, irradiated with IR and assessed for their ability to form colonies. Cells with reduced MDC1 exhibited hypersensitivity to the killing effects of IR (Fig. 5b) when compared with the control cells. Furthermore, transfection with MDC1-specific siRNA also reduced the number of colonies formed from undamaged cells when compared with control cells, indicating that MDC1 function may be required to maintain cell viability.

The sensitivity to IR suggests an important role in responding to DNA damage; therefore we checked the integrity of cell-cycle checkpoints in these cells. To test whether MDC1 could function in regulating the intra-S-phase checkpoint, cells were either transfected with control siRNA or MDC1-specific siRNA oligonucleotides. After 48 h, cells were exposed to 15 Gy of IR and the rate of incorporation of tritiated thymidine was determined. Control cells exhibited a decrease of approximately 50% in the rate of DNA synthesis. In contrast, cells treated with MDC1 siRNA oligonucleotides 4 or 5 only decreased the rate of DNA synthesis by 25% (Fig. 5c). This demonstrates a role for MDC1 in regulating S-phase progression after DNA damage.

We next examined the integrity of the G2/M checkpoint. Cells treated with MDC1-specific siRNA were irradiated and labelled with an anti-phospho-histone H3 antibody as a marker of mitotic cells. A clear reduction in phospho-H3-positive cells was observed in the control-treated cells after IR exposure, whereas a significant number of the cells lacking MDC1 entered mitosis (Fig. 5d), indicative of a defect in the ability to arrest the cell cycle in G2 phase.

To gain insight into the precise mechanism by which MDC1 mediates both the S-phase and G2/M damage-activated cell-cycle checkpoints, cells were treated with MDC1-targeted siRNA and assessed for their ability to properly phosphorylate key effector molecules known to be critical for efficient checkpoint activation after DNA damage. We observed decreased phosphorylation of SMC1 S996 (more pronounced at 2 h) and Chk1 S345 in response to IR, and SMC1, Chk1 and RPA2 in response to UV (Fig. 5e). Nbs1 and Chk2 were not affected although we observed a small decrease in Chk2 T68 phosphorylation in response to UV in some experi-

ments (data not shown). Chk1 is required for the G2/M checkpoint arrest and this defect is likely to explain the checkpoint defect we observed. Both SMC1 and Chk1 have been implicated in the intra-S-phase checkpoint, and their defective regulation is likely to explain these defects as well. The fact that UV-induced signalling is impaired suggests that the ATR pathwaydependent on the specific DNA damage response, possibly through the recruitment of MRN and CHK2.
transfected with a control or MDC1 siRNA and harvested at 48 h. Three different siRNAs to consistent with the late role of ATR in IR-induced phosphorylation shares an intimate relationship with H2AX. In response to phosphorylation and foci formation, and UV radiation and the formation of phospho-H2AX significantly affected the phosphorylation of H2AX after both IR responsive proteins, we sought to examine whether it might have a siRNA-control or MDC1 siRNA doubles with siRNAs and irradiated 72 h later. The immunoblot with SMC1 as a As a new member of this family, MDC1 is also likely to be involved flow cytometry. e, Reduced MDC1 expression results in IR sensitivity. Control or siRNA-mediated reduction of protein expression. Cells were transfected with a control or MDC1 siRNA and harvested at 48 h. Three different siRNAs to MDC1 were used. An asterisk indicates a cross-reacting band used as a loading control. of substrates shared by ATM. MDC1 may play a role in the phosphorylation. a, siRNA-mediated reduction of phosphorylation. b, Reduced MDC1 expression results in IR sensitivity. Control or MDC1 siRNA-treated cells were plated at low density, irradiated and harvested after 14 days. c, MDC1 prevents ROS. DNA synthesis was assessed 30 min after 15 Gy IR in MDC1 cells transfected with a control or MDC1 siRNA oligonucleotides. d, Analysis of the G2/M checkpoint. Cells were untreated or γ-irradiated as indicated, then incubated for 1 h at 37 °C before fixation. Mitotic cells were defined by phospho-histone H3 staining and flow cytometry. e, MDC1 is required for DNA damage checkpoint signalling. Cells were transfected twice with siRNAs and irradiated 72 h later. The immunoblot with SMC1 as a control for protein loading shows a reduced mobility in extracts from damaged cells rather than increased protein loaded on the gel. f, MDC1 is required for H2AX phosphorylation. Control or MDC1 siRNA-treated cells were irradiated and the levels of H2AX phosphorylation detected by western blot and immunofluorescence 1 h post-irradiation. of the DNA damage checkpoint signal.

As MDC1 controls the phosphorylation of several checkpoint-responsive proteins, we sought to examine whether it might have a role in H2AX phosphorylation. Depleting cells of MDC1 protein significantly affected the phosphorylation of H2AX after both IR and UV radiation and the formation of phospho-H2AX foci (Fig. 5f). Thus MDC1 and H2AX share a mutual dependency for phosphorylation and foci formation.

In this study, we have identified a new DNA damage checkpoint protein, MDC1, which transduces the DNA damage signal and shares an intimate relationship with H2AX. In response to DNA damage, MDC1 and H2AX exist in a complex, are phosphorylated and co-localize to sites of DNA damage, all in a mutually dependent fashion. Both proteins are required for optimal formation of MRN, 53BP1 and BRCA1 foci. Furthermore, peptides representing the tail of H2AX specifically recruit MDC1 and 53BP1 protein in a phosphorylation-dependent manner, suggesting a model in which H2AX is phosphorylated in response to damage and the phospho-H2AX recruits MDC1 leading to its phosphorylation. However, we cannot determine whether MDC1 controls the initial phosphorylation of H2AX, or merely protects H2AX from dephosphorylation through formation of a complex with H2AX. It is possible that there is a self-reinforcing loop whereby MDC1 and H2AX mutually stimulate each other’s phosphorylation and help larger complexes of proteins associate at these foci. Furthermore, this effect on H2AX phosphorylation may, in part, help to explain why the recruitment of 53BP1, BRCA1 and Nbs1 to damage foci partly depends on MDC1. The ability of MDC1 to form complexes with MRN and 53BP1 may also contribute to this dependency. Regardless, it is clear from the data presented here that H2AX and MDC1 are intimately related and work together to mediate the DNA damage response.

Although MDC1 and H2AX have many common features, there are some important differences. MDC1 is required for the intra-S-phase checkpoint and the G2/M DNA damage checkpoint. H2AX has recently been shown to have a mild G2/M defect in response to low doses of IR, much less severe than that observed in MDC1. MDC1 exists in complexes with several checkpoint-signalling proteins such as the MRN complex, SMCl, 53BP1 and FANC D2. Furthermore, MDC1 is required for the efficient phosphorylation of several checkpoint-signalling proteins including SMCl, RPA2 and the crucial checkpoint kinase Chk1. The defect in Chk1 phosphorylation explains the defects in both the intra-S-phase and G2/M checkpoints. The defect in MDC1 checkpoint signalling is more pronounced in response to UV than IR. This, together with the regulation of Chk1, suggests that MDC1 plays an important role in the ATR signalling pathway that controls Chk1 phosphorylation and a less important role in the ATM pathway. This may explain the mild defect in the IR-induced phosphorylation of SMCl, which is primarily ATM dependent. The mild SMCl phosphorylation defect becomes more pronounced at later times in the absence of MDC1, consistent with the late role of ATR in IR-induced phosphorylation of substrates shared by ATM. MDC1 may play a role in the recruitment of checkpoint kinases such as ATR to substrates such as H2AX and the pathway controlling Chk1, which may include BRCA1 and Claspin. Therefore, MDC1 plays a critical role in the DNA damage checkpoint response. MDC1 represents the third member of the mediator family of proteins in mammals. Each of these proteins plays important and potentially overlapping roles in transducing the DNA damage signal to promote genomic stability. As a new member of this family, MDC1 is also likely to be involved in tumorigenesis.

**Methods**

**Cells**

Lymphoblastoid cell lines (LCLs) were routinely maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS), glutamine and penicillin and streptomycin (pen/strep). 129/Sv cells were maintained in McCoy's 5A medium supplemented with 10% FBS, glutamine and pen/strep. All MEF cell lines were maintained in DMEM medium supplemented with 10% FBS, glutamine and pen/strep.

**siRNA**

The siRNA duplexes were 21 base pairs with a 2-base deoxynucleotide overhang (Dharmacon Research). The sequences of MDC1 siRNA4 and siRNA5 oligonucleotides were GUCCUGCCGAAGAAGAGAATGAC and ACAGUUGUCCCCGACGGCCTT, respectively. The control siRNA used was CGUACGCGGAUAUCUCGUAAT against LacZ. Cells were transfected with siRNA duplexes by using Oligofectamine (Invitrogen), following the manufacturer's instructions.

**MDC1 antibody generation**

A full-length MDC1 clone (K3A9H170) was generously provided by the Kazusa DNA Research Institute. Fragments of the cDNA were subcloned into a glutathione S-transferase (GST) expression vector (Amersham Biosciences), expressed in bacteria and
purified by using glutathione-coupled sepharose beads (Amersham Biosciences). Purified GST fusion proteins were injected into rabbits and the antisera were affinity purified by using the respective antigen (Bethyl Laboratories). Ab-1 was generated to a fragment of MDC1 encompassing amino acids 326-429, Ab-2 was generated to a fragment of MDC1 encompassing amino acids 643-1015 and Ab-3 was generated to a fragment of MDC1 encompassing amino acids 1-335.

**Immunoblot analysis**

Cells were sonicated in UTB buffer (8 M urea, 150 mM β-mercaptoethanol, 50 mM Tris/HCl pH 7.5) and cellular debris removed by centrifugation. Protein concentration was determined by using the BioRad Bradford Protein determination reagent. Proteins were fractionated in 6% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose, and immunoblots were performed by using the appropriate antibody. Antibodies to Nbs1, hMre11 and Rad50 were obtained from Genetex, the phospho-γH2AX (serine-139) antibody was obtained from Cell Signaling and the anti-phospho-H2AX (serine-139) antibody was obtained from Cell Signaling. Antibodies to Nbs1, P. Carpenter supplied antibodies to 53BP1. (2000), antibodies were purchased from Santa Cruz.

**Immunoprecipitation and phosphate treatment**

LCG (4 × 10^6) were either mock irradiated or irradiated with 10 Gy of ionizing radiation and incubated for 1 h at 37°C. The cells were then lysed for 30 min in NEPT lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40 supplemented with protease inhibitors (Roche) and Benzonase (Novagen)). The clarified extract was pre-cleared with the appropriate IgG (Dako) and protein A or G beads (Amersham Biosciences) for 1 h at 4°C. Immunoprecipitating antibody (5 μg) was added with protein A or G beads to the pre-cleared supernatant and incubated for 3 h at 4°C. The immunocomplexes were washed four times in NETN lysis buffer (containing 0.5% NP40), boiled in SDS sample buffer, loaded on an SDS-polyacrylamide gel. Proteins were analysed by immunoblotting using standard methods and detected as described above. For phosphate treatment of cell extract, 1,200 units of X protein phosphatase (New England Biolabs) were added to the extract in the presence of MnCl2 and incubated for 30 min at 30°C. The phosphate was then heat inactivated.

**H2AX peptide pull-down assay**

Two peptides to the last C-terminal 10 amino acids of human H2AX (CKATQASQEY) were synthesized (Bethyl, one of which was phosphorylated on the serine residue. The peptides were coupled to beads by using the SulBlk link (Pierce). 20 μg of coupled peptide were used per pull down. The assay was essentially identical to the immunoprecipitation protocol described above.

**H2AX peptide in vitro binding assay**

MDC1 construct (2 μg) was translated in vitro for 90 min at 30°C by using the TNT-coupled reticulocyte lysate system (Promega) containing 20 μCi of 35S-labelled methionine (Amersham Biosciences). Half of each reaction was added to be a volume of NETN lysis buffer containing 1% NP40. Coupled H2AX peptide (20 μg) was added and incubated for 1 h at 4°C. The beads were washed thoroughly with NETN and the bound proteins analysed by western blotting.

**Colony-forming assay**

U2OS cells transfected with siRNA were seeded at low density and irradiated with various doses of ionizing or UV radiation. Cells were left for 14 days at 37°C to allow colonies to form. Colonies were stained with 2% methylene blue/50% ethanol and counted. Colonies were defined as containing 50 or more cells.

**Radio-resistant DNA synthesis assay**

The XDS assay was done as described above. Briefly, cells were transfected with siRNA oligonucleotides, and 4 h later were placed into McCoy’s 5A medium containing 10 μCi of 3H-labelled thymidine (NE Laboratories Products Inc.) per milliliter overnight. The medium containing 3H-labelled thymidine was then replaced with normal McCoy’s 5A medium, and the cells were incubated for another 24 h. Cells were irradiated, incubated for 30 min at 37°C, and then pulse-labelled with 2.5 μCi [3H]thymidine (NE Laboratories Products Inc.) per milliliter for 15 min. Cells were harvested, washed twice with PBS, and fixed with 70% methanol for 30 min. After the cells were transferred to Whatman filters and fixed sequentially with 70% and then 90% methanol, the filters were air-dried and the amount of radioactivity was assessed in a liquid scintillation counter. The resulting ratios of 3H counts per minute to 3H counts per minute, corrected for those counts per minute that were the result of channel crossover, were a measure of DNA synthesis.

**Immunofluorescence**

Cells were fixed with 3% paraformaldehyde/5% sucrose for 10 min, permeabilized with 0.5% Triton X-100 solution, and then immunostained with primary antibodies against various proteins and the appropriate Alexa488- (green, Molecular Probes) and Cy3- (red) conjugated secondary antibodies (Amersham Biosciences). Images were taken with a Zeiss confocal microscope.
Role for the BRCA1 C-terminal Repeats (BRCT) Protein 53BP1 in Maintaining Genomic Stability*

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p53-binding protein-1 (53BP1) is phosphorylated in response to DNA damage and rapidly relocates to presumptive sites of DNA damage along with Mre11 and the phosphorylated histone H2A variant, γ-H2AX. 53BP1 associates with the BRCA1 tumor suppressor, and knockdown experiments with small interfering RNA have revealed a role for the protein in the checkpoint response to DNA damage. By generating mice defective in m53BP1 (m53BP11cre), we have created an animal model to further explore its biochemical and genetic roles in vitro. We find that m53BP11cre animals are growth retarded and show various immune deficiencies including a specific reduction in thymus size and T cell count. Consistent with a role in responding to DNA damage, we find that m53BP11cre mice are sensitive to ionizing radiation (γ-IR), and cells from these animals exhibit chromosomal abnormalities consistent with defects in DNA repair. Thus, 53BP1 is a critical element in the DNA damage response and plays an integral role in maintaining genomic stability.

DNA damage-response mechanisms ensure the fidelity of chromosomal transmission, and their failure may lead to the development of diseases such as cancer (1). In response to γ-IR, pharmacological-like kinases (PIKs) such as ATM (mutated in ataxia-telangiectasia) transduce damage signals to the nucleus, phosphorylate histones, and recruit various DNA repair factors to these sites, 53BP1 included. By generating mice defective in product of breast cancer susceptibility gene 1 (BRCA1), at Ser-1423 (3, 4), BRCA1 is a major target of the DNA damage response, and mutations in BRCA1 contribute to nearly 50% of familial forms of breast and ovarian cancer (5). BRCA1 had been found associated with RNA polymerase II (6), chromatin-remodeling factors (7), and a variety of DNA repair and replication factors (8–10). Indeed, BRCA1 has been shown to function in genomic stability by controlling homologous recombination, transcription-coupled repair of oxidative DNA damage, and cell cycle checkpoints (11–14).

One protein that contains numerous (S/T)AQ motifs and two C-terminal BRCT repeats is p53-binding protein 1 (53BP1). 53BP1 was discovered as a p53-interacting factor in a two-hybrid screen (15) and was subsequently proposed to function as a transcriptional co-activator of p53 (16). Although the relationship between 53BP1 and p53 has not been fully established, 53BP1 and p53 from both Xenopus and humans have been shown to interact either directly or indirectly in experimental settings that express high levels of 53BP1 protein from plasmids or that naturally occur in eggs (15, 17). We, as well as others, have demonstrated previously that 53BP1 is involved in the DNA damage-response network (17–20). 53BP1 proteins are phosphorylated in response to γ-IR, and this is likely governed by the action of PIKs like ATM (17, 19, 20). γ-IR also induces 53BP1 to rapidly relocate to DNA repair foci, and this response is delayed or inhibited by treatment with the PIK inhibitors caffeine and wortmannin. 53BP1 foci also overlap with those formed by the Mre11 complex, BRCA1, and the phosphorylated form of the histone variant H2AX (γ-H2AX; see Refs. 18–20). As both the Mre11 complex and γ-H2AX are believed to localize to physical sites of DNA damage (21–23) and to recruit various DNA repair factors to these sites, 53BP1 has been inferred to localize to these sites as well. This notion is further supported by the fact that γ-H2AX recruits 53BP1 to nuclear foci and physically interacts with 53BP1 (20, 24). Recent studies have revealed a role for 53BP1 in cell cycle checkpoints (25–27) as well as in maintaining p53 levels in response to γ-IR (27). Here we show that a 380-amino-acid region of 53BP1 that includes a recently described kinase-histone-binding domain (28) is necessary for the formation of irradiation-in
duced foci. We further deciphered the role of 53BP1 in the DNA damage response by generating mice defective in m53BP1. We report that murine animals expressing a truncated form of m53BP1 (m53BP1<sup>tr trunc</sup>) exhibit a pleiotropic phenotype that includes growth retardation, immune deficiencies including defects in T cell maturation, sensitivity to γ-IR, as well as increased chromosomal aberrations. Taken together, these results reveal that 53BP1 is an integral component of the DNA damage-response network and indicate that the protein plays an important role in maintaining genomic stability.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Indirect Immunofluorescence**—Three antibodies that recognize both the human and murine 53BP1 proteins were generated for this study. We found that our 53BP1 antibodies recognize both the murine and human proteins. Polyclonal antibodies raised against glutathione S-transferase fusion proteins encoding the first 524 amino acids of human 53BP1 (m53BP1) or the last 200 residues of the protein (a53BP1-C) were affinity-purified by established procedures and used as described in the text. a53BP1-N is a polyclonal, anti-peptide antibody that was raised against an N-terminal sequence GVELLSQSGQDEES that is conserved between human and murine 53BP1 proteins. Poly- and monoclonal antibodies were affinity-purified by standard methods. Anti-HA antibodies were purchased from Covance, and anti-ATR antibodies were obtained from Oncogene Research Products.

**Mouse Genetics and Genotyping of m53BP1<sup>tr trunc</sup> Animals**—Murine animals defective in m53BP1 (m53BP1<sup>tr trunc</sup>) were generated with a random retrovirus as described previously (29). Genomic DNA was isolated from mouse tail snips by standard methods. Insertion of VICTR5 expression vector pCMH6K53BP1 (16) was confirmed by Southern analysis and by restriction enzyme digestion of tail DNA (not shown). 53BP1 localizes to the kinetochore during mitosis (28). How-
Erase transgenic animals heterozygous in m53BP1 (m53BP1+/+)
and m53BP1tr/tr as described previously (29). Southern blotting with DNA isolated from tail biopsies confirmed the disruption in m53BP1 and was used to genotype the animals (Fig. 2B; see "Experimental Procedures"). Crosses between heterozygous animals appeared in heterozygous and mutant extracts but not in wild-type ones (Fig. 2E). The levels of m53BP1tr appeared much lower than in wild-type and heterozygous and mutant extracts but not in wild-type ones (Fig. 2E). We observed that a53BP1-N cross-reacted with m53BP1tr (not shown).

Figure 1. Dynamic nuclear localization of human 53BP1 during interphase, association with ATR, and structural requirements for DNA damage inducible focus formation. As shown in A, immunofluorescence analysis with an antibody specific for 53BP1 (a53BP1; see "Experimental Procedures") reveals the nuclear staining pattern for cells unambiguously assigned to the G1-phase (left), S-phase (middle), and G2-phase (right) of the cell cycle as determined by DNA content with a lasing scanning cytometer. Black and white images were captured with a ×100 objective on a Zeiss Axiohot and pseudocolored in Adobe PhotoShop. As shown in B, ATR and 53BP1 co-localize to nuclear foci in response to hydroxyurea (2.0 mM). Left panel, immunostaining for 53BP1. Middle, immunostaining with an antibody specific for ATR (see "Experimental Procedures"). Right, merged images to show co-localization between 53BP1 and ATR. As shown in C, ATR and 53BP1 physically interact before and after DNA damage. K562 cells were grown and either left untreated (lanes 1 and 2) or treated with 2.0 mM hydroxyurea (HU; lane 3) for 18 h prior to the preparation of nuclear extracts for immunoprecipitation with an antibody against 53BP1 (left panel) or ATR (right panel). Immunoblotting was then performed with the reciprocal antibodies as shown. D, schematic representation of primary structure of 53BP1 (not drawn to scale). Hatched lines represent locations of (S/T)Q sites mutated in 15AQ. KINET, kinetochore-binding region (28); NLS, nuclear localization signal (23). E, identification of a region of 53BP1 required for irradiation-induced focus formation. Wild-type, HA-tagged 53BP1 and various mutant derivatives were transfected into MC7 cells and treated with 10 Gy of ionizing radiation prior to fixation and immunostaining with an antibody specific for the HA tag (Covance). The following constructs expressing in-frame 53BP1 deletions or mutations were made: ΔBRCT (deletes amino acid residues 1,786–1,964); ΔKINET (deletes amino acid residues 1,296–1,616); ΔNH3 (deletes the first 1,294 amino acids residues except for the initiation codon), and 15AQ, a construct with mutations in 15 (S/T)Q sites. The following serine or threonine residues were mutated to alanines in 15AQ: Ser-6, Ser-13, Ser-25, Ser-29, Ser-105, Ser-166, Ser-176, Ser-178, Thr-302, Ser-452, Ser-523, Ser-543, Ser-625, Ser-784, Ser-892. All constructs were verified by DNA sequencing and expressed in either 293T or MCF7 cells (not shown).

Figure 2. Inactivation of m53BP1 in mouse embryos results in growth retardation, altered body proportions, and defects in hematopoiesis. A, immunostaining with antibodies specific for m3BP1 (a53BP1) and ATR (aATR) on cryosections of E12.5 embryos. B, numbers of embryos and postnatal animals obtained from mating of females with males. The data represent the results of two independent experiments. C, DNA extraction and genotyping from tail biopsies of mice using specific sets of primers. Males are indicated by arrows. D, Southern blot analysis with a 1.7-kb XbaI fragment of VICTR54 containing neomycin from VICTR54 was spliced adjacent to truncated in m53BP1tr/tr animals (Fig. 2, E). Rather, the "artificial" exon containing neomycin from VICTR54 was spliced adjacent to exon 13 as verified with primers specific for exon 13 and the neomycin gene (primer set D/A; Fig. 2C). Sequencing of a cloned RT-PCR product spanning the insertion event revealed that the natural coding sequence of m53BP1 had stopped after residue 1,205, where it then fused to 21 residues derived from VICTR54 before terminating (Fig. 2D). Therefore the disrupted allele of m53BP1 encodes a truncated 1,226 residue protein (m53BP1tr), and notably, m53BP1tr is missing over 700 residues including its functional nuclear localization signal, kinetochoore binding domain (KINET), and two BRCT motifs (Fig. 2D). To determine whether m53BP1 was expressed, we performed immunoprecipitation/Western blotting (IP/WB) analysis from brain extracts derived from m53BP1tr/tr, m53BP1tr/tr, and m53BP1tr/tr animals. By using antibodies specific for the N and C termini of 53BP1 (a53BP1-N and a53BP1-C, respectively; see "Experimental Procedures"), we determined that a truncated m53BP1 protein corresponding to m53BP1tr appeared in heterozygous and mutant extracts but not in wild-type ones (Fig. 2E). The levels of m53BP1tr appeared much lower than the full-length protein and, in some cases, we observed an apparent isoform of m53BP1 in wild-type and heterozygous animals (Fig. 2E). The disappearance of full-length m53BP1 in the mutant samples was accompanied by the appearance of a smaller protein corresponding to m53BP1tr (Fig. 2E). We observed that a53BP1-N cross-reacted with m53BP1tr but not with a53BP1-C, demonstrating that m53BP1 is indeed truncated in m53BP1tr/tr animals (Fig. 2, E and F).

Figure 3. m53BP1tr/m53BP1tr mice—We observed that m53BP1tr/tr animals were growth-retarded as the males and females were found on average to weigh 25 and 15% less, respectively, than their wild-type littermates (Fig. 3A). We found that thymuses derived from m53BP1tr/tr animals were significantly smaller and possessed fewer cells than those from m53BP1tr/tr animals (Fig. 3B). This suggests that defects in m53BP1 may contribute to immune deficiencies, a result that has been observed for various DNA damage-response factors, including H2AX (24). We found that the lymphoid organ architecture of thymuses, as assayed by hematoxylin and eosin staining of tissue sections, appeared normal in m53BP1tr/tr mice (data not shown). In addition, flow cytometric analysis
**53BP1 and Genomic Stability**

**Fig. 2.** Generation and characterization of mice defective in m53BP1 (m53BP1\textsuperscript{tr/tr}).

**A**, schematic diagram of insertion event in m53BP1 (not drawn to scale). The thick horizontal lines represent positions of probes for Southern blotting as described in B. Arrows represent the position and orientation of PCR primers used in C. The insertion of VICTR54 was determined by DNA sequencing to reside within the intron preceding exon 14 at nucleotide position 1,730 (marked by *). Splicing of the neomycin gene and flanking DNA produces a transcript that potentially disrupts the proper splicing of exons 13 and 14. **B**, top, Southern blotting to determine the genotype of m53BP1-defective animals. 10 

**C**

**D**

**E**

**F**

**IP:** a53BP1-N

**WB:** a53BP1-N

**IP:** a53BP1-N

**WB:** a53BP1-C

**FIG. 2.** Generation and characterization of mice defective in m53BP1 (m53BP1\textsuperscript{tr/tr}). **A**, schematic diagram of insertion event in m53BP1 (not drawn to scale). The thick horizontal lines represent positions of probes for Southern blotting as described in B. Arrows represent the position and orientation of PCR primers used in C. The insertion of VICTR54 was determined by DNA sequencing to reside within the intron preceding exon 14 at nucleotide position 1,730 (marked by *). Splicing of the neomycin gene and flanking DNA produces a transcript that potentially disrupts the proper splicing of exons 13 and 14. **B**, top, Southern blotting to determine the genotype of m53BP1-defective animals. 10 gg of genomic DNA was digested with XbaI and was probed with a radiolabeled fragment (see "Experimental Procedures") capable of discerning wild-type (WT) and mutant alleles as discussed under "Experimental Procedures." Bottom, a 700-bp probe derived from the neomycin gene was used to help genotype the animals. +/+, wild type; +/tr, heterozygous; tr/tr, homozygous. As shown in C, RT-PCR analysis indicates that improper splicing occurs between exons 13 and 14 in m53BP1\textsuperscript{tr/tr} mice. Positions and orientation of primers for PCR are indicated in A. Control reactions without reverse transcriptase showed essentially no amplified products (not shown). As shown in D, m53BP1\textsuperscript{tr/tr} encodes a truncated protein of 1,226 amino acids. RT-PCR products derived from primer set A/D (as shown in C) using RNA isolated from m53BP1\textsuperscript{tr/tr} animals as template were cloned into the TA vector (Invitrogen). DNA sequencing and conceptual translation indicated that m53BP1\textsuperscript{tr/tr} animals potentially encode a truncated m53BP1 protein (m53BP1\textsuperscript{tr}) of 1,205 natural residues along with an additional 21 residues derived from the VICTR54 vector. m53BP1\textsuperscript{tr} is missing over 700 C-terminal residues, including those that specify the kinetochore binding domain (KINET; amino acids 1,220–1,601), the nuclear localization signal (NLS; mapped to amino acids 1,601–1,703; ref), and the C-terminal BRCT repeats (amino acids 1,665–1,957). The small, vertical rectangle in m53BP1\textsuperscript{tr} represents the additional vector-derived 21 residues. **E** and **F**, detection of m53BP1\textsuperscript{tr} protein by IP/WB analysis. 1.0 mg of total brain protein extracts derived from +/+, +/tr, and tr/tr animals as determined in B. IP/WB analysis shows the presence of m53BP1\textsuperscript{tr} in m53BP1\textsuperscript{tr/tr} and m53BP1\textsuperscript{tr} animals but not of m53BP1\textsuperscript{tr/tr} ones. In some cases, we observed an apparent isoform of m53BP1 in brain tissue, as designated by the asterisk. **F**, WB with a53BP1-C, an affinity-purified antibody against the C-terminal 200 residues of human 53BP1. As shown, a53BP1-C recognizes full-length m53BP1 but fails to immunoreact with m53BP1\textsuperscript{tr}, indicating that the protein is indeed missing C-terminal residues of m53BP1.
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A. 40 +/- m53BP1

B. M53BP1 +1+

C. D. m53BP1

FIG. 3. Growth retardation and immune deficiencies in mice defective in m53BP1. A, mean body weight, in grams, of m53BP1+/+ and m53BP1tr/tr mice. (n = 10). As shown in B, reduced thymus size in m53BP1tr/tr animals results in lower tissue cell count. Mean cell numbers are x 10^6 (per mouse) of bone marrow (two femurs per mouse), spleens, and thymuses from m53BP1+/+ and m53BP1tr/tr mice (n = 4). C, defective T cell development in m53BP1tr/tr mice as revealed by double-negative thymocyte populations. CD4− and CD8− cells were removed by gating, leaving DN1 (CD44−CD25−), DN2 (CD44−CD25+), DNIII (CD44−CD25+), and DNIV (CD44−CD25−) thymocytes. Numbers in the histogram quadrants are average percentages for four mutant or control animals. D, defective B cell development in m53BP1tr/tr animals. Immature (IgM+IgD−), transitional (IgM−IgD−), and mature (IgD−IgM−) B cells in m53BP1+/+ and m53BP1tr/tr mouse spleens. Numbers in the histogram quadrants are average percentages for four mutant or control mice.

with a variety of markers (e.g. B220, CD43, Gr-1, CD11a, and Ter119) revealed that bone marrow pro-B, pre-B, myeloid, and erythroid progenitor populations were normal in m53BP1tr/tr mice (not shown). Although CD4 and CD8 T cell populations were proportionately similar in m53BP1+/+ and m53BP1+/+ thymuses, we observed that progression out of the DNIII stage of early thymocyte development was impaired in m53BP1tr/tr animals (Fig. 3C), the stage at which β-gene rearrangement occurs. This indicates that m53BP1 participates in proper T cell development, a process known to require various DNA repair factors (30). We also found that spleens derived from m53BP1tr/tr animals were similar in size and organ architecture to those from m53BP1+/+ animals and that the lack of functional m53BP1 did not affect the proportions of B and T lymphocytes (data not shown). We did observe, however, that m53BP1tr/tr spleens were deficient in mature B cells (IgM+IgD−; Fig. 4D), suggesting that deficiencies in m53BP1 may also result in defective B lymphocyte development.

Genomic Instability in m53BP1tr/tr Mice—Mice with defects in double-stranded break repair are highly sensitive to γ-IR. To evaluate whether m53BP1 contributes to increased sensitivity to DNA damage, we treated m53BP1tr/tr or wild-type animals with 7 Gy of γ-IR. After this whole body irradiation treatment, we found that 100% of the mutant animals died between 9 and 15 days post-irradiation in contrast to only 16% of the control littermates (Fig. 4A). This shows that animals defective in m53BP1 are highly sensitive to γ-IR, a result that parallels previous observations with H2AX-deficient mice (24). Despite this, we found that m53BP1+/+ animals treated with lower doses of γ-IR (1.5 Gy) remained viable (Fig. 4B). To further explore m53BP1 function, we generated embryonic fibroblasts (MEFs) from wild-type and m53BP1+/+ animals. m53BP1+/+ MEFs proliferated more slowly than their wild-type counterparts (Fig. 5A). Immunofluorescence analysis indicated that the truncated m53BP1 protein expressed in m53BP1tr/tr animals failed to form foci in response to DNA damage as it was essentially absent from the nucleus (data not shown). This result is consistent with our transfection studies, which have shown that C-terminal determinants (ΔKINET) are necessary for focus formation. The relative growth of the mutant and the wild-type MEFs was reminiscent of what has been recently described for H2AX (24). To further characterize cells defective in m53BP1, we examined the cytological consequences of impaired m53BP1 function in early passage MEFs derived from m53BP1+/+ and m53BP1+/+ animals. For this, exponentially growing MEFs (passage 2) were treated with 0, 0.5, or 1.5 Gy of γ-IR, and metaphase preparations were examined 2.5 h post-irradiation. Untreated MEFs derived from m53BP1+/+ or wild-type mice showed increased levels of chromatin gaps, breaks, and, to a lesser extent, exchanges when compared with those derived from m53BP1+/+ mice, suggesting an intrinsic genomic stability defect in the mutant cells (Fig. 5, B and C). More strikingly, irradiated MEFs derived from m53BP1+/+ animals showed an ∼2-fold increase in levels of chromatin breaks and gaps when compared with MEFs derived from wild-type mice (Fig. 5, B and C). Although MEFs from m53BP1+/+ animals showed relatively high chromatin exchange rates at 0.5 Gy when compared with those from m53BP1+/+ animals, this difference was
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less apparent at 1.5 Gy, perhaps due to the limited progression to mitosis of the most damaged cells from both populations during this time frame. One possible explanation for the increased frequencies of chromosomal aberrations observed in the m53BP1tr/tr MEFs following irradiation might be a deficiency in a G2 checkpoint response whereby more damaged cells would still be permitted to enter mitosis and would be available for chromosome analysis. In fact, recent reports have implicated 53BP1 in the G2/M checkpoint (25–27). To examine this in our MEFs, either m53BP1tr/tr or wild-type MEFs were treated with 0, 1.5, or 10 Gy of γ-IR, and cultures were analyzed for the fraction of cells showing phospho-histone H3 immunostaining (mitotic cells) either after 1 or 16 h post-irradiation (in the presence of colcemid). Although all cell types showed evidence of a partial G2/M block following irradiation, MEFs derived from m53BP1tr/tr mice showed only a slight decrease, if any, in the G2 block when compared with MEFs derived from wild-type mice (data not shown). The minimal effects on the G2/M checkpoint observed in our m53BP1tr/tr MEFs may be due to the nature of the truncated protein produced from the m53BP1tr allele that is expressed in our mutant animals described here.

53BP1 interacts with a variety of factors known to be involved in the maintenance of genomic stability including ATR, p53, H2AX, BRCA1, Chk2, and ATM (15, 20, 25, 27). The generation of murine animals defective in m53BP1 provides a valuable tool to further understand the role of the protein in the DNA damage response. The m53BP1tr allele expresses a truncated version of m53BP1, and this likely represents a

Fig. 4. Characterization of animals and cells defective in m53BP1tr. A, survival of 4–6-week-old m53BP1tr and m53BP1tr+ mice after exposure to 7 Gy of γ-IR. Six animals from each genotype were used in the experiment. B, survival of 4–6-week-old m53BP1tr animals after exposure to 1.5 Gy of ionizing radiation.

Fig. 5. Chromosomal abnormalities in m53BP1tr+ cells. A, growth curve of MEFs derived from m53BP1tr (open diamonds) and m53BP1tr+ (closed circles). B, metaphase preparation of mutant MEF following 1.5 Gy of γ-IR. Note the presence of a chromatid gap, two chromatid breaks, and one chromatid exchange in the metaphase sample. C, relative frequencies of chromatid gaps, breaks, and exchanges in metaphases of wild-type and mutant MEFs following 0, 0.5, and 1.5 Gy of ionizing radiation.
significant impairment in some aspects of its function. m53BP1
trr mice is missing over 700 amino acids including the nuclear localization signal, the C-terminal BRCT motifs, and a kinetochore-binding domain. We have observed that this domain is also necessary for forming irradiation-induced nuclear foci. Indeed, the lack of detectable, irradiation-induced foci in mutant MEFs suggests that the protein cannot fully perform its functions as a DNA damage-response element. Moreover, with the metaphase spreads. We are grateful to David Cortez for providing useful suggestions during the course of the project. We are also indebted to Mike Blackburn, Bob Kirken, Jeff Frost, Hays Young, and Jose Molina for technical advice. We thank Jungie Chen for communicating results prior to publication.

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