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PRINCIPAL INVESTIGATOR: Bin Wang, Ph.D.
Stephan Elledge

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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

Bin Wang, Ph.D.
Stephan Elledge

Baylor College of Medicine
Houston, Texas 77030

bwang@bcm.tmc.edu

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

We have shown that 53BP1, a tumor suppressor p53 binding proteins, 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 Brca1. 53BP1 plays an important role in p53 stabilization, phosphorylation of Chk2 and Brca1 in response to IR.
Introduction

We have shown that 53BP1, a tumor suppressor p53 binding proteins, 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

Task1. 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 report, 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 generated a series of 53BP1 deletion mutants and overexpressed them in mammalian cells to localize the region of 53BP1 that Chk2 or p53 binds to. We found 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. We are seeking to understand whether the binding of p53 to the BRCT motifs of the 53BP1 is necessary for the stabilization of p53.

Task2. 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 playes (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.

Task3. To determine the role of 53BP1 in development and tumor suppression (1-36)
   a. Generation of 53BP1-deficient mince (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). We are collecting data on the tumor progression of the 53BP1-/- mice. We are also assessing the role of 53BP1 in tumor suppression by crossing 53BP1-/- with mutant mice of known tumor suppressors, such as p53, Chk2 and Brac1.

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

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. We are also investigating whether 53BP1 is directly involved in tumor suppression using mouse models. These studies will allow us gain further insights into the DNA damage response pathway and the tumor suppression pathway in mammalian cells.

Appendices


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 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 its 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.

References and Notes
17. Materials and methods are available as supporting material on Science Online.
18. M. Chatterjee, J. Gobineau, G. Bestetti, Y. Chang, P. S. Moore, data not shown.

53BP1, a Mediator of the DNA Damage Checkpoint
Bin Wang,1 Shuhei Matsuoka,1 Phillip B. Carpenter,4 Stephen J. Elledge1,2,*

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, G1-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.

1Department of Biochemistry and Molecular Biology, 2Department of Molecular and Human Genetics, 3Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030, USA. 4Department of Biochemistry and Molecular Biology, University of Texas Health Science Center, Houston, TX 77030, USA.

*To whom correspondence should be addressed. E-mail: selledge@bcm.tmc.edu

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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 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 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 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 pS3 and Chk2 in response to IR. (A) IR-induced pS3 stabilization. U2OS cells were transfected with siRNA oligos targeting 53BP1 or control oligos for 2 days, then exposed to 10-Cy-GIR. 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 pS3 (Oncogene, San Diego, CA). (B) Chk2 phosphorylation at Thr68 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 T68-Chk2 (14). (C) IR-induced phospho-Chk2 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, permeabilized 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 G2-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 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.

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 Thr68 and forms foci in response to IR (15, 17). Quantification of the ratio of Chk2 phosphorylated on Thr68 to the total amount of Chk2 revealed that Chk2 phosphorylation at Thr68 was reduced twofold after 2 hours in response to IR in the 53BP1-inhibited cells (Fig. 2B). The reduction of Chk2 phosphorylation at Thr68 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 permeabilized 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 synchronized 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 53BP1 siRNA, 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 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 2% of 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. S3BP1 regulation of Brca1. (A) Brca1 phosphorylation is reduced in the absence of S3BP1. U2OS cells were treated with siRNA oligos targeting S3BP1 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 (Novus, Littleton, CO), and S3BP1. 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 S3BP1 or control oligos for 2 days, then treated with different doses of irradiation. Cell lysates were prepared at 2 hours after irradiation. (C) S3BP1 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 S3BP1 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 S3BP1 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). The loss of S3BP1 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 S3BP1 at 50-Gy IR, these cells still failed to form foci (18).

Next we examined whether S3BP1 associated with BRCA1. BRCA1 interacts with S3BP1 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 S3BP1's ability to regulate both Chk2 and BRCA1 function in response to DNA damage.

An important finding of these studies is that S3BP1 is a critical transducer of the DNA damage signal and is required for both the intra-S-phase and G2-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, S3BP1 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 S3BP1 foci (20), and S3BP1 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 S3BP1 in control of phosphorylation of these or other proteins. Regardless of the mechanism, it is clear that S3BP1 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).

References and Notes
11. Material and methods are available as supporting material on Science Online.
20. Antibodies to Chk2EGFP provided by J. Chen; S3BP1, T. D. Halazonetis; and yH2AX, W. M. Bonner.
21. 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 3CyG. 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

Grant S. Stewart*, Bin Wang*, Colin R. Bignell†, A. Malcolm R. Taylor‡ & Stephen J. Elledge§

* Verna & Mars McLean Department of Biochemistry and Molecular Biology, † Department of Molecular and Human Genetics, ‡ Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030, USA
§ MRC Institute for Cancer Studies, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

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(1). 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(2,3). Here we identify a novel BRCA1 carboxy-terminal (BCRT) and forhead-associated (FHA) domain-containing protein, MDC1 (mediator of DNA damage checkpoint 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 γH2AX 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.

BRCAl 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 phosphatidylinositol-3-OH kinase-like kinase phosphorylation motifs (SQ/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 shaded boxes indicate conserved amino acids and grey boxes indicate similar amino acids. c, Recognition of these 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. R-treated cell extracts were also incubated with and without λ protein phosphatase (λ Pase). 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 search for proteins involved in various aspects of DNA repair, 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 damage16–19. 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 investigate the role of MDC1 in the DNA damage response, three polyclonal antibodies generated to non- overlapping fragments of the MDC1 open reading frame (ORF) all specifically recognized three bands (I, II and III) of approximately 250 kDa, which were not recognized by the pre-immune sera (Fig. 1c). All three bands diminished in intensity when cells were pre-treated with three different MDC1-specific siRNA oligonucleotides but not control siRNAs demonstrating specificity (data not shown and see Fig. 5a).

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 a reduced mobility after exposure of cells to ionizing radiation (IR), ultraviolet radiation (UV) and hydroxyurea (HU) (Fig. 1d). The altered mobility was ablated by phosphatase treatment, indicating that MDC1 is modified by phosphorylation after DNA damage (Fig. 1d). Furthermore, 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).

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**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 anti-MDC1 antibodies at the times indicated. **b**, Co-localization of MDC1 and γ-H2AX foci after 10 Gy IR. **c**, Inhibition of MDC1 results in defective BRCA1, 53BP1 and Nbs1 foci formation after IR exposure. U2OS cells were transfected with a control or MDC1 siRNA and irradiated with 10 Gy IR. Cells were fixed at 2 h post-irradiation and stained with the 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.
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 antiseraum. MDC1 rapidly formed foci in 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. 53BP1, BRCA1 and Mre11/NBS1/hRad50 complex are subsequently recruited to γ-H2AX-positive repair foci. To determine the kinetics of MDC1 foci compared with other foci-forming proteins, co-localization studies were done. Strikingly, within 15 min of exposure to ionizing radiation, MDC1 foci extensively overlap with γ-H2AX foci and remain co-localized throughout the time course (Fig. 2b). MDC1 foci also significantly co-localize with 53BP1 and BRCA1 foci at 2 h post-irradiation, and at later times also exhibited co-localization with Nbs1 foci (Fig. 2c). The number of MDC1 foci per cell were in excess of 53BP1, BRCA1 and Nbs1 foci at corresponding times.

Several proteins that show co-localization in foci exist as pre-assembled complexes. Therefore, we determined whether MDC1 could bind to the components of the MRN complex and other known damage response proteins. MDC1 could be shown to co-precipitate with Nbs1, hRad50 and hMre11 before and after DNA damage and to a lesser extent with ATM and the FANCDD2 protein (Fig. 3). The prior exposure of cells to IR appears to reduce the interaction of MDC1 with these proteins slightly. Given that MDC1 binds tightly to chromatin after DNA damage (data not shown), it is likely that the reduction of co-immunoprecipitated protein after exposure to IR is due to a reduced ability to efficiently solubilize MDC1 protein before immunoprecipitation. MDC1 could also be shown to interact strongly with 53BP1 and SMc1 in a manner unaffected by DNA damage. It is therefore likely that MDC1 resides within a large complex consisting of several DNA repair proteins and cell-cycle checkpoint regulatory proteins.

There appears to be a hierarchy of proteins involved in the assembly of damage-responsive foci. H2AX is required for the formation of 53BP1, NBS1 and BRCA1 foci. To determine whether MDC1 functions in this pathway, we asked if MDC1 was required for the ability of these repair proteins to form foci. Cells were treated with a control siRNA or MDC1 siRNA, irradiated and stained for 53BP1, BRCA1 and Nbs1 foci. Cells lacking MDC1 showed a significant reduction in the number of cells with Nbs1 foci, with the number of residual foci in each cell also being reduced (Fig. 2c). This suggests that MDC1 is required for efficient localization of the MRN complex after DNA damage. In addition, both 53BP1 and BRCA1 foci were similarly affected in the MDC1 siRNA-treated cells compared with the control siRNA-treated cells (Fig. 2c).

These data demonstrate that MDC1 is likely to function upstream of 53BP1, BRCA1 and the MRN complex, possibly acting as a scaffold for protein recruitment. Consistent with this hypothesis, MDC1 foci were not affected in NBS or ATLD cells or when cells were treated with 53BP1-specific siRNA (data not shown). It should be noted that inhibitory of MRN and 53BP1 foci was not complete, suggesting that either the siRNA treatment leaves residual functional MDC1 capable of limited foci formation, or that a partly redundant pathway exists to localize these proteins.

Because MDC1 and H2AX extensively co-localize and display 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 embryonic 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 foci. To examine this we used an S139-phosphorylated and unphosphorylated peptide from the C-terminal tail of H2AX coupled to agarose to test 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-phosphorylated peptide 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.
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 using a rabbit reticulocyte lysate system and incubated with the H2AX-phosphopeptide-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. 4c). 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- 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 S396 (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 experiments (data not shown). Chk1 is required for the G2/M checkpoint arrest24,25 and this defect is likely to explain the checkpoint defect we observed. Both SMC124,25 and Chk125 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 pathway depends in part upon MDC1 function, as ATR is the major regulator of Chk1 phosphorylation.
Figure 5 MDC1 inhibition results in defective IR-induced checkpoin
ts and Chk1 and H2AX phosphorylation. a, siRNA-mediated reduction of MDC1 protein expression. Cells were transfecte
d with a control siMDC1 or a 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.
b, Reduced MDC1 expression results in IR sensitivity. Control or siMDC1 siRNA-treated
cells were plated at low density, irradiated and colonies counted after 14 days. c, MDC1
prevents RDS. DNA synthesis was assessed 30 min after 15 Gy IR in U2OS cells
transfected with a control or siMDC1 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 determined by phospho-histone H3 staining and
flow cytometry. e, MDC1 is required for DNA damage checkpoint signaling. Cells were
transfected twice with siRNAs and irradiated 72 h later. The Immunoblot with SMCA1 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 siMDC1 siRNA-treated cells were irradiated and the levels of H2AX
phosphorylation detected by western blot and immunofluorescence 1 h post-irradia
tion. Together, these results indicate that MDC1 is a central
transducer 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 phosphor-
ylation of H2AX, or merely protects H2AX from dephosphoryla
tion 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 IR3, much less severe than that observed in MDC1.
MDC1 exists in complexes with several checkpoint-signalling pro-
teins such as the MRN complex, SMCA1, 53BP1 and FANCDC2.
Furthermore, MDC1 is required for the efficient phosphorylation
of several checkpoint-signalling proteins including SMCA1, 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 observation of Chk1, suggests that MDC1 plays an important role in
the ATR signalling pathway that controls Chk1 phosphorylation5
and a less important role in the ATM pathway. This may explain the
mild defect in the IR-induced phosphorylation of SMCA1, which is
primarily ATM dependent. The mild SMCA1 phosphorylation defect
becomes more pronounced at later times in the absence of MDC1,
consistent with the late role of ATR in IR-induced phosphoryla
tion 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 tumorgenesis.

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). U2OS 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'-deoxynucleotide overhang
(Dharmacon Research). The sequences of MDC1 siRNA4 and siRNA5 oligonucleotides
were GCCUCCCAAGGACACUGAAGTdT and AACAGUGUGUCCCACACCGGGT,dT,
respectively. The control siRNA used was CUGACGGCGAUAUCCUGGATdT against
Luc2. Cells were transfected with siRNA duplexes by using Oligofectamine (Invitrogen),
following the manufacturer’s instructions.

MDC1 antibody generation

A full-length MDC1 clone (KIAA0170) was generously provided by the Kazusa DNA
Research Institute. Fragments of the cDNA were cloned into a glutathione
S-transferase (GST) expression vector (Amersham Biosciences), expressed in bacteria and

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purified by using glutathione-coupled rephase beads (Amerham 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 326-469, 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.

Immuno blot 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 hRad50 were obtained from GenoTech, the phospho-Nbs1 (serine-345) antibody was obtained from Cell Signalling and the anti-phospho-H2AX (serine-139) antibodies were obtained from Upstate Biotechnology. The anti-FANC-D2 and anti-Chk2 antibodies were purchased from Santa Cruz. J. Qin provided antibodies to SMCI and phosphorylated SMCI (ref. 21). P. Carpenter supplied antibodies to 53BP1.

Immunoprecipitation and phosphatase treatment
LCRs (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 NETN lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40 supplemented with protease inhibitors (Roche) and benzamidine (Norgen)). The clarified extract was pre-cleared with the appropriate IgG (Dako) and protein A or G beads (Amerham 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 phosphatase treatment of cell extract, 1,200 units of α-protein phosphatase (New England Biolabs) were added to the extract in the presence of MgCl2 and incubated for 30 min at 30°C. The phosphatase was then heat inactivated.

H2AX peptide pull-down assay
Two peptides, the last C-terminal 10 amino acids of human H2AX (CKATQASQYEV) were synthesized (Bethyl), one of which was phosphorylated on the serine residue. The peptides were coupled to beads by using the SulfoLink kit ( Pierce). 20 μg of coupled peptide were used per pull-down. The assay was essentially identical to the immunoprecipitation protocol as 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 (Amerham Biosciences). Half of each reaction was added to 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 fed 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 RDS assay was done as described above. Briefly, cells were transfected with siRNA oligonucleotides, and 6 h later were placed into McCoy's 5A medium containing 10 μCi of [3H]thymidine (NEN Life Science Products Inc.) per milliliter overnight. The medium containing [3H]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-labeled with 2.5 μCi [3H]thymidine (NEN Life Science Products Inc.) per milliliter for 15 min. Cells were harvested, washed twice with PBS, and fixed in 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 assayed in a liquid scintillation counter. The resulting ratios of [3H]counts per minute to [3C]counts per minute, corrected for those counts per minute that were the result of channel crossover, were a measure of DNA synthesis.

Supplementary Information accompanies the paper on Nature's website (http://www.nature.com/nature).

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Competing interests statement
The authors declare that they have no competing financial interests.
Role for the BRCA1 C-terminal Repeats (BRCT) Protein 53BP1 in Maintaining Genomic Stability*


From the †Department of Biochemistry and Molecular Biology, University of Texas Health Sciences Center, Houston, Texas 77030, the ‡Department of Experimental Therapeutics, University of Texas M. D. Anderson Cancer Center, Houston Texas 77030, and the Werna and McLean Department of Biochemistry and Molecular Biology, ††Department of Molecular and Human Genetics, and ‡‡Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030

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 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 (m53BP11111), we have created an animal model to further explore its biochemical and genetic roles in vivo. We find that m53BP11111 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 m53BP11111 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, phosphoinositide-like kinases (PIKs) such as ATM (mutated in ataxia-telangiectasia) transduce damage signals to kinases, transcription factors, and DNA repair proteins by targeting (S/T/Q) motifs (2). A second PIK, ATR (ATM- and Rad3-related), also responds to γ-IR, but it appears to respond primarily to agents that create replication stress (i.e., hydroxyurea and aphidicolin) (2). ATM and ATR have distinct but overlapping substrate specificities including the ability of both enzymes to target p53 serine residue 15 (Ser-15) as well as the 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 DNA 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 genominc 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/Q) 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 kinetochore-binding domain (28) is necessary for the formation of irradiation-in-

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**A Sophie Caroline Stieves Distinguished Professor in Cancer Research.

¶An investigator with the Howard Hughes Medical Institute, an Ellison Medical Foundation Senior Scholar, and the Welch Professor of Biochemistry.

#An Ellison Medical Foundation Junior Scholar who is grateful for their support. To whom correspondence should be addressed. Fax: 713-500-0652; E-mail: Phillip.B.Carpenter@uth.tmc.edu

†The abbreviations used are: γ-IR, ionizing radiation; PIK, phosphotyrosyl phosphatase-like kinase; BRCT, BRCA1 C-terminal repeats; MEF, mouse embryonic fibroblast; ATM, mutated in ataxia-telangiectasia; ATR, ATM- and Rad3-related; RT, reverse transcription; HA, hemagglutinin; IP, immunoprecipitation; WB, Western blotting; DN, double negative.

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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 (m53BP1trm) 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 (a53BP1) 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 GVLELSQQSQVEE that is conserved between human and murine 53BP1 proteins. Polyclonal antibodies were affinity-purified by standard methods. Anti-HA antibodies were purchased from Covance, and anti-ATR antibodies were obtained from Oncogene Research Products.

Molecular Genetics and Genotyping of 53BP1+/− Animals—Murine animals defective in m53BP1 (m53BP1trm) were generated with a random retrovirus as described previously (29). Genomic DNA was isolated from mouse tail snips by standard methods. Insertion of VICTR54 introduces new XbaI sites into the intron preceding exon 14 of the wild-type allele. Therefore, a disrupted m53BP1 allele will be broken into multiple XbaI fragments including a 5′-proximal, 1.5-kb fragment. To detect this fragment, we PCR-amplified and labeled with [α-32P]dCTP a probe downstream of the 5′ naturally occurring XbaI site but upstream of the one introduced by VICTR54, as shown in Fig. 2A. The primers used to amplify the 700-bp probe for Southern analysis were 5′-CTTACCATCATCTGTCGGC-3′ and 5′-CTACCTCTAGACCTAGAGGCTC-3′. The sequences of the primers used for RT-PCR analysis were as follows: A, 5′-CCCTAAGAGCGGAAATACTA-3′, B, 5′-GCTCTTCGTCG CACCCGAAACTC-3′, C, 5′-GCTGGCGATCGGAGAATGTCCT-3′, D, 5′-GCCAGAAGCGGAAAGGCTG-3′, and E, 5′-CTTACGCTAGATCACTAGAGGCTC-3′. Either poly(A) + total RNA was isolated by standard methods and used to prepare cDNA with the Superscript one-step PCR system (Invitrogen).

Immune System Analysis—Bone marrow, thymus, and spleen tissue were analyzed in 8-week-old male and female mice. Bone marrow was flushed with Ham’s balanced salt solution (Invitrogen) from femurs and tibias of mice aged 8 weeks. Cells were counted in 3.0% acetic acid with a hemocytometer. Bone marrow cells were stained in Hank’s balanced salt solution/2.0% fetal bovine serum, with Fc block, CD11b (Mac-1), Gr-1, ter119, CD19, anti-IgM, CD45RB2B30, and CD43 (all from Pharmin- gen). Flow cytometric analysis was performed on a BD Biosciences FACScan, with CellQuest software, and appropriate negative isotype control antibodies (Pharmingen) were used in all analyses. Spleens and thymuses were excised and gently crushed through 100-μm cell strainers (Falcon) in Hank’s balanced salt solution/fetal bovine serum. Red blood cells were lysed with ACK buffer (0.15 mM NH4Cl, 1.0 mM KHCO3, 0.1 mM EDTA, pH 7.2) (5 mL/please or thymus, 5 min at room temperature). After centrifugation and washing with phosphate-buffered saline (PBS), 2% fetal bovine serum, the cells were counted as above and stained for flow cytometric analysis. Thymic cells were stained with CD4, CD8, CD25, and CD3 (Pharmingen). Flow cytometry was performed as above.

Chromosome Aberration Studies—Exponentially growing passage 2 MEFs were irradiated at room temperature with 0.5, 1.0, 1.5 Gy of γ-IR using a Naslar irradiation, returned to 37 °C for 30 min to allow cells irradiated in mitosis to exit, and then incubated with 1 μg/ml colcemid (ICN) for 1 h. The cells were then harvested, hypotonically treated with 0.075 M KCl, fixed with 3:1 methanol/glacial acetic acid), and metaphase spread preparation. Dried slide preparations were stained with Giemsa and examined for the presence of chromatid gaps, breaks, and exchanges by light microscopy. Between 50 and 100 metaphases were scored for each treatment.

RESULTS AND DISCUSSION

Dynamic Nuclear Localization of 53BP1 in the Absence and Presence of DNA Damage—It has been recently shown that 53BP1 localizes to the kinetochore during mitosis (28). However, the behavior of 53BP1 during interphase in the absence of extrinsic DNA damage has not been fully investigated. To examine the interphase behavior of 53BP1 during the course of normal, unperturbed MCF-7 cell cycles, we used a laser-scanning cytometer to determine the nuclear localization of 53BP1 in the cellular DNA content for any given cell. In G1, 53BP1 exists in a diffuse nuclear pattern as well as in large nuclear "dots" (Fig. 1A). In S-phase, 53BP1 can be found in a discrete, punctate pattern (Fig. 1A). The nuclear distribution pattern of 53BP1 in G2 cells appeared in two types, one similar to S-phase but with fewer foci (Fig 1A) and one that exhibited few, if any, large dots (not shown). It is well established that 53BP1 relocates to nuclear foci in response to DNA damage (17–20). We found that 53BP1 and ATR co-localized to nuclear foci in response to hydroxyurea (Fig. 1B). We also found that 53BP1 physically associates with ATR in nuclear extracts derived from K562 cells (Fig. 1C). 53BP1 can be detected in ATR immunoprecipitates and ATR is present in 53BP1 immunoprecipitates, and the association occurs independently of DNA damage (Fig. 1C). Moreover, ATR phosphorylates 53BP1 in vitro (not shown). Thus, 53BP1 interacts with various factors implicated in genomic stability including ATR, p53, H2AX, BRCA1, and Chk2. To address which structural elements of 53BP1 are required for the formation of irradiation-induced foci, we created a series of mutant constructs in the 53BP1 expression vector pCMH65K53BP1 (16). We generated mutant forms of 53BP1 that deleted the C-terminal BRCT motifs (ABCR'T), the kinetochore-binding region (AIKEN'T), the N-terminal 1,234 residues except for the initiation codon (ΔNHI), and a protein mutated at 15 potential phosphorylation sites (Δ15AQ), some of which are known to be targeted during the DNA damage response. All constructs maintained the nuclear localization signal. Transient transfections with these various constructs into MCF-7 cells revealed that the mutant proteins were being expressed (not shown). We confirmed that the wild-type, HA-tagged version of 53BP1 encoded by pCMH65K53BP1 generated nuclear foci in response to DNA damage when immunostained with an antibody specific for the HA tag (Fig. 1D). Untransfected cells were found to stain negatively with anti-HA antibodies (not shown). Our results indicate that the majority of 53BP1 appears dispensable for DNA damage-inducible focus formation, including the N-terminal 1,234 residues (which includes numerous S/T/Q motifs) as well as the C-terminal BRCT motifs (Fig. 1D). Surprisingly, AIPK'T, a 380-amino acid deletion (residues 1,236–1,615) that removes the kinetochore binding region (28) of 53BP1, failed to form irradiation-induced foci as the protein persisted in a diffuse nuclear pattern after irradiation (Fig. 1D).

Generation of Mice Defective in m53BP1 (m53BP1trm)—To begin to decipher the functional role for 53BP1 in the DNA damage response, we identified embryonic stem cells (OST94324) from Omnnibact (29) containing a single, ~5.0-kb retroviral insertion (VICTR54; Fig. 2A) in murine 53BP1 (m53BP1; 1,957 amino acids; 80% identity to human 53BP1; see Ref. 28). VICTR54 was found inserted within a 4.9-kb intron located between exons 13 and 14 (Fig. 2A). VICTR54, and its related vectors, are usually found within introns and contain splice acceptor (SA) and donor (SD) splice sites such that a neo-neo, hygro resistance gene and flanking sequences are spliced into the mature transcript as an exon (Fig. 2A) (29).

These transcriptional fusions disrupt the coding sequence through the introduction of premature stop codons. Such gene trapping methodologies have been applied previously to under-
Fig. 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 (α53BP1; 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 laser scanning cytomter. Black and white images were captured with a x100 objective on a Zeiss Axioshot 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 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 5’/3’ sites mutated in 15AQ. KINET, kinetochore-binding region (28); NLS, nuclear localization signal (28). 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 MCF7 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: ABRCT (deletes amino acid residues 1,786–1,964), ΔKINET (deletes amino acid residues 1,236–1,615), ΔN3H (deletes the first 1,334 amino acid residues except for the initiation codon), and 15AQ, a construct with mutations in 15 (STTQ) sites. The following serine or threonine residues were mutated to alanines in 15AQ: Ser-6, Ser-13, Ser-17, Ser-35, Ser-37, Ser-52, Ser-523, Ser-543, Ser-565, Ser-784, Ser-892. All constructs were verified by DNA sequencing and expressed in either 293T or MCF7 cells (not shown).

erate transgenic animals heterozygous in m53BP1 (m53BP1+/−) 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 produced m53BP1+/- progeny born at the expected frequencies. The m53BP1+/- animals were found to be fertile, but we did observe that crosses between mutant animals produced smaller litters as some embryos spontaneously aborted and were reabsorbed by the mother (data not shown). RT-PCR analysis with various primers 5’ and 3’ to the insertion demonstrated that exon 13 failed to properly splice next to exon 14 in the m53BP1+/- mice (Fig. 2C). 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 1,236 residue protein (m53BP1*), and notably, m53BP1* is missing over 700 residues including its functional nuclear localization signal, kinetochore 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 m53BP1*/*, m53BP1+/-, and m53BP1+/- animals. By using antibodies specific for the N and C termini of 53BP1 (α53BP1-N and α53BP1-C, respectively; see ’Experimental Procedures’), we determined that a truncated m53BP1 protein corresponding to m53BP1* appeared in heterozygous and mutant extracts but not in wild-type ones (Fig. 2E). The levels of m53BP1* 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 m53BP1* (Fig. 2F). We observed that α53BP1-N cross-reacted with m53BP1* but not with α53BP1-C, demonstrating that m53BP1 is indeed truncated in m53BP1+/- animals (Fig. 2, E and F).

Immune Deficiencies in m53BP1+/- Mice—We observed that m53BP1+/- 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 m53BP1+/- mice were significantly smaller and possessed fewer cells than those from m53BP1+/* 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 m53BP1+/* mice (data not shown). In addition, flow cytometric analysis
Fig. 2. Generation and characterization of mice defective in m3BP1 (m3BP1<sup>mut</sup>). A, a schematic diagram of insertion event in m3BP1 (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 VICTRS4 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. LTR, long-terminal repeat; NEO, neomycin resistance gene; PGK, phosphoglycerate kinase-1; BTK, Bruton’s tyrosine kinase; SA and SD, splice acceptor and donor, respectively. B, top, Southern blotting to determine the genotype of m3BP1-defective animals. 15 µg 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 m3BP1<sup>mut</sup> 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, m3BP1<sup>mut</sup> 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 m3BP1<sup>mut</sup> animals as template were cloned into the TA vector (Invitrogen). DNA sequencing and conceptual translation indicated that m3BP1<sup>mut</sup> animals potentially encode a truncated m3BP1 protein (m3BP1<sup>mut</sup>) of 1,205 natural residues along with an additional 21 residues derived from the VICTRS4 vector. m3BP1<sup>mut</sup> is missing over 700 C-terminal residues, including those that specify the kinetochore binding domain (KIN27; amino acids 1,620–1,661), the nuclear localization signal (NLS; mapped to amino acids 1,601–1,703; ref), and the C-terminal BRCT repeats (amino acids, 1,665–1,967). The small, vertical rectangle in m3BP1<sup>mut</sup> represents the additional vector-derived 21 residues. E and F, detection of m3BP1 protein by IP/WB analysis. 1.0 mg of total brain protein extracts derived from +/-tr, +/-tr, and tr/tr animals was immunoprecipitated with 5 µg of affinity-purified antibody (a3BP1-N) raised against an N-terminal peptide sequence (see “Experimental Procedures”) and split into two equal parts. One part (E) was blotted with a5BP1-N, and the other (F) was immunoblotted with an affinity-purified antibody specific for the C terminus (a3BP1-C). E, lane 1, IP from 100 µg of MCF-7 nuclear extract. Lane 2, IP with nonspecific IgG control. Lanes 3–5, IP with +/-+, +/-tr, and tr/tr animals as determined in B. IP/WB analysis shows the presence of m3BP1<sup>mut</sup> in m3BP1<sup>mut</sup> and m3BP1<sup>mut</sup> animals but not of m3BP1<sup>mut</sup> ones. In some cases, we observed an apparent isoform of m3BP1 in brain tissue, as designated by the asterisk. F, WB with a5BP1-C, an affinity-purified antibody against the C-terminal 200 residues of human m3BP1. As shown, a5BP1-C recognizes full-length m3BP1 but fails to immunoreact with m3BP1<sup>mut</sup>, indicating that the protein is indeed missing C-terminal residues of m3BP1.
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 m35BP1tr/tr mice (not shown). Although CD4 and CD8 T cell populations were proportionately similar in m35BP1tr/tr and m35BP1+/+ thymuses, we observed that progression out of the DNIII stage of early thymocyte development was impaired in m35BP1tr/tr animals (Fig. 3C), the stage at which B-gene rearrangement occurs. This indicates that m35BP1 participates in proper T cell development, a process known to require various DNA repair factors (30). We also found that spleens derived from m35BP1tr/tr animals were similar in size and organ architecture to those from m35BP1+/+ animals and that the lack of functional m35BP1 did not affect the proportions of B and T lymphocytes (data not shown). We did observe, however, that m35BP1tr/tr spleens were deficient in mature B cells (IgM+IgD+, Fig. 4D), suggesting that deficiencies in m35BP1 may also result in defective B lymphocyte development.

Genomic Instability in m35BP1tr/tr Mice—Mice with defects in double-stranded break repair are highly sensitive to γ-IR. To evaluate whether m35BP1 contributes to increased sensitivity to DNA damage, we treated m35BP1tr/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 within 15 days post-irradiation in contrast to only 16% of the control littermates (Fig. 4A). This shows that animals deficient in m35BP1 are highly sensitive to γ-IR, a result that parallels previous observations with H2AX-deficient mice (24). Despite this, we found that m35BP1tr/tr animals treated with lower doses of γ-IR (1.5 Gy) remained viable (Fig. 4B). To further explore m35BP1 function, we generated embryonic fibroblasts (MEFs) from wild-type and m35BP1tr/tr animals. m35BP1tr/tr MEFs proliferated more slowly than their wild-type counterparts (Fig. 5A). Immunofluorescence analysis indicated that the truncated m35BP1 protein expressed in m35BP1tr/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 m35BP1, we examined the cytological consequences of impaired m35BP1 function in early passage MEFs derived from m35BP1tr/tr and m35BP1+/+ 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 m35BP1tr/tr animals showed increased levels of chromatid gaps, breaks, and, to a lesser extent, exchanges when compared with those derived from m35BP1+/+ mice, suggesting an intrinsic genomic stability defect in the mutant cells (Fig. 5, B and C). More strikingly, irradiated MEFs derived from m35BP1tr/tr animals showed an ~2-fold increase in levels of chromatid breaks and gaps when compared with MEFs derived from wild-type mice (Fig. 5, B and C). Although MEFs from m35BP1tr/tr animals showed relatively high chromatid exchange rates at 0.5 Gy when compared with those from m35BP1+/+ 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 m53BP1trtG 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 m53BP1trtG 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 m53BP1trtG 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 m53BP1trtG MEFs may be due to the nature of the truncated protein produced from the m53BP1trtG 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 m53BP1trtG allele expresses a truncated version of m53BP1, and this likely represents a

![Graph A](image)

Fig. 4. Characterization of animals and cells defective in m53BP1trtG. A, survival of 4–6-week-old m53BP1trtG and m53BP1trtG 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 m53BP1trtG animals after exposure to 1.5 Gy of ionizing radiation.

![Graph B](image)

![Graph C](image)

Fig. 5. Chromosomal abnormalities in m53BP1trtG cells. A, growth curve of MEFs derived from m53BP1trtG (open diamonds) and m53BP1trtG (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*tr 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 DNA double-strand breaks in mutant MEFs suggests that the protein cannot fully perform its functions as a DNA damage-response element. Moreover, the lethality observed for m53BP1*tr mice at higher doses of radiation (7 Gy) suggests that there are no other factors acting redundantly with m53BP1 with respect to this aspect of radiation resistance and indicates that m53BP1 is a critical element for double-stranded break repair. Therefore, the C-terminal 700 amino acids of m53BP1 encode important, functional determinants of the protein.

We observed that m53BP1*tr mice are growth-retarded as the males weigh, on average, 25% less than their wild-type littermates. The decreased thymus size, reduced T cell count, immature B cell population, and lack of progression of DNIII for thymus T cells reveal that m53BP1*tr animals are immune-deficient. How m53BP1 contributes to this process remains to be established, but one possibility is that the protein participates in the maturation of T-cell receptors and immunoglobulins during VDJ recombination, a process known to utilize DNA repair proteins (30). This is particularly interesting given the involvement of m53BP1 in double-stranded break repair as revealed by several factors including, most notably, the sensitivity of m53BP1*tr animals after exposure to 7 Gy of ionizing radiation. Indeed, sensitivity to ionizing radiation often correlates with impaired VDJing (30). Moreover, H2AX defective-animals are also immune-deficient (24). As H2AX is required for the formation of 53BP1 foci and because it physically associates with 53BP1 (20), it is possible that an ordered pathway of assembly of DNA damage-response proteins at these programmed breaks may facilitate VDJ recombination and maximize antibody diversity.

Our results show that genetic defects in m53BP1 result in a pleiotropic phenotype consistent with defects in DNA repair and checkpoint control. The phenotype of 53BP1-defective animals is quite similar to H2AX-deficient ones, consistent with the notion that H2AX operates upstream of 53BP1 in a DNA damage-response pathway. When such pathways are defective, cells cannot properly repair damaged DNA, a situation that may lead to increased genomic instability and the development of diseases such as cancer. For example, given the immune deficiencies in m53BP1*tr mice, one might anticipate the generation of lymphomas. In light of this, we have not observed the development of any cancerous phenotypes in our m53BP1-defective mice. Although there are a variety of possible reasons for this (i.e. genetic background, allele, etc.), it is interesting to note that mice nulligous for H2AX also apparently fail to generate cancers. As H2AX and 53BP1 are not required for viability, it is possible that mutations in 53BP1, when combined with other mutations in critical DNA damage-response elements (i.e. H2AX, ATM, and p53) will lead to more severe defects in genomic stability, a process that may then lead to the development of cancer. The analysis of cells derived from these crosses is likely to provide more insight into how 53BP1 functions in the DNA damage response in concert with its various interacting partners.

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