The DNA damage response mediators, 53BP1 and MDC1, play a central role in checkpoint activation and DNA repair. Here we establish that human 53BP1 and MDC1 interact directly through the tandem BRCT domain of MDC1 and residues 1288–1409 of 53BP1. Following induction of DNA double strand breaks the interaction is reduced, probably due to competition between γ-H2AX and 53BP1 for the binding of the tandem BRCT domain of MDC1. Furthermore, the MDC1 binding region of 53BP1 is required for focus formation by 53BP1. During mitosis the interaction between 53BP1 and MDC1 is enhanced. The interaction is augmented in a phospho-dependent manner, and the MDC1 binding region of 53BP1 is phosphorylated in vivo in mitotic cells; therefore, it is probably modulated by cell cycle-regulated kinases. Our results demonstrate that the 53BP1-MDC1 interaction per se is required for the recruitment of 53BP1 to sites of DNA breaks, which is known to be crucial for an efficient activation of the DNA damage response. Moreover, the results presented here suggest that the interaction between 53BP1 and MDC1 plays a role in the regulation of mitosis.

The cellular response to DNA damage maintains genomic stability by reacting to the presence of DNA damage in cells. The response includes activation of cell cycle checkpoints, repair of the damage and transcriptional regulation and activation of apoptosis. Several groups of proteins play a role in the DNA damage response (DDR): the “sensors” sense the damage and transmit signals to the “transducers,” a group of kinases that convey the signals with the help of “mediators” to downstream “effectors,” which in turn execute the response (1).

The DNA damage mediators function as adaptor proteins that recruit different members of the DDR to sites of DNA damage and introduce them to the transducer kinases, thereby facilitating the signal transduction following DNA damage. Upon induction of DNA double strand breaks (DSBs) this group of mediators relocates rapidly to sites of breaks, forming discrete nuclear foci, and its members are phosphorylated by the DDR transducer kinases (1). One class of mediators includes p53-binding protein 1 (53BP1), mediator of DNA damage checkpoint 1/nuclear factor with BRCT domain protein 1 (MDC1/NFBD1), and breast cancer 1 (BRCA1); all are large proteins that contain two consecutive BRCA1 C-terminal (BRCT) domains at their C terminus (1). BRCT domains are protein-protein interaction modules that are found in many proteins that regulate the DDR (2), and tandem BRCT domains (tBRCT domain) were demonstrated to be phospho-protein binding modules (3–5).

53BP1 was originally identified in a yeast two-hybrid screen as a p53-binding partner (6). In addition to the tBRCT domain of 53BP1, this protein contains tandem Tudor domains (tTudor domain of 19 consecutive repeats and a forkhead-associated domain (FHA) domain (7)). 53BP1 contains, in addition to its tBRCT domain, a central region of 19 consecutive repeats and a forhead-associated (FHA) domain (18–21). FHA domains are phospho-protein binding modules commonly found in signaling pathway proteins (22). Upon DNA DSB induction, MDC1 plays a role in the activation and in the correct localization of DDR factors at sites of damage. Additionally, MDC1 has a role in DNA DSB repair and is required for the establishment of the G2/M and intra-S phase checkpoints (18–20, 23–28). MDC1 is also phosphorylated in cells arrested in mitosis after nocodazole treatment (29, 30).

Real-time microscopy in live cells revealed that the assembly of 53BP1 at DNA DSB lags behind MDC1 and that MDC1 is required for the assembly and the maintenance of 53BP1 at DNA DSBs (31). Additionally, MDC1 is required for focus formation by 53BP1 (20, 26, 27, 32). Recently it was demonstrated that the interaction between MDC1 and the ring finger protein 8 (RFN8) is required for focus formation by 53BP1 (26, 27).
Endogenous 53BP1 and MDC1 were previously shown to be part of the same complex by immunoprecipitation (IP) (20) with the C-terminus of MDC1 mediating this interaction (29). However, it was not clear if these proteins interact directly with each other and, if they do, what the biological role of such an interaction might be. Here we have determined that 53BP1 and MDC1 interact directly and mapped the regions in both proteins that mediate the interaction. We also demonstrated that the 53BP1-MDC1 interaction is reduced following the induction of DNA DSBs and that 53BP1 competes with the phosphorylated form of histone H2AX (γ-H2AX), a known marker of DNA DSBs. Furthermore, we observed that the interaction increases in cells arrested in mitosis. The interaction is augmented by one or more phosphorylation events that occur in vivo on 53BP1 and relies on cell cycle-regulated kinases. Our results demonstrate that the 53BP1-MDC1 interaction is required for the recruitment of 53BP1 to sites of DNA breaks and provide new insights into the interplay between the DNA damage mediators, 53BP1 and MDC1, in the DDR and during mitosis.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Peptides**—Full-length HA-53BP1, HA-53BP1-ΔGAR, fragment numbers 2–9 (Fig. 1C), GST-53BP1:1052–1709, and GST-53BP1:667–1709 were previously described (8, 12). HA-53BP1:1052–1709 4A mutant was generated from HA-53BP1:1052–1709, and HA-53BP1:1052–1709 were previously described (8, 18, 33). Biotinylated histone H2AX peptides (SGSTVGPKAPSGGGKKATQASQEY and SGSTVGPKAPSGGGKKATQASQAY) were a kind gift from M. Stucki (University of Zurich, Switzerland).

**Cell Culture, Extract Preparation, Protein Expression, and Purification**—293T, HeLa, and MCF7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1-glutamine, penicillin, and streptomycin. Anti-MDC1 antibodies included rabbit and sheep anti-MDC1 directed against the FHA and tBRCT domains of MDC1 (18) and mouse anti-MDC1, clone MDC1–50 (Sigma-Aldrich). Anti-sheep IgG antibodies (Sigma-Aldrich) were used as controls.

**Immunoprecipitation and GST Pulldown Assays**—GST pull-down assays were done with 20 μg of the indicated bacterially expressed and purified GST fusions and glutathione-Sepharose 4 Fast Flow beads (Amersham Biosciences). IPs were done with the indicated antibodies and protein A- or G-Sepharose beads (Santa Cruz Biotechnology or Roche Applied Science). High salt protein extracts (1–2 mg) were added to the IP or GST pulldown assays. Beads were washed extensively with wash buffer (20 mM HEPES, pH 7.4, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2% Triton X-100, 150 mM NaCl) and bound proteins were subjected to SDS-PAGE and Western blots. For the peptide-competition assay, 100 pmol of peptide was added to the GST fusions and beads prior to incubation with the extract.

**In Vitro Binding Assay between Recombinant Proteins**—100 μg of recombinant GST-MDC1-tBRCT or -tBRCTm was incubated with glutathione-Sepharose 4 Fast Flow beads for 1 h at 4 °C. The GST was cleaved from MDC1-tBRCT or -tBRCTm by adding thrombin (BD Health Care) for 16 h at 16 °C. The supernatant was incubated with 20 μg of GST-53BP1:1052–1709 or GST-53BP1:667–1052, which were preincubated with glutathione-Sepharose 4 Fast Flow beads. After incubation for 2 h at 4 °C and intensive washes thrombin was added to the reaction for 16 h at 16 °C. Supernatant was subjected to SDS-PAGE and Western blot analysis with anti-MDC1 antibodies.

**Blot Overlay**—HA-53BP1:1052–1709 or HA-53BP1:1483–1972 were overexpressed in 293T cells, and protein extracts were prepared. Equal amounts of extracts were resolved by SDS-PAGE. Separated proteins were then transferred to a nitrocellulose membrane (Pall Corp.) and blocked overnight in 8% milk powder (Marvel) in TBST (25 mM Tris, pH 8.0, 140 mM NaCl, 3 mM KCl, 0.1% Tween 20) at 4 °C. The membrane was then incubated for 1 h at room temperature with recombinantly expressed GST-MDC1-tBRCT or GST-MDC1-tBRCTm at a concentration of 10 μg/ml in 4% milk powder in TBST. Next, the membrane was rinsed with TBST and incubated with antibodies directed against MDC1-tBRCT. Then the membrane was washed in TBST and incubated with a peroxidase-conjugated secondary antibody. Peroxidase activity was detected with the EZ-ECL chemiluminescence system (Biological Industries).

**In Vivo Phosphorylation Analysis**—HA-53BP1:1288–1409 was overexpressed in 293T cells, and protein extract was prepared 16 h after addition of nocodazole. Anti-HA antibody was used to IP HA-53BP1:1288–1409 from the extract. After extensive washing, the beads were split into two tubes, and λ-phosphatase was added to one sample. Both samples were incubated at 37 °C for 30 min, sample buffer was added, and samples were separated by SDS-PAGE. The proteins were visualized by Coomassie Blue staining (PageBlue protein staining solution, Fermentas), and both bands (corresponding to HA-53BP1:1288–1409) were excised for mass spectrometry analysis. Peptides were extracted from the gel with 60% CH3CN, 1% CHOOH, evaporated to dryness, rehydrated with 1 μl of CH3CN, 1%
**Direct Interaction between 53BP1 and MDC1**

**FIGURE 1.** 53BP1 and MDC1 interact through residues 1288–1409 of 53BP1. A and B, endogenous 53BP1 and MDC1 interact. Antibodies directed against MDC1 (A) or 53BP1 (B) were used to IP MDC1 and 53BP1, respectively, from protein extracts prepared from HeLa cells. Proteins were separated and analyzed by Western blots using antibodies directed against 53BP1 (A) or MDC1 (B). Control antibodies were anti-HA (A) and sheep IgG (B) antibodies. C, schematic representation of 53BP1 and its derivatives fused to an HA tag and their ability to interact (+) or not (−) with MDC1. The constructs are numbered 1–10, and the locations of the nuclear localization signal (NLS), the tTudor and tBRCT domains and the GAR motif are indicated. Numbers indicate amino acid residues. D and E, mapping the MDC1 binding region of 53BP1. Protein extracts were prepared from 293T cells overexpressing constructs 1–10 illustrated in C. The HA-tagged proteins (D) or MDC1 (E) were immunoprecipitated using antibodies directed against the HA tag or MDC1, respectively. Proteins were separated and analyzed by Western blots using antibodies directed against MDC1 (D) or HA (E).

CHOOH, and then diluted with 9 μl of 1% formic acid. The peptide mixture was solid-phase-extracted with a C18 resin-filled tip (ZipTip Millipore, Billerica, MA) and nanosprayed into an Orbitrap mass spectrometry (MS) system (ThermoFinnigan) in 50% CH3CN, 1% CHOOH solution using a nanospray attachment. Data analysis was done using the Bioworks package (ThermoFinnigan), and data base searches were performed with the Mascot package (Matrix Science). Peptides were positively identified by analysis of MS spectra and subsequent MS/MS spectra.

Immunochemistry—MCF7 cells were grown on poly-l-lysine (Sigma-Aldrich)-coated coverslips and fixated in methanol and acetone after exposure to IR at the indicated times. The coverslips were rinsed in phosphate-buffered saline and blocked for 1 h in 10% fetal bovine serum in phosphate-buffered saline. Cells were then immunostained with specific primary antibodies and with fluorescein isothiocyanate or Rhodamine Red-X-conjugated secondary antibodies (Jackson Laboratories). The DNA was stained with 2 μg/ml 4′,6-diamidino-2-phenylindole. Coverslips were placed on microscope slides using fluorescent mounting medium (DakoCytomation). Slides were viewed using the Eclipse TE2000-E inverted fluorescence microscope (Nikon, Japan) equipped with a 60×/1.4 oil-immersion objective, photos were obtained using a Vosskühler COOL-1300Q camera (VDS, Germany) and analyzed via the NIS elements AR software version 2.3. Slides were also viewed using the FV-1000 confocal microscope (Olympus, Japan) equipped with an IX81 inverted microscope and a 60×/1.4 oil-immersion objective. After scanning, a maximum intensity projection was generated from the serial sections, using the Olympus software version 1.6. Photos were subsequently processed using the Adobe Photoshop Elements software version 2.0.

**RESULTS**

53BP1 and MDC1 Interact via a Central Region in 53BP1—Previous work demonstrated that MDC1 co-IPs 53BP1 from cell protein extracts (20). We repeated this experiment and obtained similar results, using various antibodies directed against MDC1 for the IP reaction and anti-53BP1 antibodies for detection of 53BP1 in Western blots (Fig. 1A and data not shown). We also preformed the reciprocal co-IP and demonstrated that antibodies directed against 53BP1 efficiently co-immunoprecipitated the different isoforms of MDC1 from protein extracts (Fig. 1B). These isoforms probably represent alternative splicing forms of MDC1 (18).

The next step was to map the binding regions in both 53BP1 and MDC1. We first analyzed which region in 53BP1 mediates the interaction with MDC1. Full-length or fragments of human
53BP1, fused to a hemagglutinin (HA) tag (illustrated in Fig. 1C) were overexpressed in cells. We performed a series of reciprocal co-IP experiments, using either an anti-HA antibody to IP the different fragments of 53BP1 and anti-MDC1 antibodies for Western blots analysis (Fig. 1D) or anti-MDC1 antibodies for the IP and an anti-HA antibody for the detection of the different 53BP1 fragments via Western blotting (Fig. 1E). These co-IP studies revealed that the minimal region that binds MDC1 is composed of amino acid residues 1288–1409 of 53BP1 (53BP1: 1288–1409, construct 10) (Fig. 1, C–E). It seems that this region is the only region in 53BP1 that binds MDC1, because 53BP1 fragments that do not include this region do not co-IP with MDC1 (Fig. 1, C–E).

The GAR Domain of 53BP1 Is Not Required for the Interaction between 53BP1 and MDC1—The MDC1 binding region of 53BP1 (53BP1: 1288–1409) lies upstream of the tTudor domain of 53BP1 and contains the GAR motif (Fig. 1C), which was shown to be methylated on arginine residues (11, 12). To determine whether the GAR motif is required for the 53BP1-MDC1 interaction we overexpressed an HA-tagged version of full-length 53BP1 in which the arginines in its GAR motif were replaced with alanines (12). We used an anti-HA antibody to IP the overexpressed proteins from whole cell extracts prepared from these cells. Similar amounts of MDC1 were co-immunoprecipitated with either wild-type or mutant 53BP1 (data not shown). We conclude that methylation of the GAR motif of 53BP1 is not necessary for the 53BP1-MDC1 interaction.

The tBRCT Domain of MDC1 (MDC1-tBRCT) Binds 53BP1:1288–1409—Subsequently, we determined which region in MDC1 mediates the interaction with 53BP1. A fragment of the C terminus of MDC1 that contains its tBRCT domain (residues 1698–2089) was previously demonstrated to bind 53BP1 (29). We used a fragment of this region, containing MDC1-tBRCT (residues 1883–2089) fused to glutathione S-transferases (GST, GST-MDC1-tBRCT) in GST pull-down assays. GST-MDC1-tBRCT was sufficient to retrieve endogenous 53BP1 or the MDC1 binding region of 53BP1 (HA-53BP1:1288–1409) from whole cell extracts (Fig. 2, A and B, respectively).

We next analyzed whether MDC1-tBRCT interacts with 53BP1 in cells. We prepared extracts from cells overexpressing MDC1-tBRCT fused to an HA tag (HA-MDC1-tBRCT). HA-MDC1-tBRCT was immunoprecipitated from the extract, using an anti-HA antibody. Endogenous 53BP1 was efficiently co-immunoprecipitated with HA-MDC1-tBRCT (Fig. 2C), demonstrating that the interaction between MDC1-tBRCT and 53BP1 occurs in vivo. IP of the FHA domain of MDC1 obtained only a very faint band of 53BP1 on Western blot (Fig. 2C). To verify that MDC1-tBRCT is the main binding region, we compared the ability of full-length MDC1 and MDC1 that lacks its tBRCT domain (MDC1-ΔtBRCT) to co-IP 53BP1. 53BP1 was co-immunoprecipitated with full-length MDC1, whereas it was only poorly co-immunoprecipitated with HA-MDC1-ΔtBRCT (Fig. 2D). The ability of HA-MDC1-ΔtBRCT to weakly co-IP 53BP1 from extracts may reflect additional weaker interactions involving other regions in MDC1, or indirect interactions, involving additional proteins that interact with both 53BP1 and MDC1. Altogether, these results reveal that MDC1-tBRCT is the main region in MDC1 that mediates the interaction with 53BP1.

53BP1 and MDC1 Interact Directly—Next we wanted to examine whether the 53BP1-MDC1 interaction is direct or mediated through additional proteins. Bacterially expressed MDC1-tBRCT and GST-53BP1:1052–1709 directly interacted (Fig. 3A). As expected, MDC1-tBRCT did not bind an irrelevant fragment of 53BP1 that we used as a negative control (GST-53BP1:667–1052 (Fig. 3A)). To further demonstrate direct interaction we performed a blot overlay assay using a recombinant GST-MDC1-ttBRCT as a probe and demonstrated that it specifically and directly bound HA-53BP1:1288–1409 on a membrane containing a mixture of cellular proteins extracted from cells overexpressing HA-53BP1:1288–1409 (Fig. 3B). As a negative control we overexpressed a C-terminal fragment of 53BP1 that does not co-IP MDC1 (HA-53BP1:1483–1972; Fig. 1C, construct 5). As expected, GST-MDC1-tBRCT did not bind this fragment (Fig. 3B).

The Interaction between 53BP1 and MDC1 Is Enhanced upon Phosphorylation—We demonstrated that MDC1-tBRCT, a phospho-binding module (3–5), mediates the binding with
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53BP1 (Figs. 2 and 3). We therefore analyzed whether the 53BP1-MDC1 interaction is modulated upon phosphorylation. We mutated one of the key residues of the phosphate-binding pocket of MDC1-tBRCT, lysine 1936, to methionine (K1936M, tBRCTm). This mutation is known to abolish the phospho-dependent interaction of MDC1-tBRCT with γ-H2AX (35) and with the anaphase-promoting complex/cyclosome (33). GST-MDC1-tBRCTm did not retrieve 53BP1 from protein extracts (Fig. 2A). Also, there was a reduction (~3-fold) in the ability of bacterially expressed MDC1-tBRCTm, to directly bind GST-53BP1:1052–1709 compared with MDC1-tBRCT (Fig. 3A). In addition, GST-MDC1-tBRCTm did not bind directly neither HA-53BP1:1052–1709 nor HA-53BP1:1483–1972 (Fig. 1C, constructs 6 and 5, respectively) in a blot overlay assay (Fig. 3B). Taken together, the phosphate-binding pocket of MDC1-tBRCT is required for an intact interaction between 53BP1 and MDC1. The weakly detected interaction between MDC1-tBRCTm and GST-53BP1:1052–1709 (Fig. 3A) probably occurs due to the fact that the experiment was done with excessive amounts of recombinant proteins.

We further analyzed how phosphorylation modulates the interaction. λ-phosphatase treatment of protein extracts, prepared from cells overexpressing HA-53BP1, reduced the ability of anti-MDC1 antibodies to co-IP HA-53BP1 (Fig. 4A, top). The ability of an anti-HA antibody to co-IP endogenous MDC1 from λ-phosphatase-treated extracts was also reduced (Fig. 4A, bottom). This effect was reversed when EDTA, which inhibits the phosphatase activity, was added (Fig. 4A). This effect was examined in three independent experiments, and the reduction in the ability of anti-MDC1 antibodies to co-IP HA-53BP1:1052–1709 from λ-phosphatase-treated extracts was found to be statistically significant (Fig. 4B). These experiments suggest that there is a basal binding between 53BP1 and MDC1 that does not require phosphorylation (Fig. 4B). In addition, in a GST pulldown assay, GST-MDC1-tBRCT retrieved HA-53BP1 less efficiently from whole cell extracts treated with λ-phosphatase compared with untreated extracts (Fig. 4C). We also observed a reduction in the ability of unphosphorylated 53BP1:1288–1409 to co-IP MDC1. This was done by the IP of HA-53BP1:1288–1409 from cell extracts using an anti-HA antibody, a treatment of the immunoprecipitated proteins with λ-phosphatase, addition of untreated cell extracts and analyzing the binding to MDC1 (Fig. 4D). A similar result was obtained by a blot overlay assay, using GST-MDC1-tBRCT as a probe, which showed that addition of λ-phosphatase to protein extracts from cells overexpressing HA-53BP1:1052–1709 resulted in a reduced signal (Fig. 4E).

As mentioned before, addition of phosphatase in all experiments did not completely abolish the interaction between 53BP1 and MDC1 but significantly reduced complex formation. This observation suggests that the 53BP1-MDC1 interaction is modulated by phosphorylation but does not rely solely on phospho-dependent interactions, which means that there is a basal binding level that is phospho-independent.

The Interaction between 53BP1 and MDC1 Is Reduced upon DNA DSB Induction—53BP1 and MDC1 play a crucial role in the cellular response to DNA DSBs (1). Thus we analyzed whether the 53BP1-MDC1 interaction is affected by induction of DNA DSBs. Notably, upon DNA DSB induction the interaction between MDC1 and 53BP1 was highly impaired (Fig. 5A). To further investigate this observation we examined the 53BP1-MDC1 interaction at different time points following IR induction. We overexpressed HA-53BP1 in cells and examined the ability of anti-MDC1 antibodies to co-IP HA-53BP1 from extracts prepared from untreated cells or from cells treated with 10 Gy of IR that were left to recover for various time points. There was a significant reduction in the ability of the anti-MDC1 antibodies to co-IP HA-53BP1 following induction of DNA DSBs (Fig. 5B, the same results were obtained with 3 Gy of IR, data not shown). The reduction in the binding appears rapidly following induction of DNA DSBs and lasts for at least 6 h.

FIGURE 3. The interaction between MDC1-tBRCT and 53BP1:1052–1709 is direct. A, recombinant MDC1-tBRCT directly binds recombinant GST-53BP1:1052–1709. GST pulldown assays were performed using recombinant proteins expressed in bacteria, GST-53BP1:1052–1709, or GST-53BP1:667–1052 and MDC1-tBRCT or MDC1-tBRCTm. After the reaction, the beads were treated with thrombin, and supernatant was separated and analyzed by Western blot using anti-MDC1-tBRCT antibodies. B, protein extracts made from 293T cells overexpressing GST-53BP1:1052–1709, GST-53BP1:667–1052, or from mock transfected cells were separated on SDS-PAGE. Blot overlay assay was performed with GST-MDC1-tBRCT or the mutant GST-MDC1-tBRCTm as probes. The probes were identified on a membrane with antibodies directed against MDC1-tBRCT (right panel). The bound proteins and antibodies were stripped off the membranes, and protein expression was examined by Western blot using anti-HA antibody (left panel).
change the ability of MDC1-tBRCT to retrieve 53BP1 from extracts as compared with control, in which no peptide was added (Fig. 5C).

The MDC1 Binding Region of 53BP1 Is Required for Focus Formation by 53BP1—Our results indicate that upon DNA DSB induction 53BP1 and MDC1 dissociate (Fig. 5, A and B). To test whether this dissociation occurs before or after the recruitment of the 53BP1-MDC1 complex to sites of damage, we analyzed the ability of different fragments of 53BP1 (HA-tagged) to form foci following DNA DSB induction. As described previously (7, 8, 37), full-length HA-53BP1 and HA-53BP1:1052–1709 (Fig. 1C, constructs 1 and 6) formed clear foci that co-localized with γ-H2AX foci following DNA DSB induction (Fig. 6A). The region containing the tTudor domain of 53BP1 alone (HA-53BP1:1480–1616, Fig. 1C, construct 9) was more diffused in the nucleus but could still be detected at foci (Fig. 6A). However, we could not detect foci when we analyzed a fragment of 53BP1 containing only the MDC1 binding region of 53BP1 (HA-53BP1:1288–1409, see Figs. 1C–E, construct 10) and 6A). Using a full-length HA-53BP1 that lacks only the binding region for MDC1, we detected a focus formation pattern similar to that of the tTudor domain alone (i.e. mostly diffused in the nucleus but still partly localized to foci (Fig. 6A)). Therefore, our results imply that the MDC1 binding region of 53BP1 is not sufficient for clear focus formation by 53BP1 and that the MDC1 binding region of 53BP1 is required

We thus conclude that the 53BP1-MDC1 interaction occurs in untreated cells and is rapidly reduced after induction of DNA DSBs.

53BP1 and γ-H2AX Bind the Same Region in MDC1-tBRCT—Because γ-H2AX also binds directly to MDC1-tBRCT (35, 36) we aimed to study the interplay between the three players of the DDR, MDC1, 53BP1, and γ-H2AX. We analyzed whether the same region in MDC1-tBRCT binds 53BP1 and γ-H2AX using a competition experiment. We performed a GST pulldown assay and showed that a phospho-peptide that corresponds to the C terminus of γ-H2AX competed with the interaction between endogenous 53BP1 and MDC1-tBRCT (Fig. 5C). As expected, the unphosphorylated derivate of that peptide did not for the complete recruitment of 53BP1 to foci.

Impaired Localization of 53BP1 to IR-induced Foci in Cells Down-regulated for MDC1 Expression—To determine whether MDC1 is required for both 53BP1 recruitment and maintenance at IR-induced foci or only for the recruitment of the protein to foci, we analyzed the percentage of γ-H2AX foci that contained 53BP1 in cells down-regulated for MDC1 expression (shRNA-MDC1), via immunofluorescence. There was a reduction in the localization of 53BP1 at DNA DSB-induced foci in shRNA-MDC1 cells compared with the control (shRNA-LacZ) cells (indicated from the reduction in 53BP1 at foci and analyzed by the co-localization between 53BP1 and γ-H2AX (Fig. 6B)). This reduction in the localization at foci in shRNA-MDC1 cells was

![Figure 4](image-url)
Direct Interaction between 53BP1 and MDC1

The Interaction between 53BP1 and MDC1 Is Enhanced in Mitotic Cells—53BP1 and MDC1 are hyperphosphorylated in mitotic arrested cells (29, 30, 17), suggesting that they may have a role in the regulation of mitosis. We therefore analyzed whether the interaction between 53BP1 and MDC1 is modulated in cells arrested in mitosis. We overexpressed HA-53BP1 in cells, and 24 h after transfection the cells were incubated with nocodazole for 16 h (∼87% mitotic cells, as determined by fluorescence-activated cell sorting analysis, data not shown). We immunoprecipitated HA-53BP1 from an extract prepared from these cells and detected a remarkable increase in the ability of MDC1 to co-IP MDC1 from the mitotic cells extract compared with untreated cells (Fig. 7A). We also detected an enhancement in the 53BP1-MDC1 interaction when we did the reciprocal experiment, using anti-MDC1 antibodies for the IP reaction and anti-53BP1 antibodies for the Western blot analysis (data not shown). This result reveals that there is an enhancement in the 53BP1-MDC1 interaction during mitosis. The effect is emphasized when comparing it to the reduction in the interaction observed following DNA DSB induction (Fig. 7A).

The MDC1 Binding Region of 53BP1 Is Phosphorylated in Vivo on Serine 1362—The minimal region that we found to bind MDC1 (53BP1:1288–1409 (Fig. 1C, construct 10)), is highly conserved among 53BP1 orthologs (Fig. 7B) and contains a high proportion of serine and threonine residues (33/122 (Fig. 7B)) that can be targets for phosphorylation events. Among the serines and threonines there are two conserved putative phosphorylation sites for the DNA damage transducer kinases (serine or threonine followed by glutamine (S/T-Q) (Fig. 7B)), and four putative phosphorylation sites for cell
sites are phosphorylated during mitosis. This result is in agreement with the fact that residue 1372 (Thr-1372) of an S/T-P site is not phosphorylated in vivo on a putative cell cycle related phosphorylation site (Fig. 7B) and that 53BP1-MDC1 interaction is enhanced in cells arrested at mitosis (Fig. 7A), we analyzed whether kinases involved in cell cycle regulation have a role in this enhancement. Mutating the four S/T-P sites (SP1288, SP1362, TP1372, and TP1393; 4A mutant), which are putative phosphorylation sites for different cell cycle-regulated kinases, in HA-53BP1:1052–1709 (Fig. 7B) reduced its ability to bind MDC1 in mitotic cells (Fig. 7C). When we mutated each one of the S/T-P sites alone, we did not detect such a reduction (data not shown). Therefore we suggest that more than one S/T-P site is phosphorylated or that the different S/T-P sites may function in redundancy with one another. When we analyzed the ability of this mutant to form IR-induced foci, as expected, we did not detect any difference from the wild-type protein (Fig. 6A). In addition, we did not observe any change in the interaction when we mutated the putative DNA damage transducer kinases phosphorylation sites (S/T-Q) in HA-53BP1:1052–1709 (data not shown). Taken together, these results imply that cycle-related kinases (serine or threonine followed by proline (S/T-P) (Fig. 7B)), of which three are conserved in 53BP1 orthologs, as well as additional putative phosphorylation sites for other kinases (Fig. 7B).

Because the 53BP1-MDC1 interaction is modulated by phosphorylation (Fig. 4) and we observed an enhancement of the interaction in mitotic arrested cells, we analyzed in vivo phosphorylation of the MDC1 binding region of 53BP1 in mitotic extracts by tandem mass spectrometry (MS/MS) analysis. The analysis revealed two relevant peptides (Fig. 7B). The first, a phospho-peptide GGPGKLsPR (phosphorylated Ser-1362 on an S/T-P site). The second peptide, containing the threonine residue 1372 (Thr-1372) of an S/T-P site is not phosphorylated during mitosis. This result is in agreement with the fact that Thr-1372 is not conserved among 53BP1 orthologs (Fig. 7B).

There are two additional S/T-P sites in the MDC1 binding region of 53BP1 that were not retrieved from the first round of the MS analysis. Further studies will determine whether these sites are phosphorylated in vivo.

**FIGURE 7.** The 53BP1-MDC1 interaction is enhanced in cells arrested at mitosis and requires the phosphorylation of S/T-P site/s. A, the 53BP1-MDC1 interaction is augmented in mitotic cells. 293T cells overexpressing HA-53BP1 were untreated, treated with 3 Gy of IR, and left to recover for 1 h or treated with nocodazole for 16 h. Co-IP experiments were performed using an anti-HA antibody, and proteins were separated and blotted using anti-MDC1 antibodies. B, serine 1362 of 53BP1 is phosphorylated in vivo. Alignments of the minimal binding region of 53BP1 in the orthologs of 53BP1 were obtained using the HomoloGene tool of NCBI. In boldface are conserved serine and threonine residues. In gray are the putative consensus phosphorylation sites for the DNA damage transducer kinases (S/T-Q) and for cell cycle-regulated kinases (S/T-P). The two relevant peptides obtained in the MS/MS analysis is done on HA-53BP1:1288–1409 immunoprecipitated from mitotic arrested cells are indicated. Serine 1362 is marked with an asterisk. C, the putative phosphorylation sites for cell cycle-regulated kinases module 53BP1-MDC1 interaction. HA-53BP1:1052–1709 (WT) or a mutant HA-53BP1:1052–1709, which contains four substitutions of S/T-P residues (S/T to alanine; 4A m), were overexpressed in 293T cells. The cells were treated with nocodazole for 16 h, and extracts were prepared. HA-tagged proteins or MDC1 were immunoprecipitated using anti-HA or anti-MDC1 antibodies, respectively. Proteins were separated and analyzed by Western blots using antibodies directed against MDC1 or HA.

**DISCUSSION**

In our study, a direct interaction between 53BP1 and MDC1 is demonstrated and analyzed. The analysis of the interaction sheds light on novel biological roles for MDC1 and 53BP1. We found that the interaction between these proteins is dynamic and changes during the DDR and in mitosis. The results presented here show that the 53BP1-MDC1 interaction per se is required for the recruitment of 53BP1 to sites of DNA DSBs and suggest a role for the interaction during the mitotic spindle checkpoint. Moreover, we have identified a serine residue in 53BP1 that is in vivo phosphorylated in mitotic cells.

**MDC1-tBRCT Mediates Different Types of Interactions**

MDC1-tBRCT binds directly to various proteins via distinct modes of binding, and the interplay between these interactions probably provides a fine-tuned mechanism for regulation of the different signals mediated by MDC1. Previous work identified
The Role of the Interaction between 53BP1 and MDC1 in Recruitment of 53BP1 to DNA DSB-induced Foci—Our results demonstrate that MDC1 directly binds 53BP1 (Fig. 3) and that the interaction is dynamic. It occurs in untreated cells, but is significantly enhanced in cells arrested in mitosis, and is rapidly reduced after DNA DSB induction (Figs. 5A, 5B, and 7A). Previous studies demonstrated that MDC1 is recruited to IR-induced foci prior to 53BP1 and that MDC1 is required for focus formation by 53BP1 (20, 26, 27, 32). In addition, it was demonstrated that the tTudor domain of 53BP1 is sufficient for focus formation by 53BP1 (7, 8). Several observations imply that the MDC1 binding region of 53BP1 is necessary for the recruitment of 53BP1 to foci. 53BP1:1052–1709 (Fig. 1, construct 6) forms foci as the wild-type protein, whereas the tTudor domain alone is not sufficient for the full recruitment of the protein to foci (Fig. 6A). When we deleted the MDC1 binding region in full-length 53BP1, focus formation was highly impaired. In addition, we could not detect the fragment of 53BP1 that contains only the MDC1 binding region at foci (Fig. 6A). We also detected a reduction in the interaction between 53BP1 and MDC1 following damage induction (Figs. 5A, 5B, and 7A), and we demonstrated that MDC1 is required for focus formation by 53BP1 (Fig. 6B). Therefore, we propose that 53BP1 is recruited to sites of DNA DSBs due to its binding to MDC1. As the proteins arrive to the breaks they dissociate, and because the MDC1 binding region of 53BP1 no longer binds MDC1, it relocalizes away from the breaks and thus does not form foci. The partial localization of the tTudor domain of 53BP1 alone at foci may be attributed to the ability of this region to bind methylated histones (7, 10), which are more exposed due to chromatin changes that occur following damage induction. Strengthening our assumption that the direct interaction between 53BP1 and MDC1 is required for the recruitment of 53BP1 to foci are the results we obtained, demonstrating that γ-H2AX and 53BP1 compete for the binding to MDC1-tBRCT (Fig. 5C) and that MDC1 is required for focus formation by 53BP1 from early time points after DNA DSB induction (Fig. 6B). This suggests that, upon arriving to DNA DSB sites, 53BP1 and MDC1 dissociate, allowing MDC1 to bind γ-H2AX (35) and probably additional proteins. It also enables 53BP1 to bind methylated lysine 79 of histone H3 (7) and dimethylated lysine 20 of histone H4 (10), and, presumably, other DDR-related proteins (Fig. 8).

Events in the Recruitment of 53BP1 and MDC1 to Sites of DNA DSBs—Our results imply that only a fraction of 53BP1 and MDC1 bind each other in untreated cells (basal binding), because we observed a large enhancement in the interaction in mitotic arrested cells (Fig. 7A). It was previously demonstrated that MDC1 is recruited to sites of damage before 53BP1 (31). We therefore suggest (Fig. 8) that, as DNA DSBs are induced in the cell, the fraction of MDC1 that does not bind 53BP1, rapidly localizes to foci, and associates with γ-H2AX (35, 36). Early chromatin changes are made as MDC1 and additional members of the DDR accumulate at sites of damage. These chromatin changes, revealing methylated residues on histones H3 and H4, may be attributed to RNF8, an E3 ubiquitin ligase, which is recruited by MDC1 and ubiquitinates histone H2A and H2AX (26–28). Subsequently, the fraction of MDC1 that is bound to 53BP1 is also recruited to sites of damage. When the two pro-
tein interaction with MDC1 is enhanced in mitotic arrested cells and is modulated by cell cycle-regulated kinases, implying a yet unknown role for these proteins during mitosis or in the mitotic spindle assembly checkpoint.

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REFERENCES

1. Motoyama, N., and Naka, K. (2004) Curr. Opin. Genet. Dev. 14, 11–16
2. Derbyshire, D. J., Basu, B. P., Serpell, L. C., Joo, W. S., Date, T., Iwabuchi, K., and Doherty, A. J. (2002) EMBO J. 21, 3863–3872
3. Manke, I. A., Lowery, D. M., Nguyen, A., and Yaffe, M. B. (2003) Science 302, 636–639
4. Yu, X., Chini, C. C., He, M., Mer, G., and Chen, J. (2003) Science 302, 639–642
5. Rodriguez, M., Yu, X., Chen, J., and Songyang, Z. (2003) J. Biol. Chem. 278, 52914–52918
6. Iwabuchi, K., Bartel, P. L., Li, B., Marraccino, R., and Fields, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6098–6102
7. Huyen, Y., Zgheib, O., Ditullio, R. A., Jr., Gorgoulis, V. G., Zacharatos, P., Petty, T. J., Sheston, E. A., Mellert, H. S., Stavridi, E. S., and Halazonetis, T. D. (2004) Nature 422, 406–411
8. Iwabuchi, K., Basu, B. P., Kysela, B., Kurihara, T., Shibata, M., Guan, D., Cao, Y., Hamada, T., Imamura, K., Jeggo, P. A., Date, T., and Doherty, A. J. (2003) J. Biol. Chem. 278, 36487–36495
9. Charrier, G., Couprie, J., Alpha-Bazin, B., Meyer, V., Quemeneur, E., Guer- ois, R., Callebaut, I., Gilquin, B., and Zinn-Justin, S. (2004) Structure (Comb.) 12, 1551–1562
10. Botuyan, M. V., Lee, J., Ward, I. M., Kim, E. J., Thompson, J. R., Chen, J., and Mer, G. (2006) Cell 127, 1361–1373
11. Adams, M. M., Wang, B., Xia, Z., Morales, J. C., Lu, X., Donehower, L. A., Bochar, D. A., Elledge, S. J., and Carpenter, P. B. (2005) Cell Cycle 4, 1854–1861
12. Boisvert, F. M., Rhee, A., Richard, S., and Doherty, A. J. (2005) Cell Cycle 4, 1834–1841
13. Wang, B., Matsuoka, S., Carpenter, P. B., and Elledge, S. J. (2002) Science 298, 1435–1438
14. DiTullio, R. A., Jr., Mochan, T. A., Venere, M., Bartkova, J., Sehested, M., Bartek, J., and Halazonetis, T. D. (2002) Nat. Cell Biol. 4, 998–1002
15. Adams, M. M., and Carpenter, P. B. (2006) Cell Div. 1, 19
16. Xu, A., Hartloderode, A., Stucki, M., Odate, S., Puget, N., Kwok, A., Nagaruja, G., Yan, C., Alt, F. W., Chen, J., Jackson, S. P., and Scully, R. (2007) Mol. Cell 28, 1045–1057
17. Jullien, D., Vagnarelli, P., Earnshaw, W. C., and Adachi, Y. (2002) J. Cell Sci. 115, 71–79
18. Goldberg, M., Stucki, M., Falck, J., D’Amours, D., Rahman, D., Pappin, D., Bartek, J., and Jackson, S. P. (2003) Nature 421, 952–956
19. Lou, Z., Minter-Dykhouse, K., Wu, X., and Chen, J. (2003) Nature 421, 957–961
20. Stewart, G. S., Wang, B., Bignell, C. R., Taylor, A. M., and Elledge, S. J. (2003) Nature 421, 961–966
21. Stucki, M., and Jackson, S. P. (2004) DNA Repair (Amst.) 3, 953–957
22. Durocher, D., and Jackson, S. P. (2002) FEBS Lett. 513, 58–66
23. Lou, Z., Chen, B. P., Asaithamb, A., Minter-Dykhouse, K., Chen, D. J., and Chen, J. (2004) J. Biol. Chem. 279, 46359–46362
24. Lukas, C., Melander, F., Stucki, M., Falck, J., Bekker-Jensen, S., Goldberg, M., Lerenthal, Y., Jackson, S. P., Bartek, J., and Lukas, J. (2004) EMBO J. 23, 2674–2683
25. Zhang, J., Ma, Z., Treszezamsky, A., and Powell, S. N. (2005) Nat. Struct. Mol. Biol. 12, 902–909
26. Huen, M. S., Grant, R., Manke, I., Minn, K., Yu, X., Yaffe, M. B., and Chen, J. (2007) Cell 131, 901–914
27. Kolas, N. K., Chapman, J. R., Nakada, S., Ylanke, J., Chahwan, R., Sweeney, F. D., Panier, S., Mendez, M., Wildenhain, J., Thompson, T. M., Pelletier, L., Jackson, S. P., and Durocher, D. (2007) Science 316, 1637–1640
28. Mailand, N., Bekker-Jensen, S., Faustrop, H., Melander, F., Bartek, J., Lukas, C., and Lukas, J. (2007) Cell 131, 887–900
29. Xu, X., and Stern, D. F. (2003) FASEB J. 17, 1842–1848
30. Xu, X., and Stern, D. F. (2003) J. Biol. Chem. 278, 8875–8883
31. Bekker-Jensen, S., Lukas, C., Melander, F., Bartek, J., and Lukas, J. (2005) J. Cell Biol. 170, 201–211
32. Minter-Dykhouse, K., Ward, I., Huen, M. S., Chen, J., and Lou, Z. (2008) J. Cell Biol. 181, 727–735
33. Coster, G., Hayouka, Z., Argaman, L., Strauss, C., Friedler, A., Brandeis, M., and Goldberg, M. (2007) J. Biol. Chem. 282, 32053–32064
34. Li, J., Williams, B. L., Haire, L. F., Goldberg, M., Wilker, E., Durocher, D., Yaffe, M. B., Jackson, S. P., and Smerdon, S. J. (2002) Mol. Cell 9, 1045–1054
35. Stucki, M., Clapperton, J. A., Mohammad, D., Yaffe, M. B., Smerdon, S. J., and Jackson, S. P. (2005) Cell 123, 1213–1226
36. Lee, M. S., Edwards, R. A., Thede, G. L., and Glover, J. N. (2005) J. Biol. Chem. 280, 32053–32056
37. Pryde, F., Khalili, S., Robertson, K., Selfridge, J., Ritchie, A. M., Melton, D. W., Jullien, D., and Adachi, Y. (2005) J. Cell Sci. 118, 2043–2055