Disassembly of MDC1 Foci Is Controlled by Ubiquitin-Proteasome-dependent Degradation*

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The orderly recruitment, retention, and disassembly of DNA damage response proteins at sites of damaged DNA is a conserved process throughout eukaryotic evolution. The recruitment and retention of DNA repair factors in foci is mediated by a complex network of protein-protein interactions; however, the mechanisms of focus disassembly remain to be defined. Mediator of DNA damage checkpoint protein 1 (MDC1) is an early and key component of the genome surveillance network activated by DNA double-strand breaks (DSBs). Here, we investigated the disassembly of MDC1 foci. First, we show that ubiquitylation directs the MDC1 protein for proteasome-dependent degradation. Ubiquitylated MDC1 associates with chromatin before and after exposure of cells to ionizing radiation (IR). In addition, increased MDC1 ubiquitylation in the chromatin fraction is observed in response to IR, which is correlated with a reduction in total MDC1 protein levels. We demonstrate that blocking MDC1 degradation by proteasome inhibitors leads to a persistence of MDC1 foci. Consistent with this observation, chromatin immunoprecipitation experiments reveal increased MDC1 protein at site-specific DSBs. Interestingly, we show that the persistence of MDC1 foci is associated with an abrogated recruitment of the downstream factor BRCA1 in a manner that is RNF8 independent. Collectively, the evidence presented here supports a novel mechanism for the disassembly of MDC1 foci via ubiquitin-proteasome dependent degradation, which appears to be a key step for the efficient assembly of BRCA1 foci.

DNA double-strand breaks (DSBs) can be exogenously generated by a variety of genotoxic agents, such as ionizing radiation (IR). They also occur endogenously during normal DNA replication (1–3). Alterations in the cellular response to DSBs often result in genomic instability, which may contribute to the development of malignancies (4, 5). To avoid the deleterious consequences of unrepaired DSBs, cells have developed a complicated signaling network to coordinate the activation of the DNA repair machinery and delay of cell cycle progression. One important aspect that determines the effectiveness of the DSB response is the recruitment of pathway components to nuclear regions containing the damaged DNA (6), which is a highly conserved process throughout evolution (7). The cytological manifestation of nuclear redistribution is the formation of so-called foci (8), which can be visualized by immunofluorescence microscopy. As various DNA damage checkpoint and repair pathways are required for the rescue of stalled or collapsed DNA replication forks (7, 9–11), many of the pathway components also form foci in proliferating cells in the absence of exogenous DNA-damaging agents. Foci are highly dynamic structures (6, 12) in which proteins are recruited, retained, and disassembled at sites of damaged DNA in a distinct order (2, 13). Recent evidence has suggested that the assembly and retention of checkpoint and repair protein foci appears to be largely controlled by a network of protein-protein interactions (14–16). However, the molecular mechanisms controlling the disassembly of protein foci remain poorly understood.

The discovery of ubiquitin–proteasome-dependent degradation has led to the recognition of cellular proteolysis as a central area of research in biology (17, 18). The majority of intracellular proteins are proteolyzed by the proteasome. In most cultured mammalian cells, ubiquitin–proteasome-dependent degradation accounts for 80–90% of protein breakdown (18, 19). The ubiquitin–proteasome system covalently modifies target proteins by the attachment of a 76-amino acid ubiquitin to lysine residue(s) of the target. The elongated poly-ubiquitination chain signals the target protein for proteasome-mediated degradation (18). Typically, lysine 48 (K48)-linked polyubiquitin chains target conjugated proteins to the proteasome for degradation, although in recent years it has been shown that ubiquitylation of a target protein may also have other consequences (18, 20–23). The 26S proteasome system exists in abundance in the cell, and all of its components distribute in both the cytoplasm and nucleus (18, 24–28). The proteasome is recruited to DSBs in yeast and to chromatin in mammalian cells, suggesting a direct role for the proteasome in DSB repair (29, 30).
Recently, treatment of cells with pharmacological inhibitors of the ubiquitin-proteasome was found to result in a delay in the formation of several DNA damage response and repair foci (31, 32) and a suppression of homologous recombination (HR) (32). Mediator of DNA damage checkpoint protein 1 (MDC1) is an early and critical mediator of the DSB response pathway. It contains an amino-terminal FHA (forkhead-associated) domain and a tandem repeat of BRCT (breast cancer susceptibility gene-1 C terminus) domains, which are frequently found in DNA damage response proteins (33, 34). Both domains have been shown to mediate protein–protein interactions (16, 35–37). MDC1 forms protein foci in proliferating undamaged cells as well as in response to exogenous DNA damage (38). MDC1 is required for cell cycle checkpoint control and DSB repair via regulating the recruitment or retention of proteins (14, 15, 39, 40).

Data demonstrating that MDC1 knockdown almost completely inhibited IR-induced BRCA1 focus formation suggests that MDC1 is critical for BRCA1 recruitment in response to DSBs (39). Recently, several laboratories have shown that MDC1 recruits the E3 ubiquitin ligase RNF8, thereby mediating ubiquitin conjugation of H2A/H2AX, which is required for BRCA1 focus accumulation at sites of damaged DNA (36, 37, 41, 42). However, whether RNF8-mediated ubiquitylation of H2A/H2AX is the sole mechanism that controls damage-induced BRCA1 recruitment remains unknown. It has been suggested that MDC1 focus formation peaked within 30 min after irradiation and then gradually decreased, while BRCA1 focus accumulated over several hours after IR exposure (43).

Recently, it was found that MDC1 physically associates with USP28, a ubiquitin protease, and decreased MDC1 protein was observed after depletion of USP28, suggesting that MDC1 degradation may depend on the ubiquitin-proteasome system (44). However, whether MDC1 is ubiquitylated and how this modification regulates the DNA damage response including BRCA1 recruitment is not known.

Here, we hypothesized that ubiquitin-proteasome dependent degradation controls the disassembly of MDC1 foci in response to DSBs. We show that MDC1 is an ubiquitylated protein that is up-regulated in response to DSBs. Disruption of MDC1 degradation by treatment with proteasome inhibitors (PIs) results in MDC1 accumulation at DSB sites, which is associated with a failure of BRCA1 recruitment through an RNF8-independent mechanism. This work provides new insight into the spatiotemporal dynamics of DNA damage response foci, which not only involves recruitment and retention of foci but also focus disassembly via the ubiquitin-proteasome system.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Plasmids, siRNA, and Transfections**—MCF-7 cells were obtained from the American Type Culture Collection and maintained as recommended. MDC1, RNF8, and control siRNA sequences have been described previously (40, 41, 45). The final siRNA concentration for transfection was 80 nM. All transfections were performed with Lipofectamine 2000 (Invitrogen). The H6-Myc-ub and mutant K48 expression vectors were obtained from the American Type Culture Collection (46). DNA plasmid transfections were performed using Lipofectamine according to the manufacturer’s recommendations (Invitrogen). The adenoviral I-SceI endonuclease Ad-SceI-NG expression vector was obtained from Kristoffer Valerie (47).

**Proteasome Inhibition Studies**—PIs MG132 (carbonyl oxy-

-lycyl-1-leucyl-1-leucyl-1-leucinal) and epoxomicin were dissolved in DMSO and applied to cells at a final concentration of 10 μM for MG132 and 5 μM for Epoxomicin. An equivalent volume of DMSO was added to control cells. PIs were added to the medium, and cells were processed for immunofluorescence staining, Western blotting, or immunoprecipitation at the indicated time points. Alternatively, PIs were added to the cells 1 h prior to exposure of cells to IR.

**Immunofluorescence Analysis**—For visualization of MDC1 protein expression, cells were grown in multiple well chamber slides, washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde (BM-155, Boston Bioproducts Inc.), and then permeabilized with PBS containing 0.5% (v/v) Triton X-100 for 5 min. Cells were then blocked using 10% fetal bovine serum in PBS and incubated with anti-MDC1 antibody at a corresponding dilution. For analysis of focus formation, a detergent extraction method was employed (48). Briefly, the cells were incubated with 0.5% Triton X-100 CSK buffer and fixed with 4% formaldehyde. The fixed cells were then permeabilized using 0.1% Triton X-100 for 15 min, followed by blocking and incubation with primary antibodies at a corresponding dilution. Bound antibodies were revealed by goat anti-mouse IgG Alexa Fluor 594 (red channel) and chicken anti-rabbit IgG Alexa Fluor 488 (green channel). The DNA was stained using DAPI at 50 ng/ml. Slides were viewed at ×1000 magnification on an Olympus fluorescence microscope (BX40 with Magna-Fire CCD camera).

**Immunoprecipitation and Immunoblotting**—For the exogenous Myc-tagged ubiquitin analysis, cells was transfected with H6-M-ub or control vector and 48 h later exposed to PIs. Cell extracts were prepared by resuspending the cells in radioimmunoprecipitation assay lysis buffer (RIPA buffer with EDTA (BP-115D) purchased from Boston Bioproducts Inc.). Ten micrograms of an MDC1 antibody AHP799 (Secrotec) were added to denatured 500–1,000 μg of protein extract and rotated for an additional 2 h at 4 °C. The immune complexes were collected with 60-μl slurry of protein A-Sepharose CL-4B beads (Amersham Biosciences AB). The complexes were washed four times with lysis buffer, fractionated by 5% SDS-PAGE overnight at 20 V, transferred to membranes by a semi-dry transfer method, and immunoblotted with an antibody against c-Myc (Oncogene). The membranes were incubated with ECL (Amersham Biosciences) and exposed to x-ray film for protein detection for 2–3 h or longer. All membranes used for anti-ubiquitin immunoblotting were autoclaved submerged in deionized water for 20 min to ensure complete denaturation of the bound ubiquitylated proteins (49).

To analyze ubiquitylated MDC1 bound to chromatin, the exogenous Myc-tagged ubiquitin was transfected as described above, and 48 h later cells were treated with PIs. Chromatin-bound proteins were isolated after 4 h using established protocols with minor modifications (38, 40, 50). Briefly, insoluble chromatin was collected by two rounds of centrifugation (4 min each at 1,700 × g and 4 °C). The final chromatin pellet was
resuspended in Laemmli buffer and sonicated for 30 s at 25% amplitude. The fractioned chromatin-bound protein was denatured by boiling the sample for 5–10 min, and analyzed by immunoprecipitation using anti-MDC1 antibody and probed with mixed anti-c-Myc antibody and anti-MDC1 antibody or anti-MDC1 antibody alone. To measure MDC1 protein levels, PI-treated MCF-7 cells were harvested at indicated time points. Immunoblotting was performed by using 5% SDS-PAGE, and Western blots were developed with a rabbit polyclonal anti-MDC1 antibody (Novus). Proteins were detected using enhanced chemiluminescence (Amersham Biosciences).

**Construction of the Chromosomal DSB Repair Substrate—**The pDR-GFP plasmid (51), which contains the 18-bp recognition site for the I-SceI endonuclease within the green fluorescence reporter (GFP), was chromosomally integrated into H1299 cells. Cells were transfected with 1–3 μg of linearized pDR-GFP using Lipofectamine (Invitrogen), and stable integrants were selected with 1.0 μg/ml puromycin (Sigma). Several H1299/DR-GFP subclones were established and infected with the I-SceI expression vector Ad-SceI-NG (47). Twenty-four hours after infection, cells were trypsinized to a single cell suspension and subjected to flow cytometric analysis. Two-color fluorescence analysis revealed the percentage of green fluorescent cells relative to the total cell number. For each analysis, 50,000 cells were processed. Subclones that exhibited I-SceI-inducible green fluorescence as a marker of HR were identified (51).

**Chromatin Immunoprecipitation (ChIP)—**The ChIP protocol was previously published and applied here with minor modifications (52, 53). H1299/DR-GFP cells were either pretreated with PIs for 2 h or remained untreated. Following infection with the I-SceI expression construct, cells were incubated at 37 °C for 12 h. Formaldehyde cross-linking and ChIP assays of cultured H1299/DR-GFP cells were performed with the CHIP-IT™ Express kit (Active Motif) following the manufacturer's instructions. Briefly, cells were treated with 1% formaldehyde for 10 min to cross-link proteins to DNA. Glycine was added to quench the reaction. Cells were collected in scraping solution. Cells were resuspended in lysis buffer and incubated on ice for 30 min. Nuclei pellets were collected, resuspended in shearing buffer, and sonicated to obtain DNA fragments using a Bioruptor™ from Diagenode. Immunoprecipitations were performed overnight with polyclonal antibodies against MDC1 (Bethyl, rabbit anti-MDC1 A300–053A). Rabbit anti-human IgG (H+L) antibody (Jackson Immunoresearch Laboratories) was used as a negative control. The crosslinks were reversed by adding reverse crosslinking buffer followed by incubation at 94 °C for 15 min. Samples were deproteinized for 1 h with proteinase K, and DNA was extracted with Gel/PCR DNA fragment extraction kit (IBI Scientific). Quantification of the amount of immunoprecipitated DNA was carried out by real-time PCR using the iQ™ SYBR Green Supermix (Bio-Rad), which contained Fast Start TaqDNA polymerase and SYBR Green Dye. For each PCR assay, 1-μl aliquots of the DNA sample were amplified in duplicate. Values were calculated as fold-enrichment compared with the IgG control versus a control locus distinct from the DSB site (52, 53).

**RESULTS**

**MDC1 Is Ubiquitylated and Degraded by the Ubiquitin-Proteasome—**It has been reported that MDC1 focus formation peaks early after induction of DNA damage and then dramatically declines (43). In addition, a recent study showed that MDC1 degradation is dependent on the proteasome system (44). We, therefore, sought to elucidate whether ubiquitin-proteasome-dependent degradation of MDC1 may occur at sites of DSBs where it would impact the assembly of other DNA damage response proteins. We first investigated whether MDC1 is post-translationally modified by ubiquitin. Exponentially growing MCF-7 cells were transfected with a Myc-tagged ubiquitin expression vector, H6-Myc-ub, or an empty control vector, which was followed by proteasome inhibition 36–48 h later (see “Experimental Procedures”). Fig. 1A shows that higher molecular weight bands were detected in anti-MDC1 immunoprecipitates from cells containing H6-Myc-ub but not in immunoprecipitates from cells transfected with the control vector (lanes 1 versus 2). This suggests that MDC1 is post-translationally modified by ubiquitylation.

Lysine 48 (K48) of ubiquitin is the most common lysine used for targeting proteins for proteasome dependent degradation. Therefore, we analyzed if a K48 mutant of ubiquitin affects MDC1 ubiquitylation. Wild-type ubiquitin, a mutant in which the lysine 48 residue had been substituted to arginine (K48R) were transfected into MCF-7 cells, and cells lysates were analyzed for ubiquitylation of MDC1. We found that overexpression of the K48R ubiquitin mutant resulted in a reduction of ubiquitylated MDC1 (Fig. 1B, compare lanes 2 and 1), consistent with an interference with the formation of a K48-dependent ubiquitin chain on MDC1. To determine if ubiquitylated...
MDC1 is targeted for proteasome-dependent degradation, we examined the effect of PIs on MDC1 protein levels. Treatment of MCF-7 cells with the PIs MG132 and epoxomicin led to a significantly increased expression of MDC1 (Fig. 1, C and D). Taken together, these data suggest that the degradation of MDC1 is an ubiquitin-proteasome mediated process, in which K48 mediates the assembly of polyubiquitin chains.

**DSBs Upregulate Ubiquitylated MDC1**—MDC1 protein is known to associate with chromatin both before and after induction of DNA damage, and no MDC1 protein is detected in the cytoplasmic and soluble nuclear fractions (40, 54) (data not shown). Therefore, ubiquitylation is expected to occur on chromatin-associated MDC1 (40, 54). To confirm this hypothesis, we investigated whether ubiquitylated MDC1 is associated with chromatin and participate in the DNA damage response. Fig. 2A shows that ubiquitylated MDC1 was present in the chromatin fraction of undamaged MCF-7 cells and that the amount of ubiquitylated MDC1 increased following exposure of cells to IR, whereas the amount of immunoprecipitated MDC1 protein was unchanged. These data indicate that IR treatment results in increased MDC1 ubiquitylation. It was not surprising that ubiquitylation of MDC1 occurs even in the absence of exogenous DNA damage, considering that DSBs are continuously produced during normal DNA metabolism in cycling cells, especially during late S and G₂/M phases (55). However, we did not further pursue the possibility that ubiquitylation of MDC1 may occur in a DNA damage-independent manner.

The observed increased MDC1 ubiquitylation is consistent with decreased MDC1 protein levels seen in response to IR in both whole cell lysates and chromatin fractions (Fig. 2 B and C). These findings do not contradict previous data suggesting that DSBs lead to an increased affinity of MDC1 to chromatin (54). DNA damage increases the binding affinity of MDC1 to chromatin and focus formation while concurrently promoting MDC1 focus disassembly by ubiquitin-proteasome-dependent degradation. In the following, we studied the implications of this dynamic model.

**Localization of Ubiquitylated MDC1 in Damage-induced Protein Foci**—To specifically monitor the localization of the ubiquitylated form of MDC1 to damaged DNA, we considered that the covalent link between the C-terminal glycine of ubiquitin to a substrate lysine, which is the final product of the ubiquitin conjugation pathway, can be detected with a monoclonal antibody (FK2). This antibody is specific for K29-, K48-, and K63-linked ubiquitin, but not free ubiquitin (see instructions by the manufacturer (BioMol)) (56). Any conjugated ubiquitylation can be detected by FK2 antibody and it has been previously used to identify conjugated ubiquitin epitopes in fixed cells (57–59). Exponentially growing MCF-7 cells were treated with Triton X-100 prior to fixation with formaldehyde in order to improve the visualization specifically of chromatin-bound protein (48, 60) (see “Experimental Procedures”). Under these conditions, efficient knockdown of RNF8 by two rounds of siRNA transfection resulted in a clear decrease of FK2 foci (Fig. 3 A and B), consistent with the known MDC1-RNF8-mediated ubiquitylation of H2A/H2AX. Importantly, even though RNF8 protein knockdown is complete, residual FK2 foci are still observed (Fig. 3C, bottom panel). In addition, not all cells with FK2 foci show RNF8 foci (Fig. 3C, upper panel, marked by arrow). Therefore, RNF8-mediated ubiquitylation of H2A/H2AX is not the only mechanism underlying the development of conjugated FK2 foci in response to DSBs. Consistent with this notion, we found that MDC1 foci showed near complete
co-localization with conjugated ubiquitin foci stained with the FK2 antibody at 0–4 h after irradiation (Fig. 3D).

We next assessed the effects of knocking down MDC1 by siRNA. Almost complete protein knockdown was achieved compared with control siRNA-transfected cells (Fig. 3, siRNA). Almost complete protein knockdown was achieved by Western blot and immunostaining. RNF8 knockdown by immunofluorescence staining analogous to A, C, residual FK2 foci in cells with RNF8 knockdown. MCF7 cells transfected with siRNA twice were irradiated with 8 Gy, and immunostaining was performed 4 h after IR. Briefly the cells were detergent-extracted with 0.5% Triton X-100 CSK buffer and fixed with 4% formaldehyde. The fixed cells were then permeabilized using 0.1% Triton X-100, which was followed by blocking and incubation with FK2 and anti-RNF8 antibodies. Foci were visualized by AlexaFluor594-labeled anti-mouse IgG secondary antibody (red) or AlexaFluor488-labeled anti-rabbit antibody (green). Cell nuclei were stained with DAPI. D, MDC1 foci co-localize with conjugated ubiquitin in both untreated and IR-treated cells. Exponentially growing MCF7 cells were mock-treated or irradiated with 8 Gy and fixed at the indicated time points. The fixed cells with and without IR treatment were co-stained with anti-MDC1 and anti-ubiquitin (FK2) antibodies. This was followed by incubation with the secondary antibodies. Co-localization is indicated by a yellow signal. E–G, MDC1 knockdown resulted in inhibition of conjugated ubiquitin foci. Exponentially growing MCF7 cells were transfected with two rounds of MDC1 or control siRNA as described in A and B. MDC1 knockdown was monitored by Western blot (E) and immunostaining (F).

Increased Amounts of MDC1 Protein at Site-specific DSBs in PI-treated Cells—Foci of DNA damage response proteins, such as MDC1, are generally thought to accumulate at sites of DSBs. However, increased levels of MDC1 in PI-treated cells may also result in unspecific binding of MDC1 to chromatin or forced protein-protein interaction. To exclude this possibility, we asked if the increased levels of MDC1 protein following PI treatment were associated with increased amounts of MDC1 protein at chromosomal DSB sites. Because IR-induced DSBs occur randomly, they are not good substrates to monitor the localization of MDC1. Therefore, we elected to study the association of MDC1 with site-directed DSBs, generated by I-SceI endonuclease cleavage in a chromosomally integrated GFP reporter. As shown in Fig. 5A, H1299/DR-GFP cells demonstrate increased frequencies of DSB repair by HR following infection of the adenoviral I-SceI expression vector, which confirms the occurrence of I-SceI DSBs. I-SceI expression was monitored by Western blot using antibody against the HA tag (Fig. 5B). H1299/DR-GFP cells were first fixed with formaldehyde and the chromatin was solubilized by sonication and purified. Immunoprecipitations were conducted with antibodies raised against MDC1. After reversal of the formaldehyde cross-links, DNA samples were deproteinized. DNA was isolated and amplified by real-time
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FIGURE 4. Kinetics of MDC1 and γ-H2AX focus formation without and with PI treatment. A, representative MDC1 foci in cells with and without PI treatment at 4 h after irradiation with 8 Gy. B, dynamics of MDC1 focus formation after irradiation of MCF7 cells with 8 Gy. The percentage of cells with more than five nuclear foci was calculated. In each experiment, 200–300 nuclei were counted per data point. Error bars indicate S.E from three independent experiments. C, representative γ-H2AX foci in cells with and without PI treatment 4 h after irradiation with 8 Gy. D, dynamics of γ-H2AX focus formation after irradiation of MCF7 cells with 8 Gy. γ-H2AX foci kinetics was determined as in B.

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PCR with primer pairs specific to regions of interest near the DSB created by I-SceI cleavage (Fig. 5C) (52). All reactions were normalized against a control primer pair for sequences near the AFP locus, which allowed us to control for DSB-independent effects on protein occupancy. It was found that Ad-I-SceI-NG infection resulted in a dramatical enrichment of MDC1 protein at DSB sites using the indicated primer pairs (Fig. 5, A and B). This observation confirmed the previous finding that generation of DNA damage by IR increased the level of MDC1 foci. This highly informative approach allowed us then to further determine the I-SceI DSB association of MDC1 upon treatment of cells with PIs, as we had observed that proteasome inhibition augmented MDC1 focus retention after IR (Fig. 4, A and B). Fig. 5E shows that in PI-treated cells an increased amount of MDC1 was DSB-associated compared with cells without PI treatment. These data suggest that the observed increased MDC1 foci were not a result of protein-protein interaction or unspecific chromatin binding. The pulled down MDC1 protein levels were monitored by immunoprecipitation using anti-MDC1 antibody (Fig. 5E, bottom panel). In cells without PI treatment, less MDC1 protein was immunoprecipitated after Ad-I-SceI-NG infection, compared with cells without Ad-I-SceI-NG infection, which is consistent with the observed decreased MDC1 expression after IR (Fig. 2) or I-SceI infection (data not shown).

Together, the results in Fig. 5 strongly suggest that MDC1 protein accumulates at DSB sites in the presence of PIs. Thus, ubiquitin-proteasome-dependent degradation occurs at DSBs, in keeping with the observations in Fig. 3.

Ubiquitin-Proteasome Inhibition Prevents the Disassembly of MDC1 Focus Formation While Abrogating BRCA1 Focus Formation—Next, we sought to address the biological significance of ubiquitin-proteasome-dependent disassembly of MDC1. The repair of DSBs is a highly dynamic process that requires careful orchestration of a multitude of enzymes, adaptor proteins, and repair factors. It has been demonstrated that the disassembly of upstream factors may be linked to the further recruitment of downstream factors. For instance, the disassembly of MRE11 is correlated with the downstream recruitment of the HR protein Rad52 (2). Because it has been suggested that MDC1 focus formation peaks early after irradiation and then gradually decreases, while BRCA1 foci accumulates over several hours after IR exposure (43), we hypothesized that the disassembly of MDC1 may be required for recruitment of BRCA1. We analyzed IR-induced BRCA1 foci in the same time frame as for the MDC1 foci in cells with and without PI treatment. It was found that PI treatment resulted in a virtual abrogation of BRCA1 focus formation, as illustrated in Fig. 6A. This observation was not a result of reduced BRCA1 protein levels in PI-treated cells (Fig. 6B). In Fig. 6C, we determined the kinetics of BRCA1 focus formation with and without PI treatment. BRCA1 foci failed to form after exposure of PI-treated cells to IR, compared with cells not treated with PIs. For example, at 4–6 h the induction of BRCA1 foci was reduced by 3.5–4.9-fold (Student's t test, p < 0.01). This observation was striking as MDC1 focus formation has been previously shown to promote BRCA1 recruitment (39).

Notably, PI treatment also differentially affected the fraction of MDC1 and BRCA1 foci in untreated cells, marked as 0-hour time point in Figs. 4B and 6C. This finding is consistent with previous reports suggesting that DSBs also arise during normal DNA replication in the absence of exogenous DNA-damaging agents and that DNA damage response pathways are required for the rescue of stalled or collapsed DNA replication forks (7, 9–11).

Impaired MDC1 Degradation and Persistent MDC1 Foci Interfere with BRCA1 Recruitment Independent of RNF8—Recently, several laboratories have shown that ubiquitylation of H2A/H2AX mediated by the MDC1-RNF8 interaction is critical for BRCA1 focus formation, and PI treatment resulted in a decreased ubiquitylation of H2A/H2AX (37, 41). Thus, PIs may impact BRCA1 focus formation not only through interfering with MDC1 degradation but also by impairing H2A/H2AX ubiquitylation. To further dissect this relationship, we examined RNF8 knockdown (see Fig. 3A). As expected, siRNA against RNF8 resulted in a reduction of damage-induced BRCA1 foci from 74 to 31% at 4-h post-irradiation (Fig. 7A). In
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FIGURE 5. Detection of the enhanced MDC1 protein accumulation at chromosomal DSB sites in the presence of PIs as determined by ChIP. A, induction of site-directed DSBs, as reflected by subsequent homology-mediated repair, was measured by dual-color flow cytometric detection of GFP-positive cells. Representative flow cytometric analyses of H1299/DR-GFP cells treated with and without infection of the adenoviral I-SceI expression construct (Ad-SceI-NG) are shown. Left panel show cells without DSB induction, with few GFP-positive cells detected. The right panel shows cells 24 h after infection with Ad-SceI-ING. B, expression of HA-tagged I-SceI endonuclease was monitored by Western blot using anti-HA antibody. H1299/DR-GFP cells were infected with and without Ad-SceI-NG, and whole cell lysate was prepared at the indicated time points. C, schematic representation of the position of primers used for real-time PCR quantification of ChiPs respective to the DSB created by I-SceI endonuclease in vivo. Real-time PCR on ChiP samples were carried out at 94–378 nucleotides from the break (52). D, ChiP of MDC1 protein on DSB after Ad-SceI-NG. -Fold enrichment represents the enrichment of MDC1 proteins compared with an IgG control and normalized to a PCR internal control for a chromosomal locus distinct from the DSB site (52). Data points represent an average of four independent repeated experiments. E, ChIP analysis of MDC1 in the presence and absence of PIs (upper panel). Samples were prepared in parallel to monitor the amount of pulled-down MDC1 proteins using immunoprecipitation. The anti-MDC1 immunoprecipitate was probed with anti-MDC1 antibody (bottom panel). Shown is one representative result from three independent repeated experiments.

In contrast, MDC1 knockdown led to a more profound abrogation of BRCA1 foci, i.e. to 9%, suggesting that a fraction of BRCA1 foci formed independently of RNF8. Accordingly, when RNF8 siRNA-transfected cells were treated with PIs, the residual BRCA1 foci were eliminated (Fig. 7B). Importantly, the effectiveness of RNF8 and MDC1 knock-down was comparable (Fig. 3) and neither manipulation affected BRCA1 protein expression levels (Fig. 7, C and D).

Taken together, these data suggest that RNF8-mediated ubiquitylation of γ-H2AX is not the only mechanism for the regulation of BRCA1 focus formation. A second mechanism that is MDC1 ubiquitylation-dependent yet RNF8-independent is required for the recruitment of damage-induced BRCA1 foci.

DISCUSSION

Recent studies have revealed that the local recruitment and retention of DNA damage response foci is a dynamic and hierarchical process (7, 14, 15, 54). However, the disassembly of protein foci remains poorly understood. In this report, we find MDC1 to be an ubiquitylated protein, which localizes at sites of DSBs (Figs. 1, 3, and 5) and is degraded in an ubiquitin-proteasome-
some-mediated process (Fig. 1). Inhibition of proteasome-mediated protein degradation leads to a striking persistence of MDC1 protein at DSB sites, as shown by both foci and ChIP studies (Figs. 4 and 5). Interestingly, the persistence of MDC1 is associated with a severe defect of BRCA1 recruitment (Fig. 6, A and C).

DNA damage response proteins assemble in multiprotein complexes and are regulated by direct protein-protein interactions in a spatiotemporal manner. However, the integration into multi-protein aggregates is expected to limit the mobility of the individual components. It has been suggested that MDC1 protein exhibits slow motility and an increased immobilization in chromatin following DSB induction (54). Thus, the local ubiquitylation-mediated degradation of MDC1 at sites of DSBs is biologically significant as it provides a novel mechanism to overcome the low mobility disadvantage of proteins accumulated in a complex. Moreover, this mechanism may also provide a greater flexibility in altering the composition of the DNA repair complex without displacing the interacting proteins that are required for the subsequent repair events, thereby ensuring that later steps can progress.

Our data integrate well with recent reports on the increasing importance of ubiquitylation in the DNA damage response (36, 37, 41, 42). The functional consequences of ubiquitylation depend in part on the type(s) of ubiquitin modification of the protein substrate. For example, a lysine 63 mediated ubiquitin addition onto a protein does not target the protein for the degradation pathway (20, 21, 62). Ubiquitylated proteins can be recognized by a multitude of ubiquitin-binding proteins and ubiquitin receptors that enable the modified proteins to interact and form complexes with other proteins and change subcellular localization. To this end, MDC1 recruitment has been shown to lead to histone ubiquitylation via RNF8 ligase and downstream recruitment of BRCA1 and 53BP1 (36, 37, 41, 42). However, the kinetics of MDC1 focus formation was not investigated in those studies, and our data demonstrate the MDC1 ubiquitylation and degradation is an important step in the DNA damage response. Disruption of RNF8 function does not seem to result in an increase in MDC1 foci (36, 37, 41). Therefore, the ubiquitin ligase acting on MDC1 remains to be identified.

Our data strongly suggest that MDC1 focus disassembly is, at least in part, required for the recruitment of BRCA1 foci (Figs. 4 and 6). On the other hand, MDC1 promotes BRCA1 recruitment through the direct interaction with RNF8, which is critical for triggering the formation of ubiquitin conjugates (36). These observations may be explained by the dynamics of focus recruitment. We note that damage-induced MDC1 foci peak after 30 min while BRCA1 foci peak at a much later time (Figs. 4B and 6C). Thus, the early presence of MDC1 is required to initiate RNF8-mediated ubiquitylation of downstream factors leading to a recruitment of BRCA1. Subsequently, MDC1 disassembly is required for further localization of BRCA1 to the site of DNA damage. Clearly, the DNA damage response is a highly dynamic process and a diverse spectrum of DNA intermediates may arise at sites of DSBs. For instance, single-stranded DNA may be generated at DSBs due to nuclease function, and BRCA1 is recruited to both DSB regions and ssDNA (63). Therefore, the mechanisms of BRCA1 recruitment in different phases of the response to DSBs are likely distinct. The notion that disassembly of MDC1 may regulate a second phase of BRCA1 recruitment in the DNA damage response is supported by a recent study showing that the proteasome is recruited into the chromatin at a relatively late stage following irradiation (30).

We acknowledge that the application of PI to disrupt MDC1 function is lacking specificity, though through the additional use of RNF8 siRNA, we have identified an ubiquitin-proteasome-dependent and RNF8-independent mechanism for BRCA1 recruitment.

To specifically inhibit MDC1 degradation, all ubiquitylation sites of MDC1 would need to be identified. However, there are 103 lysines distributed over the length of this large protein (2,089 amino acids), and it is known that ubiquitylation occurs redundantly in complex patterns. Further, even if the primary lysine were identified and mutated, it is likely that this mutation will force ubiquitylation of other lysine sites, which is a well-established mechanism. Thus, such an Ub-defective mutant will likely appear wild-type. While it is beyond the scope of our report to identify the likely multiple targets of ubiquitin-proteasome activity, we have described one of these targets, MDC1. Intriguingly, a picture is emerging in which ubiquitylation not only coordinates direct protein-protein interactions in DNA repair and replication (36, 37, 41, 42), but also causes rapid MDC1 degradation to facilitate the recruitment and retention of repair factors such as BRCA1. Thus, our data help us to understand the molecular basis and the biological significance of the disassembly of damage induced protein foci.

**Acknowledgment**—We thank Dr. Stephen Jackson for his useful comments and suggestions.

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