Mediator of DNA damage checkpoint 1 (MDC1) plays an important role in the DNA damage response (DDR). MDC1 functions as a mediator protein and binds multiple proteins involved in different aspects of the DDR. However, little is known about the organization of MDC1 complexes. Here we show that ataxia telangiectasia, mutated (ATM) phosphorylates MDC1 at Thr-98 following DNA damage, which promotes its oligomerization. Oligomerization of MDC1 is important for the accumulation of MDC1 complex at the sites of DNA damage. Mutation of Thr-98 (T98A) would abolish its oligomerization and result in a defect in DNA damage checkpoint activation and increased sensitivity to irradiation. Taken together, these results suggest that the oligomerization of MDC1 plays an important role in DDR and help understand the formation of proteins complexes at the sites of DNA damage.

In eukaryotic cells, DNA double strand breaks activate the DNA damage response (DDR), which is a complex signaling network that regulates cell cycle progression, transcription, DNA repair, and cell survival (1). A number of factors involved in DDR are also recruited to the double strand breaks to help DNA repair. Mediator of DNA damage checkpoint protein 1 (MDC1) is emerging as an important nodule that helps recruit DDR factors and act as docking sites for NBS1 and RNF8, respectively (18–21). The SDT and TQXF motifs become phosphorylated and act as docking sites for NBS1 and RNF8, respectively (18–24), whereas the PST repeats could interact with DNA-PK (25).

These results suggest that oligomerization of MDC1 at double strand breaks is important for the assembly of DDR factors and DNA damage response.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MDC1<sup>+/+</sup> and MDC1<sup>−/−</sup> mouse embryonic fibroblasts (MEFS) were grown in DMEM (Invitrogen) supplemented with 15% fetal bovine serum (Invitrogen). U2OS cells were grown in McCoy's 5A (ATCC) supplemented with 10% fetal bovine serum (Invitrogen). HEK293T cells were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). MDC1<sup>−/−</sup> reconstituted with WT MDC1 or T98A were grown in standard MDC1<sup>−/−</sup> MEFS medium supplemented with 200 μg ml<sup>−1</sup> of hygromycin (Invitrogen). Cells infected with MDC1 shRNA virus were grown in standard medium supplemented with 200 μg ml<sup>−1</sup> of puromycin (Invitrogen).

**Plasmids, shRNA, and siRNA**—MDC1 was cloned into pKTo2c-linker-hygR (a gift from Karl J. Clark (Mayo Clinic)), and FLAG or HA was inserted in the N terminus of MDC1. The sequences encoding corresponding regions of MDC1 were cloned into pGEX-4T-1 (GE Healthcare). Deletion and mutations were generated by site-directed mutagenesis (Stratagene).

pKTo.1-based shRNA targeting MDC1 was purchased from Sigma. MDC1 siRNAs were synthesized as described before (10). (The coding strand of MDC1 siRNA1 was UCCAGUGAUCUCUGAGGUGdTdT, and the coding strand of MDC1 siRNA2 was ACAACUGCAGAGAUUGAAdTdT.)
Antibodies—The rabbit anit-MDC1 antibody was generated as described previously (10). Prebleeds from rabbits were used as controls in immunoprecipitation experiments. Anti-phospho-Thr-98 MDC1 antibody was generated in rabbit using phosphorylated peptides (CGSLNG(p)TQLRPP).

The following antibodies were used in Western blotting: FLAG (Sigma, 1:1000), β-actin (Sigma, 1:2000), HA (Covance, 1:2000), 53BP1 (generated against the C terminus of 53BP1, 1:1000), pSMC1 (Genescript, 1:1000), SMC1 (Epitomics, 1:1000), pS345 CHK1 (Cell Signaling, 1:1000), Chk1 (Santa Cruz Biotechnology, 1:500), Ku80 (Santa Cruz Biotechnology, 1:1000), BRCA1 (Santa Cruz Biotechnology, 1:500), GST (Santa Cruz Biotechnology, 1:1000), pATM (Rockland, 1:1000), and ATM (GeneTex, 1:1000). The following antibodies were used in immunofluorescence: MDC1 (Millipore, 1:1000), FLAG (Sigma, 1:1000), pS345 Chk1 (Cell Signaling, 1:1000), Chk1 (Santa Cruz Biotechnology, 1:500), BRCA1 (Santa Cruz Biotechnology, 1:1000), pATM (Rockland, 1:1000), and ATM (GeneTex, 1:1000). The following antibodies were used in immunoblotting and immunostaining: MDC1 (Millipore, 1:1000), FLAG (Sigma, 1:1000), pS345 CHK1 (Cell Signaling, 1:1000), Chk1 (Santa Cruz Biotechnology, 1:500), Ku80 (Santa Cruz Biotechnology, 1:1000), BRCA1 (Santa Cruz Biotechnology, 1:500), GST (Santa Cruz Biotechnology, 1:1000), pATM (Rockland, 1:1000), and ATM (GeneTex, 1:1000).

In Vitro Peptide Pulldown Assay—Biotinylated non-phospho- or phosphopeptides were synthesized by the Mayo Proteomics Core Facility. The peptides were coupled to streptavidin-Sepharose and incubated with GST-MDC1 fragments or cell lysates. Proteins retained on the Sepharose were eluted with biotin (2 mM) and subjected to SDS-PAGE and immunoblotting with anti-GST-antibodies or indicated antibodies.

Phospho-H3 Staining—Cells were γ-irradiated (1.5 Gy) or left untreated, then stained with anti-phospho-H3 antibodies, and phospho-H3-positive cells were evaluated by counting.

DNA Synthesis Assay—Cells were irradiated at the indicated dose (0, 10, or 20 Gy) or left untreated. 1 h later, cells were incubated with BrdU and collected after additional 1 h. The BrdU incorporation into DNA was detected by immunostaining and analyzed under a microscope.

In Vitro Kinase Assay—GST fusion proteins covering different regions of MDC1 were used as substrates. Cells were harvested after irradiation, and lysates were subjected to immunoprecipitation using anti-ATM antibody (GeneTex). The ATM immunoprecipitates and substrate were incubated in kinase buffer (25 mM Tris-HCl (7.5 pH), 5 mM β-glycerophosphate, 2 mM DTT, 0.5 mM Na2VO4, 10 mM MgCl2, 0.2 mg ml−1 BSA, 10 μM ATP, and 5 μCi of γ-32P label) for 30 min at 30 °C. Reactions were stopped by addition of SDS sample buffer. Samples were separated by SDS-PAGE, and incorporation of 32P was examined by autoradiography. The gel was stained with Coomassie Blue to show the amounts of proteins were included in these reactions.

Colony Formation Assay—MEFs were exposed to ionizing radiation and left for 10–14 days at 37 °C to allow colony formation. Colonies were stained with 5% GIEMSA and counted. Results were normalized to plating efficiencies.

RESULTS

ATM Phosphorylates MDC1 on Thr-98 following DNA Damage—ATM transduces its signals through its kinase activity. In addition to phosphorylation and activation of the downstream kinases Chk1 and Chk2, ATM also phosphorylates proteins with no kinase activity, such as BRCA1 and NBS1 (26–31). Phosphorylation of BRCA1 and NBS1 by ATM are important for DNA damage-induced checkpoint activation (27–31). We and others have previously shown that MDC1 is phosphorylated in response to DNA damage, in an ATM-dependent manner (7–10). Several phosphorylation sites on MDC1 by ATM have been reported, such as Thr-752 and Thr-719 (21–23), which are important for the recruitment of RNF8. To map other sites on MDC1 that can be phosphorylated by ATM, we expressed MDC1 fragments in vitro and performed in vitro kinase assay using ATM purified from cells. We found that, besides the region (481–823) containing the TQXF motifs, the N terminus of MDC1 was strongly phosphorylated (Fig. 1A), suggesting that the N terminus of MDC1 contains ATM phosphorylation sites.

ATM preferentially phosphorylates the Ser/Thr-Gln (S/TQ) motif. Examination of the sequence of N-terminal MDC1 reveals two candidate sites, Thr-4 as well as Thr-98 within the FHA domain. Mutagenesis analysis further showed that mutation at Thr-98 (Fig. 1B), but not Thr-4 (data not shown), significantly decreased MDC1 phosphorylation in vitro by ATM. Thr-98 is conserved across species and could be found in the corresponding position of MDC1 in many species, including chimpanzees, monkey, pig, mouse, and rat, implying that this site could have important functions.

To confirm that Thr-98 of MDC1 is phosphorylated in vivo, we generated specific antibodies against phosphorylated Thr-98 (pT98). As shown in Fig. 1C, anti-pT98MDC1 antibodies recognized MDC1 from irradiated cells, but not from un-irradiated cells. When MDC1 from irradiated cells was treated with λ-phosphatase, it was no longer recognized by anti-pT98 antibodies. These results suggest that Thr-98 of MDC1 is phosphorylated in vivo following DNA damage.
To confirm the phosphorylation of MDC1 Thr-98 is ATM-dependent in vivo, we examined Thr-98 phosphorylation in ATM-proficient (C3ABR) and ATM-deficient (L3) cells. As shown in Fig. 1D, Thr-98 phosphorylation was defective in ATM-deficient cells. Furthermore, pre-treatment of cells with the ATM inhibitor KU55933 also inhibited Thr-98 phosphorylation (Fig. 1E). These results confirm that MDC1 Thr-98 phosphorylation following IR is ATM-dependent.

Phosphorylation on MDC1 Thr-98 by ATM following DNA Damage Is Essential for Its Oligomerization—Because MDC1 mainly acts as an adaptor during the DDR, we tried to examine whether the phosphorylation on Thr-98 contributes to interactions between MDC1 and other proteins involved in DDR.

We conjugated the phospho- or nonphospho-Thr-98 peptide of MDC1 to streptavidin-Sepharose and analyzed the interaction with DDR factors by pulldown assay. Surprisingly, we found that the phospho-Thr-98, not nonphospho-Thr-98 peptide, could interact with MDC1 itself (Fig. 2A). This result suggests that MDC1 phosphorylation at Thr-98 might promote MDC1 homo-oligomerization. To confirm this, we performed a co-immunoprecipitation assay and found that MDC1 indeed became oligomerized following DNA damage in both 293T cells and MEFs (Fig. 2, B and C). We also performed FPLC to analyze MDC1 complex purified from cell lysates before and after DNA damage. As shown in supplemental Fig. S1A, MDC1 formed higher molecular weight complex following DNA damage suggesting a dimer formation. However, the experiment procedure we performed before FPLC analysis might disrupt the complex. The chromatin environment in vivo might be optimal for the MDC1 complex. Therefore, MDC1 might form oligomers with more than two MDC1 molecules in vivo.

We then examined whether the phosphorylation on Thr-98 is required for the oligomerization of MDC1. As shown in Fig. 2D, the T98A mutation significantly disrupted the oligomerization of MDC1. These results suggest that the phosphorylation on Thr-98 of MDC1 is essential for the oligomerization of MDC1.

**FHA Domain Is Required for the Oligomerization of MDC1—** We next examined how phosphorylation of Thr-98 promotes MDC1 oligomerization. MDC1 contains the FHA and BRCT domains that can bind phosphorylated Ser/Thr. We examined whether the FHA or BRCT domain of MDC1 could bind phosphorylated Thr-98. We found that phospho-Thr-98 peptide of MDC1 could directly interact with the FHA domain, but not the BRCT domain of MDC1 in vitro (Fig. 3A). The FHA domain preferentially binds to pTXA(I/L) (32), which fits perfectly with sequence following Thr-98. This led us to hypothesize that the FHA domain of MDC1 binds pT98 and contributes to MDC1 oligomerization. To confirm this, we expressed GST-FHA in vitro and performed a GST-pulldown assay. GST-FHA coupled to Sepharose could pull down WT MDC1 from cell lysates in a DNA damage-inducible manner but could not pull down the T98A mutant (Fig. 3B), supporting that the pT98-FHA interaction mediates MDC1 oligomerization. Furthermore, a series of HA-tagged MDC1 internal-deletion mutants was coexpressed with FLAG-tagged full-length MDC1 in HEK 293T cells to investigate MDC1 oligomerization following DNA damage in cells. The oligomerization was dramatically decreased in cells expressing the mutant with deletion of the FHA domain (Fig. 3C). These results suggest that the interaction between FHA and pT98 is both required and sufficient for the oligomerization of MDC1 following DNA damage.

**Disruption of MDC1 Oligomerization Compromises the Intra-S Phase Checkpoint and G2/M Checkpoint after DNA Damage—** MDC1 regulates DNA damage checkpoint activation following DNA damage. One checkpoint function of...
MDC1 is the intra-S-phase checkpoint (7–9), which inhibits DNA replication following DNA damage. To evaluate the functional significance of MDC1 oligomerization, we tested whether the phosphorylation of Thr-98 is required for the intra-S-phase checkpoint activation. When the intra-S-phase checkpoint is activated, DNA synthesis will be suppressed. This could be revealed by a decrease in BrdU incorporation, which acts as a marker for DNA synthesis. When the intra-S-phase checkpoint is impaired however, DNA synthesis would not be significantly suppressed. As shown in Fig. 4A, mutation at Thr-98 resulted in a partial defect in the S-phase checkpoint. These results suggest that phosphorylation at Thr-98 is involved in the activation of intra-S-phase checkpoint.

In addition to regulating the S-phase checkpoint, MDC1 regulates the G_{2}/M checkpoint, which, when activated, arrests cells at G_{2} phase and prevents cells from entering M phase,
resulting in a decrease in the mitotic population (mitotic index).

We also examined whether mutation at Thr-98 affects the G2/M checkpoint. As shown in Fig. 4B, cells expressing MDC1 T98A also had a partial defect in the G2/M checkpoint following DNA damage.

Furthermore, we found that T98A mutation affected cellular sensitivity to DNA damage. As shown in Fig. 4C, MEFs stably expressing T98A were more sensitive to irradiation than those expressing wild-type MDC1.

One caveat of the findings above is that Thr-98 localized in the FHA domain and T98A mutation might affect the overall conformation of the FHA domain. To exclude this possibility, we examined the MDC1-Chk2 interaction, which has been shown to be dependent on the FHA domain of MDC1 (10). As shown in Fig. 4D, both WT MDC1 and MDC1T98A could interact with Chk2 following DNA damage, suggesting the FHA domain structure is intact in MDC1T98A. Overall, these results suggest that MDC1 oligomerization plays an important role in DNA damage checkpoint activation and cellular sensitivity to DNA damage.

Phosphorylation on Thr-98 Is Important in the DNA Damage Response Pathway Signaling—Next, we examined how MDC1 oligomerization affects its function. When we examined the focus formation of MDC1T98A following DNA damage, we found that the MDC1T98A could still form foci (Fig. 5A); however, both the number and intensity of foci were decreased compared with those of WT MDC1. Although the decreased accumulation of MDC1 T98A could be a defect in initial recruitment, we thought this is unlikely the case, as the initial recruitment of MDC1 requires the interaction between the MDC1 BRCT domain and γH2AX and is independent of the FHA domain of MDC1 (5, 6, 33). As shown in Fig. 5B, the focus formation of MDC1 and 53BP1 in the early time point (30 s) was similar in cells expressing MDC1 WT and T98A mutant, however WT MDC1 foci became brighter and bigger, while MDC1T98A foci did not at the later time points. These results suggest that MDC1 oligomerization could affect the sustained accumulation of MDC1 and other DDR factors at the sites of DNA damage. We confirmed our findings in a chromatin retention assay. As shown in Fig. 5C, the accumulation of MDC1T98A on chromatin was weaker than that of WT MDC1. Similarly, 53BP1 and BRCA1 chromatin binding...
FIGURE 5. Disruption of MDC1 oligomerization comprises the signaling transduction in DDR. A, MDC1−/− MEFs reconstituted with FLAG-WT MDC1 or T98A MDC1 were left untreated or treated with IR (2 Gy). The focus formation of MDC1, 53BP1, and BRCA1 were then examined by immunofluorescence. Left panel: typical figures of different foci in cells expressing WT or T98A MDC1. Right panel: quantification of percentage of cells with different kinds of foci as indicated. **, \( p < 0.01; \), \( p < 0.05 \). Error bars represent ± S.E. of three independent experiments. B, cells were treated as in A. The focus formation of MDC1 and 53BP1 was examined at the indicated time points. C, cells from Fig. 5A were left untreated or treated with IR and then subjected to nuclear fractionation. The chromatin retention, non-chromatin, and whole cell lysates were analyzed by Western blot using the indicated antibodies. D, cells were treated as in A. The focus formation of 53BP1 was examined at the indicated time points. Both strong and weak foci were counted. **, \( p < 0.01 \). Error bars represent ± S.E. of three independent experiments. E, U2OS cells infected with MDC1 shRNA virus were transfected wild-type or T98A MDC1 constructs. 48 h later, cells were irradiated. 1 h later, cells were harvested and subjected to immunoblotting with indicated antibodies. Blots were quantified using ImageJ.
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were also weaker in cells expressing MDC1T98A. These results suggest that MDC1 oligomerization is important for the effective accumulation of DDR factors at the sites of DNA damage.

The accumulation of DDR factors at DNA damage sites gradually decreases with time as DNA damage gets repaired. To test DNA repair capability of cells expressing MDC1T98A, we examined the persistence of 53BP1 foci (Fig. 5D). Although the accumulation of 53BP1 (both strong and weak foci) in cells expressing MDC1 T98A is lower compared with MDC1 WT at 1 h after IR, the 53BP1 foci was more persistent in T98A cells in the later time points, indicating more unrepaired DNA damage remained. This suggests that the MDC1 oligomerization would affect the efficiency of DNA repair.

We have shown previously that MDC1 facilitates the amplification of ATM signaling following DNA damage. Our results in Fig. 4 also showed that disruption of MDC1 oligomerization comprises the S-phase and G2/M checkpoint activation following DNA damage. Therefore, we hypothesized that disruption of the oligomerization of MDC1 affects ATM signaling. As shown in Fig. 5E, phosphorylation of Chk1 and SMC1 were decreased in cells expressing MDC1 T98A, indicating a partial defect in the ATM signaling when MDC1 fails to oligomerize. We did not observe significant defect in ATM phosphorylation in cells expressing MDC1 T98A (data not shown). These data suggested that disruption of MDC1 oligomerization partially affects its recruitment to the DNA damage sites as well as the downstream signaling transduction in the DDR.

DISCUSSION

We showed here that the DNA damage-induced phosphorylation on MDC1 Thr-98 by ATM is required for the oligomerization of MDC1. The oligomerization plays an important role in DDR such as the accumulation of MDC1, 53BP1, and BRCA1 at the sites of DNA damage as well as downstream signaling transduction.

Previous studies have shown that oligomerization of other DDR factors are involved in DDR signaling. For example, ATRIP and ATR homo-oligomerization are important for ATR signaling, although these oligomerizations seem constitutive and not subjected to regulation by DNA damage signaling (34). The yeast Rad9 protein is also reported to form oligomer during DDR. A two-hybrid assay identified a specific interaction between the Rad9 BRCT domain and Rad9 itself. Furthermore, it was demonstrated that the Rad9-Rad9 interaction occurred with a much greater affinity when Rad9 was hyperphosphorylated and was inhibited by the BRCT point mutations (35). Disruption of Rad9 oligomerization resulted in a defect in the maintenance of DNA damage checkpoint (36). We believe that MDC1 oligomerization functions in a similar fashion in mammalian cells.

The disruption of the oligomerization of MDC1 resulted in smaller and dimmer foci of MDC1, BRCA1, and 53BP1. There are two not mutually exclusive possibilities to explain the effect of MDC1 oligomerization on DDR: one is that oligomerization of MDC1 facilitates the amplification of ATM signaling by bringing ATM and downstream DDR factor close to the sites of DNA damage, resulting in a positive feedback loop. Disruption of oligomerization would disrupt the positive feedback loop and limit ATM signaling. The other possibility is that the oligomerization might affect the stability of MDC1 complex. Because MDC1 mainly functions as a platform to recruit other DDR factors, the oligomerization of MDC1 may help stabilize the large complex at the sites of DNA damage and sustain DDR damage signaling. Either way, disruption of MDC1 oligomerization would negatively affect efficient DDR signaling (Fig. 6).

Taken together, our data demonstrate that MDC1 oligomerization plays an important role in facilitating the signaling transduction in the DNA damage checkpoint pathway and reveal new insights into the complex forming at the DNA damage sites.

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