Mdm2 Binds to Nbs1 at Sites of DNA Damage and Regulates Double Strand Break Repair*

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The M11, Nbs1, and Rad50 proteins form a complex (the M-N-R complex) that is essential in maintaining DNA integrity by functioning in double strand break repair, meiotic recombination, and telomere maintenance (1). Mre11 is the catalytic subunit of the complex with 3′-5′ exonuclease, single-stranded DNA endonuclease, and DNA unwinding activities (2). M11 binds both Nbs1 and Rad50 (3, 4). Rad50 is an SMC (structural maintenance of chromosome) family member and, with its ATPase motifs, provides the energy source for the M-N-R complex (2, 5). A forkhead-associated domain and a breast cancer C-terminal domain at the N terminus of Nbs1 are critical for proper nuclear localization of the M-N-R complex to nuclear foci or sites of DNA strand breaks (6). The breast cancer tumor suppressor protein BRCA1 binds to Rad50 at nuclear foci and reportedly inhibits nuclease activity of the M-N-R complex (7, 8). Therefore, a multimeric complex, including M11, Nbs1, Rad50, and other proteins, is necessary to repair broken DNA. However, there is currently a paucity of information on the regulation of the M-N-R complex and the function of the proteins at nuclear foci.

The biological significance of the M-N-R complex in maintaining DNA integrity was revealed with the identification of human genetic disorders resulting from mutations in M11 and Nbs1 (4, 9, 10). Humans with mutations in Nbs1 develop Nijmegen breakage syndrome (NBS) and have a very high incidence of cancer (4, 10, 11). An ataxia telangiectasia (A-T)-like disorder (ATLD) develops in individuals with M11 mutations (9). This disorder is similar to that seen in humans harboring mutations in ataxia telangiectasia mutated (ATM) (12), a protein that is required for phosphorylation of Nbs1, M11, the p53 tumor suppressor, and the p53 regulator Mdm2 following γ-irradiation (1, 13–18). Cells isolated from patients with the NBS, ATLD, or A-T disorders exhibit radiation sensitivity, chromosomal instability, and defective cell cycle checkpoints (1).

The p53 tumor suppressor is critical for the checkpoint response to DNA damage by blocking cell cycle progression until repairs are made (19). If DNA is unrepaired, p53-dependent apoptotic signaling pathways are activated. Mdm2 regulates p53 by binding to and inhibiting p53-dependent transcription (20). In addition, Mdm2 ubiquitylates p53 (21), targeting p53 for proteosomal degradation (22, 23). Mdm2 also functions to shuttle p53 out of the nucleus into the cytoplasm (24). Genetic evidence that Mdm2 restricts p53 function was revealed when the early embryonic lethality of Mdm2−/− mice was rescued with loss of p53 (25, 26). The multifaceted regulation of p53 by Mdm2 is controlled by the tumor suppressor, p14ARF(human)/p19ARF(mouse), which binds to and inhibits Mdm2 (27). Recent evidence suggests that threshold levels of p53, Mdm2, and ARF are required to maintain a proper balance between apoptosis and cancer development (28–30).

In addition to p53-dependent functions, mounting evidence...
suggests Mdm2 also acts independent of p53. Mdm2 transgenic mice lacking p53 have an increased incidence of malignancies as compared with mice deficient in p53 alone (31). Mice overexpressing Mdm2 in breast epithelial cells had increased numbers of polyploid cells regardless of whether p53 was present or absent (32). Another report showed that expression of an alternatively spliced variant of Mdm2 increased the proliferation of p53-null MEFs and increased cancer incidence in mice (33). Additionally, lymphomas arising in humans and mice that have inactivated p53 also frequently overexpress Mdm2 protein (28, 34, 35). Finally, tumor cells lacking p53 died when treated with Mdm2 antisenese (36). Combined, these reports suggest that Mdm2 has functions independent of p53 that contribute to transformation. Therefore, we sought to identify novel Mdm2-binding proteins that influenced tumor development independent of p53. We determined that Mdm2 bound to the M-N-R DNA repair complex in a p53-independent manner at sites of DNA double strand breaks and that Mdm2 inhibited efficient DNA repair, which was dependent on the Nbs1 binding domain in Mdm2. This finding suggests a novel role of Mdm2 in compromising DNA integrity.

MATERIALS AND METHODS

Silver Staining and Mass Spectrometry—HeLa cells were Dounce-homogenized in complete lysis buffer (20 mM Tris-HCl, pH 7.3, 300 mM KC1, 0.2 mM EDTA, 0.1% Nonidet P-40, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.4 units/ml aprotinin, 1 mM NaF, 10 mM β-glycero-phosphate, and 0.1 mM Na3VO4). Total cellular protein (60 µg) was rotated with anti-Mdm2-conjugated beads (SMP14; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 °C, washed in complete lysis buffer, and separated by SDS-PAGE. Following silver staining, protein bands were excised and digested as described previously with slight modifications (37). After separation on a reversed phase LC column, eluted fractions were analyzed on a Q-TOF Ultima tandem mass spectrometer with electrospray ionization (Micromass/Waters, Toronto, Canada). The MS/MS data were processed using Masslynx software (Micromass), and the Mascot (Matrix Science, London, UK) search engine was used to search the NCBI nonredundant database protein identities were based on a minimum random probability score of 25 and with a mass accuracy of 0.1 daltons.

Cell Culture Conditions—HeLa, 293T, MCF7, MDA-MB-231, HT1080, HCT116, CCL, K562, NIH3T3, and IMR90 cell lines were cultured as described by the American Type Culture Collection (Manassas, VA). p53+/− ARPE-17, p53−/− Mdm2−/−, and p53−/− MEFs were isolated as described previously (38) and maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Atlanta Biologicals) at 37 °C in a humidified atmosphere of 5% CO2. Additionally, lymphomas arising in humans and mice that have inactivated p53 also frequently overexpress Mdm2 protein (28, 34, 35). Finally, tumor cells lacking p53 died when treated with Mdm2 antisenese (36). Combined, these reports suggest that Mdm2 has functions independent of p53 that contribute to transformation. Therefore, we sought to identify novel Mdm2-binding proteins that influenced tumor development independent of p53. We determined that Mdm2 bound to the M-N-R DNA repair complex in a p53-independent manner at sites of DNA double strand breaks and that Mdm2 inhibited efficient DNA repair, which was dependent on the Nbs1 binding domain in Mdm2. This finding suggests a novel role of Mdm2 in compromising DNA integrity.
DNA Damage Quantitation—Following infection (36 h) of NIH3T3 cells or p53−/− MEFs with wild-type Mdm2, Mdm2-(198–489), Mdm2-(298–489), or Mdm2-(198–400) encoded or empty MSCV-IRES-GFP retroviruses (see above), cells were irradiated with 5 Gy from a cesium-137 source. Cells were incubated at 37 °C for specific intervals and harvested for comet assay analysis. Comet assays were performed under neutral conditions to optimize for detection of DNA double strand breaks, as directed by the manufacturer’s protocol (Trevigen, Gaithersburg, MD). Samples were blinded, and DNA was stained with ethidium bromide. Multiple pictures per sample were taken by fluorescence microscopy (Nikon E600, Melville, NY) using a TRITC-HYQ filter. DNA breaks, as directed by the manufacturer’s protocol (Trevigen, Gaithersburg, MD). Samples were blinded, and DNA was stained with ethidium bromide. Multiple pictures per sample were taken by fluorescence microscopy (Nikon E600, Melville, NY) using a TRITC-HYQ filter. DNA damage quantitation was performed by computer scoring at least 50 cells per interval per cell line with TriTekCometScore™ software (TriTek Corp., Summertuck, VA). Tail moments were calculated by the software and represent the length of the comet tail multiplied by the percentage of DNA in the comet tail. Tail moment is a measurement of DNA double strand breaks (41, 42). Following data collection and tabulation, codes identifying each set of pictures were broken to reveal the identity of each sample.

RESULTS

The Mre11-Nbs1-Mre50 Complex Is Associated with Mdm2

A small difference in protein loading (see β-actin; Fig. 2A) and do not represent differences of the endogenous levels of these proteins in the different MEFs analyzed. Evaluation of human (MDA-MB-231, 293T, Saos-2, CLL, and K562) and murine tumor cell lines that lack functional p53 showed that Mdm2 was associated with the M-N-R complex (Fig. 2B, lanes 2, 4, 5, 8, and 9; and data not shown). Similarly, in whole cell lysates from human and mouse cells lacking ARF expression (MCF7, HT1180, HCT116, and NIH3T3), Mdm2 co-immunoprecipitated Nbs1, Mre11, and Rad50 (Fig. 2B, lanes 1, 6, and 7; data not shown). In addition, the M-N-R complex precipitated with Mdm2 in primary diploid human fibroblasts (IMR90; Fig. 2B, lane 10) and in immortal murine fibroblasts (p53−/−MEFs; Fig. 2A), indicating that the association of Mdm2 with the M-N-R complex is not due to cellular transformation. The small variations in the levels of Nbs1, Mre11, and Rad50 co-immunoprecipitating with Mdm2 in the various cell lines (Fig. 2B) were repeatedly observed and appear to reflect differences in one or more of these proteins between the cell lines. Together, these results demonstrated that the interaction between Mdm2 and the M-N-R complex does not require p53 or ARF and is not specific to human cells or cells of a particular tissue type or transformation status.

Nbs1, Mre11, BRCA1, and Mdm2 are phosphorylated by ATM (13–18, 43), and BRCA1 can associate with Rad50 (7, 8). Therefore, we questioned whether functional ATM and BRCA1 were required for Mdm2-M-N-R-R binding. To determine this, we utilized an A-T lymphoblastoid cell line (GM11261) and HCC1937 ductal breast carcinoma cell line, containing mutations in ATM and BRCA1, respectively, that inactivate the proteins (12, 44). Nbs1, Mre11, and Rad50 co-immunoprecipitated with Mdm2 in both cell lines (Fig. 2C). Therefore, Mdm2-M-N-R complex association does not require functional ATM or BRCA1.

Amino Acids 198–314 of Mdm2 Bind to the M-N-R Complex—Specific domains in Mdm2 are required to mediate binding to p53, ARF, and other proteins (Fig. 3A) (45). To identify the region of Mdm2 required for M-N-R complex association, we expressed a series of HA-tagged deletion mutants of Mdm2 in 293T cells (Fig. 3A). Expression of all HA-Mdm2 mutants and the control wild-type HA-Mdm2 protein was verified by Western blot analysis (Fig. 3B, top panel). Anti-HA immunoprecipitated wild-type HA-Mdm2 along with the M-N-R complex (Fig. 3B, lane 1). Similarly, the HA-Mdm2 mutant 198–489, lacking the p53 binding domain, still interacted with the M-N-R complex, since Nbs1, Mre11, and Rad50 were readily detected by Western blot (Fig. 3B, lane 2). Another Mdm2 mutant lacking both the p53 binding domain and the ring finger ubiquitin ligase domain (residues 198–400) also retained binding to the M-N-R complex (Fig. 3B, lane 7). In contrast, the M-N-R complex did not co-immunoprecipitate with HA-Mdm2 mutants containing just the p53 binding domain (residues 1–192) or the ring domain (residues 349–489), despite high levels of expression of these mutants (Fig. 3B, lanes 6 and 4). Thus, neither the N nor C terminus of Mdm2 is required to mediate the binding of Mdm2 to the M-N-R complex.

To further narrow down the M-N-R binding domain in Mdm2, we utilized two overlapping HA-Mdm2 mutants (1–314 and 298–489) (Fig. 3A). The 1–314 HA-Mdm2 mutant bound to the M-N-R complex (Fig. 3B, lane 5), whereas the HA-Mdm2 mutant 298–489 was unable to precipitate the M-N-R complex (Fig. 3B, lane 3). Therefore, we postulated that since amino acids 198–400 and 1–314 of Mdm2 did and amino acids 298–489 of Mdm2 did not co-immunoprecipitate the M-N-R complex, amino acids 198–314 were likely the domain that...
Nbs1, Mre11, and Rad50, respectively, and are in 80-kDa bands were homologous to Rad50, mass spectrometry for the 150-, 95-, and bands excised and analyzed by mass spectrometry. Peptide sequences identified by the polypeptide bands indicates the polypeptide bands excised and analyzed by mass spectrometry. Peptide sequences identified by mass spectrometry for the 150-, 95-, and 80-kDa bands were homologous to Rad50.

HeLa cell lysate. Following separation by SDS-PAGE, proteins were silver-stained. The arrows indicate the polypeptide bands excised and analyzed by mass spectrometry. Peptide sequences identified by mass spectrometry for the 150-, 95-, and 80-kDa bands were homologous to Rad50.

...binds to the complex. As predicted, the HA-Mdm2 mutant 198–314 (Fig. 3A) did bind to the M-N-R complex and precipitated equivalent levels of Nbs1, Mre11, and Rad50 as compared with wild-type HA-Mdm2, despite apparently low levels of expression of this Mdm2 mutant (Fig. 3B, lane 8). Interestingly, amino acids 198–314 overlap the domain in Mdm2 that binds to ARF and ribosomal proteins (45). Thus, the minimal essential domain of Mdm2 required for M-N-R association lies within amino acids 198–314.

...indicate that wild-type Nbs1 is required for Mre11 and Rad50 association with Mdm2. To test this possibility, we generated and purified from bacterial cultures GST-linked Nbs1 protein. The addition of purified GST-Nbs1 protein to NBS fibroblast cell lysate restored Mre11 and Rad50 co-immunoprecipitation with Mdm2 (Fig. 4D, lane 3), whereas the control GST purified protein did not (Fig. 4D, lane 2). Therefore, these results demonstrate that Nbs1 is the protein mediator between Mdm2 and Mre11/Rad50.

...Nbs1 appears to mediate the interaction of the M-N-R complex with Mdm2. Most NBS patients (95%) express a mutant form of Nbs1 that is severely truncated and lacks the Mre11 binding domain (10).

To determine whether Nbs1 is the protein in the M-N-R complex that mediates the binding of the complex to Mdm2, Mdm2 was immunoprecipitated from an NBS fibroblast cell line that contains the common 657del5 mutation. Although endogenous Mdm2 was immunoprecipitated from the NBS cell line, C-terminal truncated Nbs1 was not associated (Fig. 4C). Notably, in the absence of wild-type Nbs1, co-precipitation of Mre11 and Rad50 with Mdm2 was also abolished (Fig. 4C). These results indicate that wild-type Nbs1 is required for Mre11 and Rad50 association with Mdm2. To test this possibility, we generated and purified from bacterial cultures GST-linked Nbs1 protein. The addition of purified GST-Nbs1 protein to NBS fibroblast cell lysate restored Mre11 and Rad50 co-immunoprecipitation with Mdm2 (Fig. 4D, lane 3), whereas the control GST purified protein did not (Fig. 4D, lane 2). Therefore, these results demonstrate that Nbs1 is the protein mediator between Mdm2 and Mre11/Rad50.

The Central Region of Nbs1 Is Required for Interaction with Mdm2—In contrast to fibroblasts, lymphoid cells from NBS patients express a C-terminal p70 Nbs1 protein generated from a second translation initiation site (47). This C-terminal Nbs1 protein (amino acids 221–754) still contains the Mre11 binding domain (Fig. 5B) and consequently retains the ability to bind to Mre11 and co-immunoprecipitate Rad50 (data not shown; see Ref. 47). To determine whether Mdm2 was associated with this p70 truncated Nbs1 protein, we immunoprecipitated Mdm2 from the lymphoblastoid NBS cell line isolated from the same patient as the NBS fibroblast cell line used in experiments shown in Fig. 4, C and D. Notably, p70 Nbs1, along with Mre11 and Rad50, were co-immunoprecipitated with Mdm2 (Fig. 5A).

Thus, the N terminus of Nbs1 appears dispensable for the...
interaction of Nbs1 with Mdm2. To further characterize the Mdm2 interaction domain of Nbs1, we generated and transiently expressed FLAG-tagged wild-type Nbs1 and mutants of Nbs1 in 293T cells (Fig. 5, B–D). Not surprisingly, wild type Nbs1 and the N terminus truncated 179–754 Nbs1 mutant, which is similar to the Nbs1 mutant expressed in the NBS lymphoblasts (Fig. 5B), co-immunoprecipitated with Mdm2 (Fig. 5C). In contrast, the C terminus of Nbs1 (amino acids 540–754) did not co-immunoprecipitate with Mdm2 (Fig. 5, B and D). Due to the low level of expression of the 540–754 Nbs1 mutant, 4 times the amount of whole cell lysate from cells with this Nbs1 mutant was used for immunoprecipitating Mdm2, as compared with the amount used for immunoprecipitating Mdm2 in cells expressing wild-type Nbs1 or vector control (Fig. 5D). Mdm2 did associate with an Nbs1 mutant lacking its C terminus, since amino acids 1–592 of Nbs1 co-immunoprecipitated with Mdm2 (Fig. 5, B and C). Therefore, neither the N nor C terminus of Nbs1 is required for binding to Mdm2, suggesting that the central part of Nbs1 (amino acids 221–540) mediates the interaction with Mdm2.

Mdm2 Binds to Nbs1—Nbs1 and Mdm2 interact in cellular lysates. To determine whether the association between Nbs1 and Mdm2 is direct or indirect, we performed a series of in vitro binding experiments with purified protein. Purified GST or GST-Mdm2 fusion protein was incubated with in vitro transcribed and translated [35S]Met-labeled proteins. As a negative control, 35S-Met-labeled Bax was added to all reactions and did not specifically bind to GST or GST-Mdm2 (Fig. 6A). As a positive control for Mdm2 binding, [35S]Met-labeled p53 was generated. In contrast to p53 (Fig. 6, A (lanes 1–3) and B) and consistent with our previous data, Mre11 did not bind to GST-Mdm2 (Fig. 6, A (lanes 7–9) and B). Importantly, Nbs1 bound specifically to GST-Mdm2 (Fig. 6, A (lanes 4–6) and B). The amount of [35S]Met-labeled protein that bound to GST and GST-Mdm2 was quantified by phosphorimaging analysis. Quantitation revealed that p53 and Nbs1 bound to GST-Mdm2.
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21–25 and 4.3–5.7 times, respectively, the amount that bound to GST (Fig. 6B). Thus, the interaction between Mdm2 and Nbs1 was specific and appears direct.

Mdm2 Co-localizes with Nbs1 at Sites of DNA Damage—In unirradiated cells, Mdm2 is ubiquitously expressed throughout the cell (45), whereas Nbs1, Mre11, and Rad50 are predominantly nuclear proteins (4). To determine whether nuclear Mdm2 can bind to the M-N-R complex, we utilized nuclear and S-100 cytosolic extracts from unirradiated HeLa cells. Nbs1, Mre11, and Rad50 co-immunoprecipitated with Mdm2 in the whole cell and nuclear extracts, but due to the low levels of the complex in the cytoplasm, none of the proteins in the M-N-R complex were detected in Mdm2 immunoprecipitations from S-100 extracts (Fig. 7A). Therefore, in the absence of DNA damage, nuclear Mdm2 is able to associate with Nbs1.

To determine whether DNA damage would alter the binding of Mdm2 to Nbs1, Mdm2 was immunoprecipitated from γ-irradiated HeLa cells. A similar amount of Nbs1 co-immunoprecipitated from cells that were unirradiated and irradiated (Fig. 7B). Although these results suggest that the amount of Mdm2 that associates with Nbs1 appears to be unaltered following DNA damage, whole cell lysates do not necessarily reflect changes in protein association if only a fraction of the total proteins is involved; nor do whole cell lysates provide information on altered cellular localization of protein complexes, as was the case for BRCA1/Rad50 association (7). To test these possibilities, we evaluated by immunofluorescence the localization of Mdm2 and Nbs1 prior to and following γ-irradiation. In unirradiated IMR90 cells, diffuse nuclear Nbs1 staining and both cytoplasmic and nuclear Mdm2 staining were detected (Fig. 7C). Merging these two images revealed faint and nondistinct regions of overlap, which indicate a lack of co-localization of Mdm2 and Nbs1 in the absence of DNA damage. The M-N-R complex forms Type II nuclear foci at locations of DNA double strand breaks and are visible within 8 h post irradiation in primary diploid human fibroblast cells (IMR90) (48). 6–8 h following γ-irradiation and as previously reported (48), Nbs1 and Rad50 co-localization was visible in DNA damage-dependent nuclear foci (Fig. 7C). Notably, Mdm2 had a more clustered appearance following γ-irradiation with Mdm2 localizing to a few distinct sites throughout the nucleus (Fig. 7C). Importantly, multiple sites of specific Nbs1 and Mdm2 co-localization at nuclear foci were also detected in single planes of γ-irradiated IMR90 cells (Fig. 7C). Mdm2/Nbs1 co-localization was also evident at reduced levels at earlier times postirradiation (data not shown). Although Mdm2 did not appear to co-localize with Nbs1 at every focus, Mdm2/Nbs1 co-localization was observed in over 40% of cells where Nbs1 nuclear foci were detected. Moreover, these data are consistent with the observation that BRCA1 binds to the M-N-R complex but does not always co-localize with the M-N-R complex following irradiation (49). Thus, these results illustrate that at a single point in time, Mdm2 co-localized with a subset of the Nbs1 DNA damage foci.

Although we previously showed that p53 was not required for Nbs1/Mdm2 association, Mdm2 and p53 do bind (45), and thus, it was possible that p53 also localized with Mdm2 to sites of DNA damage. Therefore, IMR90 cells were γ-irradiated, fixed, and fluorescently stained for Nbs1 and p53. In unirradiated IMR90 cells, p53 is expressed at very low levels and appears diffuse throughout the cell (Fig. 7D). Following γ-irradiation, p53 is stabilized, and consequently the protein levels of p53 were higher, particularly in the nucleus where p53 acts as a transcription factor (Fig. 7D). However, p53 staining does not appear to overlap Nbs1 staining at nuclear foci following DNA damage (Fig. 7D). These results indicate that p53 does not co-localize with Nbs1 at sites of DNA damage and may also suggest that Mdm2/Nbs1 binding at double strand breaks is independent of p53.

Mdm2 Overexpression Delays DNA Double Strand Break Repair—Nbs1, together with Mre11 and Rad50, repairs DNA double strand breaks induced by γ-irradiation (1). To evaluate whether Mdm2 expression would influence Nbs1-mediated DNA repair, we took advantage of the comet assay, which under neutral conditions measures double strand breaks on a per cell basis (41, 42). In these comet assays, the length and darkness of broken DNA (spreading from the nucleus like the tail of a comet) directly correlates with the severity of DNA damage (41, 42). DNA damage, as detected by a comet assay, can be measured visually or by computer analysis, and both methods are reported to be comparable (41). Computerized scoring calculates tail moments, which are an accurate measurement of the amount of broken DNA per cell (41). In our experiments, tail moments of greater than 4 denote extensive DNA damage, and tail moments of 0–4 represent slightly damaged and repaired and unbroken DNA, in comparison with visual scoring.

We first analyzed the impact on DNA repair of Mdm2 overexpression in NIH3T3 cells, which lack ARF expression. NIH3T3 cells were infected with a control GFP-expressing MSCV retrovirus or an MSCV retrovirus encoding murine Mdm2 and, in cis, GFP, as previously described (39). GFP+ NIH3T3 cells were γ-irradiated, and DNA damage was analyzed by comet assays. At 5 min postirradiation, severe DNA damage in the vast majority of vector control and Mdm2-overexpressing cells was detected as tail moments of ~4 (Fig. 8A). At 60 min following irradiation, an expected decrease in the percentage of tail moments of ~4 and an increase in the percentage of tail moments of <4 were observed in the cells in-
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FIG. 5. The N and C terminus of Nbs1 are dispensable for Mdm2 binding. A, whole cell lysates from an NBS lymphoblastoid cell line were immunoprecipitated with an Mdm2-specific antibody. Immunoprecipitated proteins (IP; 2 mg) and a whole cell lysate (WCL; 250 pg) were Western blotted with antibodies specific for the proteins indicated to the left of each panel. B, schematic diagrams of the Nbs1 mutant expressed in NBS lymphoblastoid cells and wild-type FLAG-tagged Nbs1 and FLAG-tagged Nbs1 deletion mutants. The white boxed areas denote the forkhead-associated, the breast cancer C-terminal, and the Mre11 binding domains. The black boxes represent the location of the three nuclear localization sequences. Amino acids are indicated above each diagram. FLAG-tagged Nbs1 mutants that did (+) or did not (−) co-immunoprecipitate with Mdm2 are indicated. C and D, following transient transfection of FLAG-tagged wild-type Nbs1, the indicated Nbs1 mutants, or vector control, immunoprecipitations (2 mg in C) were performed with an Mdm2-specific antibody (Mdm2 IP) or an isotype control antibody (Isotype IP). Whole cell lysates (WCL; 100 pg in C) were run to show the level of expression of each FLAG-tagged protein. Proteins were Western blotted with anti-FLAG. The locations of immunoglobulin heavy chain (IgH) and light chain (IgL) are indicated. Four times (×4; 4 mg versus 1 mg in the IP lanes and 200 pg versus 50 pg in the WCL lanes) or the same (×1; 50 pg) amount of cell lysate from 540–754 mutant Nbs1-expressing cells as compared with the amount used for vector control and wild-type expressing cells is indicated in D.

Fig. 5. The N and C terminus of Nbs1 are dispensable for Mdm2 binding. A, whole cell lysates from an NBS lymphoblastoid cell line were immunoprecipitated with an Mdm2-specific antibody. Immunoprecipitated proteins (IP; 2 mg) and a whole cell lysate (WCL; 250 pg) were Western blotted with antibodies specific for the proteins indicated to the left of each panel. B, schematic diagrams of the Nbs1 mutant expressed in NBS lymphoblastoid cells and wild-type FLAG-tagged Nbs1 and FLAG-tagged Nbs1 deletion mutants. The white boxed areas denote the forkhead-associated, the breast cancer C-terminal, and the Mre11 binding domains. The black boxes represent the location of the three nuclear localization sequences. Amino acids are indicated above each diagram. FLAG-tagged Nbs1 mutants that did (+) or did not (−) co-immunoprecipitate with Mdm2 are indicated. C and D, following transient transfection of FLAG-tagged wild-type Nbs1, the indicated Nbs1 mutants, or vector control, immunoprecipitations (2 mg in C) were performed with an Mdm2-specific antibody (Mdm2 IP) or an isotype control antibody (Isotype IP). Whole cell lysates (WCL; 100 pg in C) were run to show the level of expression of each FLAG-tagged protein. Proteins were Western blotted with anti-FLAG. The locations of immunoglobulin heavy chain (IgH) and light chain (IgL) are indicated. Four times (×4; 4 mg versus 1 mg in the IP lanes and 200 pg versus 50 pg in the WCL lanes) or the same (×1; 50 pg) amount of cell lysate from 540–754 mutant Nbs1-expressing cells as compared with the amount used for vector control and wild-type expressing cells is indicated in D.

FIG. 6. Mdm2 binds to Nbs1. A, in vitro transcribed and translated [35S]Met-labeled p53, Nbs1, Mre11, and, as a negative control, Bax were added to GST- or GST-Mdm2-conjugated glutathione-Sepharose beads. Input, 12% of the protein added to each binding reaction. Bound proteins were separated by SDS-PAGE and visualized by phosphorimaging analysis. B, three independent in vitro binding assays were quantified by phosphorimaging analysis and expressed as an average of the number of decays/min/mm² (dpm/mm²). The error bars represent one S.D. The numbers above the bars indicate the amount of p53 and Nbs1 input that specifically bound to GST-Mdm2 protein relative to GST.

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infected with the vector control retrovirus. In contrast, 60 min postirradiation, cells overexpressing Mdm2 showed little change in the percentage of tail moments that were >4 and no increase in the tail moments that were <4 (Fig. 8A), suggesting an inhibition of DNA repair in these cells. Similar results were obtained when DNA damage and repair were measured with visual comet scoring (data not shown). Although in repeated experiments, γ-irradiation of NIH3T3 cells overexpressing Mdm2 consistently led to longer tail lengths for an extended period of time when directly compared with cells infected with
vector control, Mdm2 overexpression did not block DNA repair. Specifically, 2 hours postirradiation, cells that overexpressed Mdm2 or vector control had similar high percentages of tail moments, indicating DNA repair occurred in the cells overexpressing Mdm2. In agreement with this, Mdm2 overexpression did not correlate to an overall decrease in cell survival or altered rates of proliferation, since a comparable number of cells appeared to survive irradiation, and similar rates of growth in short term assays were observed whether cells overexpressed Mdm2 or vector control (data not shown). Combined, these results indicate that Mdm2 overexpression delays DNA repair independent of ARF without appearing to alter cell growth or survival.

To determine whether p53 is required to mediate this effect of Mdm2 overexpression, we repeated these studies in p53−/− MEFs. In accordance with the previous results, Mdm2 overexpression in cells lacking p53 also showed a delay in DNA repair. 90 min following γ-irradiation, over 80% of the p53−/− MEFs overexpressing Mdm2 had tail moments >4, whereas less than 50% of the vector control-infected p53−/− MEFs had tail moments of >4 (Fig. 8B). Moreover, there was a large increase in the percentage of tail moments <4 in the vector control-expressing cells and only a slight increase in the percentage of tail moments <4 in cells overexpressing wild-type Mdm2 (Fig. 8B). Similarly, when the 198–489 mutant Mdm2 lacking the p53 binding domain and containing the Nbs1 interaction domain (Fig. 3A) was retrovirally expressed in NIH3T3 cells, mean tail moments of >4 and <4 were not significantly different 60 min following γ-irradiation, whereas there was significant DNA repair in the vector control-infected cells (Fig. 8C). Thus, the delay by Mdm2 of DNA repair does not require the p53-binding domain of Mdm2 and is independent of p53 expression.
We postulated that if Mdm2/Nbs1 interaction was necessary for the inhibition of DNA repair, then deletion of the Nbs1 binding domain in Mdm2 should restore the rate of DNA repair. Therefore, p53–/– MEFs were infected with retroviruses expressing the 298–489 Mdm2 mutant, which lacks both the p53 binding domain and the Nbs1 binding domain (Fig. 3A), wild-type Mdm2, or vector control. p53–/– MEFs were chosen, since expression of the 298–489 Mdm2 mutant inhibits proliferation and induces apoptosis in cells that have wild-type p53 (39). As predicted, GFP– MEFs expressing the 298–489 Mdm2 mutant did not have a delay in DNA repair, since the disappearance of tail moments of >4 and the appearance of tail moments of <4 occurred at rates similar to those of GFP– vector control cells (Fig. 8D). Although there appeared to be a slight increase in the initial amount of DNA damage in the MEFs overexpressing the 298–489 Mdm2 mutant in comparison with the DNA damaged sustained in MEFs with empty vector, the percentage decrease in tail moments >4 and percentage increase in tail moments <4 between the two were analogous. Specifically, between 5 and 60 min postirradiation in MEFs overexpressing the 298–489 Mdm2 mutant and in MEFs with empty vector, there was a 29.4 and 32.4% decrease in tail moments >4 and a 28.2 and 27.9% increase in tail moments <4, respectively. In contrast, there was only a 7.8% decrease in tail moments >4 and a 5.7% increase in tail moments <4 in MEFs overexpressing Mdm2. These results indicate that wild-type Mdm2 delayed DNA repair, whereas Mdm2 lacking the Nbs1 binding domain did not. Therefore, Mdm2 overexpression delays DNA double strand break repair, and this requires the Nbs1 binding domain in Mdm2.

Mdm2 is an E3 ubiquitin ligase that regulates the expression and activity of p53 and other proteins by ubiquitinylating them (21–23, 45). To determine whether the ubiquitin ligase activity of Mdm2 is required for the delay in DNA repair imposed by Mdm2, we retrovirally infected p53-null MEFs with the Mdm2 mutant (residues 198–400) that lacks the entire ubiquitin ligase domain as well as the p53-binding domain but that still retains the Nbs1 binding domain (Fig. 3A). To our surprise, overexpression of the 198–400 Mdm2 mutant delayed DNA repair to a similar extent as wild-type Mdm2 (Fig. 8D). At 5 and 60 min postirradiation, the percentage of tail moments >4 and <4 in GFP– MEFs overexpressing the 198–400 Mdm2 mutant paralleled those of MEFs that overexpressed wild-type Mdm2 (Fig. 8D). There was a 12.5% decrease in tail moments >4 and a 9.2% increase in tail moments <4 in the MEFs that overexpressed the 198–400 Mdm2 mutant, which were similar to the percentages quantified for MEFs overexpressing Mdm2 (see above). These data indicate that the loss of the ubiquitin ligase domain did not prevent Mdm2 from delaying DNA repair, and therefore, the ubiquitin ligase activity of Mdm2 is not required for its ability to inhibit DNA repair.

**DISCUSSION**

Mdm2 has p53-dependent and -independent functions (28, 31–34, 36), both of which are likely to contribute separately to tumorigenesis. Mdm2 regulation of p53 is firmly established (45), whereas p53-independent Mdm2 activity is not well characterized. For example, overexpression of Mdm2 in mice alters DNA ploidy and has resulted in transformation that is independent of p53 expression (31–33), yet the pathways that mediate these Mdm2-initiated cellular processes are unknown. Here we describe the identification and characterization of the novel interaction of Mdm2 with the DNA repair protein Nbs1. The binding of Mdm2 to Nbs1 was direct and independent of
both p53 and ARF. These results provide critical new insights on Mdm2 and the p53-independent oncogenic pathways that Mdm2 may influence. In particular, our finding that Mdm2 and Nbs1, but not p53, bind at Nbs1 nuclear foci implies Mdm2 in a DNA repair response distinct from the DNA damage checkpoint response regulated by p53. Moreover, a previous report showed that overexpression of Mdm2 increased DNA instability and chromosomal abnormalities, which was attributed to the inhibition by Mdm2 of p53 (50). However, in light of our results, Mdm2 overexpression also appears to negatively impact the DNA repair function of the M-N-R complex, which could lead to loss of DNA integrity or generation of chromosomal abnormalities. Mdm2 inhibition of DNA double-strand break repair was independent of p53 and ARF expression and relied on the domain in Mdm2 that binds to Nbs1. Additionally, Mdm2 was reported to bind to and alter the activity of DNA polymerase 𝜖, an enzyme that is important in DNA repair (51). Therefore, Mdm2 may regulate DNA repair through its interactions with the M-N-R complex, DNA polymerase 𝜖, and possibly other proteins necessary for restoring broken DNA. Thus, Mdm2 would impact the DNA damage response in two complementary ways: first by controlling the activity of p53, the sensor of DNA damage, and second by regulating Nbs1 and DNA polymerase 𝜖, proteins that repair DNA.

Formation of Nbs1 foci at sites of DNA damage involves many proteins, including BRCA1, ATM, the phosphorylated histone protein H2AX, Mre11, and many others, whose expression is critical for maintaining DNA integrity (1, 7, 12, 52). Although BRCA1 and ATM are necessary for DNA repair, a deficiency in BRCA1 or ATM does not inhibit Nbs1 focus formation (7, 48). In contrast, loss of H2AX inhibits the formation of Nbs1 foci and co-localization of BRCA1 to foci (52), suggesting that H2AX expression is requisite for Nbs1 and BRCA1 to localize to sites of DNA damage. Furthermore, the forkhead-associated and breast cancer C-terminal domains of Nbs1 are required for Nbs1 localization with H2AX at sites of DNA strand breaks (6, 53). However, these N-terminal domains of Nbs1 are dispensable for interaction with Mre11 and Mdm2 (6). The C terminus of Nbs1 directly binds to Mre11 and controls the cellular localization of Mre11 (6). Mre11 is cytotoxic in unirradiated cells from NBS patients, whereas Mre11 is primarily nuclear in cells expressing wild-type Nbs1 (9). In contrast, there was no detectable difference in Mdm2 localization in unirradiated NBS cells (2). Similar to Nbs1 control of Mre11/Rad50 localization, Nbs1 may be involved either directly or indirectly in relocating Mdm2 to foci upon DNA damage. On the other hand, it is interesting to speculate that the shuttling functions of Mdm2 (24) may be important for Nbs1 localization following DNA damage, since the ubiquitin ligase activity of Mdm2 does not appear to be necessary for its ability to inhibit DNA repair. Alternatively, Nbs1 may retain Mdm2 at sites of DNA damage rather than either protein bringing the other protein to DNA breaks, although even when Nbs1 was expressed, Mdm2 localization was not observed at every Nbs1 focus. These data are consistent with the observation that BRCA1 did not always co-localize with the M-N-R complex, which was attributed to the order of recruitment of BRCA1 and the M-N-R complex to sites of DNA damage (49). Moreover, in yeast, a whole host of proteins are recruited to sites of DNA damage much later than the M-N-R complex, which has been shown to be one of the earliest protein complexes to arrive at DNA breaks (54). Similarly, the kinetics of Mdm2 localization to DNA strand breaks may differ from that of Nbs1 in that Mdm2 may localize to DNA damage at a later time than Nbs1 does. On the other hand, Mdm2 may only transiently associate with Nbs1 and only bind to Nbs1 at specific times in the DNA repair process, which is dynamic and unsynchronized with other sites of DNA damage. In addition, it has been recently postulated that the larger and more brightly staining foci at later times following DNA damage represent DNA breaks that are irreparable or delayed in their repair (55). In accordance with this theory, Mdm2 may associate with Nbs1 at foci that are having difficulty repairing their DNA, as Mdm2/Nbs1 co-localization was more prominent at later time points than in the first 2 h following DNA damage. Although since early foci are fainter and more difficult to detect, Mdm2 localization at early foci may be underestimated. Nevertheless, in a single cell at one point in time, Mdm2 co-localization in a subset of Nbs1 foci would be expected, as we observed. However, further analysis is required to define the regulation of Mdm2 localization to nuclear foci, the release of Mdm2 from these sites, and the role Nbs1/Mdm2 interactions have in these processes. In addition, investigations are needed to determine whether Mdm2 overexpression delays DNA repair by inhibiting Nbs1 localization to or function at foci.

The association of Mdm2 with Nbs1 does not require Mre11, ATM, BRCA1, or p53, although all of these proteins are necessary for a normal cellular response to ionizing irradiation and to maintain DNA integrity (1, 45). ATM is required to signal the DNA damage response by phosphorylating many target proteins. Following γ-irradiation, ATM phosphorylates Mdm2 and p53 (13, 17). Phosphorylation of Mdm2 inhibits Mdm2 from targeting p53 for degradation (13), whereas phosphorylation of serine 15 on p53 activates p53 (17). BRCA1 is also phosphorylated by ATM in response to γ-irradiation (43). Recently, a complicated regulatory mechanism between Nbs1, Mre11, and ATM activation has been elucidated. Nbs1 and Mre11 are phosphorylated by ATM following γ-irradiation (14–16), and Nbs1 and Mre11 are necessary to activate ATM (56–58). However, Nbs1 is not required for ATM-dependent phosphorylation of p53 (57). Whether Nbs1 is required for Mdm2 phosphorylation by ATM remains to be determined. Of note, phosphorylation of serine 395 in Mdm2 by ATM is reported to be at least partially responsible for destabilizing Mdm2 following γ-irradiation (59). However, we observed similar amounts of Mdm2 bound to Nbs1 prior to and following γ-irradiation, suggesting that the Mdm2 associated with Nbs1 may be stable. Thus, additional experiments are needed to determine whether phosphorylation of Nbs1 and/or Mdm2 alters Mdm2 and Nbs1 binding and the stability of this interaction. For example, preliminary mass spectrometry analysis revealed that in unirradiated cells, Nbs1 is phosphorylated at a site distinct from the ATM phosphorylation sites when bound to Mdm2 (2). Therefore, post-translational modifications are likely to regulate the association of Mdm2 and Nbs1 and the localization and activity of this complex.

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