The Mre11-Rad50-Nbs1 Complex Acts Both Upstream and Downstream of Ataxia Telangiectasia Mutated and Rad3-related Protein (ATR) to Regulate the S-phase Checkpoint following UV Treatment*

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The Mre11-Rad50-Nbs1 (MRN) complex is required for mediating the S-phase checkpoint following UV treatment, but the underlying mechanism is not clear. Here we demonstrate that at least two mechanisms are involved in regulating the S-phase checkpoint in an MRN-dependent manner following UV treatment. First, when replication forks are stalled, MRN is required upstream of ataxia telangiectasia mutated and Rad3-related protein (ATR) to facilitate ATR activation in a substrate- and dosage-dependent manner. In particular, MRN is required for ATR-directed phosphorylation of RPA2, a critical event in mediating the S-phase checkpoint following UV treatment. Second, MRN is a downstream substrate of ATR. Nbs1 is phosphorylated by ATR at Ser-343 when replication forks are stalled, and this phosphorylation event is also important for down-regulating DNA replication following UV treatment. Moreover, we demonstrate that MRN and ATR/ATR-interacting protein (TRIP) interact with each other, and the forkhead-associated/breast cancer C-terminal domains (FHA/BRCT) of Nbs1 play a significant role in mediating this interaction. Mutations in the FHA/BRCT domains do not prevent ATR activation but specifically impair ATR-mediated Nbs1 phosphorylation at Ser-343, which results in a defect in the S-phase checkpoint. These data suggest that MRN plays critical roles both upstream and downstream of ATR to regulate the S-phase checkpoint when replication forks are stalled.

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The Mre11-Rad50-Nbs1 (MRN)3 complex plays essential roles in the maintenance of genome stability (1, 2). Mutations in the NBS1 and MRE11 genes lead to two human recessive disorders, Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia-like disorder (ATLD), respectively (3, 4). Mutant cells from patients with these disorders are extremely sensitive to radiation, fail to suppress DNA replication following ionizing radiation (IR), and exhibit a high degree of genome instability. The Mre11 complex is involved in both DNA repair and checkpoint activation, which provides a molecular basis for the genome instability observed in NBS and ATLD patients.

Substantial evidence suggests that the Mre11 complex plays a key role in the cellular responses to double strand breaks (DSBs) (5, 6). It migrates to DSBs immediately after these are generated, forming nuclear foci that co-localize with γH2AX (7–9). MRN binds directly to ATM stimulating ATM kinase activity to promote phosphorylation of multiple substrates (10–15). Nbs1 itself is a substrate of ATM, and ATM-dependent phosphorylation of Nbs1 at serine residue 343 is important for mediating the S-phase checkpoint when DSBs are generated after IR (16–19). The Mre11 complex carries exonuclease activity and acts on different types of double-stranded DNA ends (20, 21). It has been demonstrated that MRN directly participates in DSB repair by participating in homologous recombination pathways (22–24).

Although MRN plays a critical role in the damage responses that are initiated when DNA DSBs are detected, a connection between MRN and the ATR pathway recently emerged. The primary function of ATR is to monitor DNA replication (25, 26). It is activated by single-stranded DNA arising when replication forks are stalled or generated when DNA ends are processed. ATR stably interacts with ATRIP, and through this interaction ATR is recruited to RPA-coated single-stranded DNA at damage sites (27, 28). Recently, it was demonstrated that following HU-induced replication arrest, Nbs deficiency leads to an impaired G2/M checkpoint and a failure to restart DNA synthesis, similar to the defects observed in ATR-deficient cells (29). Rad50 depletion also causes hypersensitivity to UV treatment, a damage-induced pathway that requires functional ATR (30). Co-purification of MRN and ATR was also recently reported (31). In some cases, MRN facilitates ATR-mediated response.

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3 The abbreviations used are: MRN, Mre11-Rad50-Nbs1; ATLD, ataxia telangiectasia-like disorder; ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3-related protein; NBS, Nijmegen breakage syndrome; DSB, double strand break; IR, ionizing radiation; HU, hydroxyurea; PIPES, 1,4-piperazineethanesulfonic acid; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; GST, glutathione S-transferase; shRNA, short hairpin RNA; RPA, RNase protection assay; FHA, Forkhead-associated; BRCT, breast cancer C-terminal; ATRIP, ATR-interacting protein; UVDS, UV-resistant DNA synthesis.
mediated phosphorylation (29, 30, 32), although it was also reported that UV-induced Chk1 phosphorylation is MRN-independent (31, 33).

Here we demonstrate that MRN acts both upstream and downstream of ATR activation to mediate the UV-induced S-phase checkpoint. We show that the requirement of MRN to facilitate ATR-mediated phosphorylation following UV and HU treatment is both dose- and substrate-dependent. Although phosphorylation of several ATR substrates is impaired in Mre11- and Nbs1-deficient cells after a low dose of UV or HU treatment, MRN is dispensable for the phosphorylation of these substrates when relatively high doses of UV light or HU are used. Interestingly, unlike other ATR substrates, ATR-mediated RPA2 phosphorylation depends on MRN even when cells are treated with relatively high doses of UV light or HU. This MRN-dependent phosphorylation of RPA2 by ATR contributes to the MRN-mediated S-phase checkpoint regulation. In addition, we show that Ser-343 of Nbs1 is directly phosphorylated by ATR when replication forks are stalled. ATR-mediated phosphorylation of Ser-343 represents another pathway distinct from RPA2 phosphorylation in S-phase checkpoint control. Furthermore, we demonstrate that ATR interacts with MRN through the FHA/BRCT domains of Nbs1, and this interaction is not only required for Nbs1 phosphorylation at Ser-343 but is also important for mediating the S-phase checkpoint following UV treatment.

EXPERIMENTAL PROCEDURES

Cell Culture, Cell Synchronization, Transfections, Retroviral Infections, and shRNA—U2OS, 293T, GM847, or GM847-KD cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and 5% super calf serum. The cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum with vitamins and nonessential amino acids (35) were cultured in minimum Eagle’s medium containing 20% fetal bovine serum with vitamins and nonessential amino acids as described (36). Wild-type Nbs1 or Mre11 was introduced into the GM07166 or the ATLD cell lines, respectively, by infection of retroviruses followed by selection of puromycin-, G418-, or hygromycin-resistant cells. T98G cells were synchronized by treating with aphidicolin in hypotonic buffer (10 mM Tris, pH 7.5, 10 mM KCl, 1.5 mM MgCl2) and homogenized with a Dounce homogenizer. After incubation in horseradish peroxidase-conjugated secondary antibodies, nuclear extracts were prepared. After washing with cold phosphate-buffered saline, cell pellets were resuspended and lysed in NETN (20 mM Tris, pH 7.5, 1.2M KCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 20% glycerol). Nuclear debris were removed by centrifugation. Nuclear extracts were diluted in NETN, and immunoprecipitates were extensively washed in lysis buffer and resuspended in 2× SDS loading buffer and boiled. Western blot analysis was performed by separating proteins by SDS-PAGE, followed by transfer to nitrocellulose membranes. Membranes were incubated overnight in primary antibodies followed by 1 h of incubation in horseradish peroxidase-conjugated secondary antibodies.

For detecting the endogenous interaction of MRN with ATR, nuclear extracts were prepared. After washing with cold phosphate-buffered saline, cell pellets were resuspended and lysed in hypotonic buffer (10 mM Tris, pH 7.5, 10 mM KCl, 1.5 mM MgCl2) and homogenized with a Dounce homogenizer. After centrifugation, nuclear pellets were resuspended in low salt buffer (20 mM Tris, pH 7.5, 20 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 20% glycerol) and homogenized. Subsequently, high salt buffer (20 mM Tris, pH 7.5, 1.2 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 20% glycerol) was added dropwise, and nuclear debris were removed by centrifugation. Nuclear extracts were dialyzed into buffer BC100 (20 mM Tris, pH 7.5, 100 mM KCl, 1.5 mM MgCl2, 0.2 mM EDTA, and 20% glycerol). Nuclear extracts were diluted in NETN, and immunoprecipitation was performed using an affinity-purified polyclonal antibody to Nbs1.

Chromatin isolation from U2OS cells was performed as described previously (40). For chromatin isolation from GM07166 or ATLD cells, cells were washed once with phosphate-buffered saline, trypsinized, collected, resuspended in a
small volume of CSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, and protease inhibitors), and incubated on ice for 2 min. Cytoplasmic proteins were separated by centrifugation, and isolated nuclei were lysed in solution B (3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol) and protease inhibitors. Soluble nuclear proteins were removed by centrifugation, and the pellet was resuspended in CSK buffer and boiled after adding 2× SDS loading buffer. To detect damage foci after UV treatment in NIH3T3 cells that were reconstituted with human Nbs1, cells were treated with 70% methanol and 30% acetone at −20 °C for 20 h, followed by immunostaining analysis as described (41).

ATR in Vitro Kinase Assay—FLAG-tagged ATR (a generous gift from R. T. Abraham) was transiently transfected into 293T cells. Cells were lysed 48 h after transfection in NET-Tween lysis buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-Cl, pH 8.0, 0.5% Tween 20) containing protease and phosphatase inhibitors. FLAG-tagged ATR was immunoprecipitated by incubating cell lysates with anti-FLAG M2 antibody (Sigma) at 4 °C for 4 h, followed by the addition of secondary antibody (MP Biomedicals) and protein A-agarose (Calbiochem) for 1 additional h. Immunoprecipitates were extensively washed (3× in NET-Tween, 2× in kinase buffer) and then incubated with glutathione-eluted GST fusion proteins in the presence of [γ-³²P]ATP in ATR kinase buffer (25 mM HEPES, pH 7.4, 50 mM NaCl, 10 mM MnCl₂, 10 mM MgCl₂, 1 mM dithiothreitol) at 30 °C for 30 min. Proteins were separated by SDS-PAGE; gels were dried and exposed to film. Coomassie-stained gels confirmed that equal amounts of substrate proteins were added to the kinase reactions.

UV-resistant DNA Synthesis—Inhibition of DNA synthesis following exposure to UV light was performed as described previously (40). Briefly, U2OS cells were grown in the presence of 0.02 µCi per ml of [¹⁴C]thymidine for 24 h. Two hours after the removal of [¹⁴C]thymidine, cells were either mock-treated or irradiated with 10 J/m² of UV light. Cells were allowed to recover 1 h and were then labeled with 2.5 µCi per ml of [³²P]thymidine for 15 min. Cells were spotted onto Whatman microfiber filters, washed extensively with trichloroacetic acid and 100% ethanol, and vacuum-dried. Radioactivity was measured using a liquid scintillation counter and a dual energy program.

Antibodies—Antibodies used in this study were purchased from Bethyl Laboratories (Smc1-S966p), Cell Signaling (Nbs1-S343p), Oncogene (Mre11, RPA1, and RPA2), R & D Systems (Chk1-S317p), Santa Cruz Biotechnology (Chk1 and Ku70), Sigma (M2), and Upstate (Rad50). The monoclonal antibodies against Nbs1 (EE15 (16)) and the polyclonal antibodies against Mre11 (D27 (42)) and Nbs1 (D29 (16)) were described previously.

RESULTS

Mre11 and Nbs1 Are Required for Mediating the S-Phase Checkpoint in Response to UV Radiation—In response to IR when DSBs are generated, ATM is activated, and MRN is involved in one of the ATM-dependent pathways required for mediating the S-phase checkpoint (43). When replication forks are stalled, the S-phase checkpoint is also activated (44), but the underlying mechanisms have not been extensively studied. To test if MRN is required for mediating the S-phase checkpoint in response to UV-induced DNA damage, we silenced endogenous Mre11 or Nbs1 in U2OS cells by retroviral infection of two shRNAs directed against Mre11 or Nbs1. These rates were expressed as percentages by normalizing to the appropriate mock-treated controls. The average of three independent experiments is shown in the graph, with error bars indicating the standard deviation from the average (top). Cells retrovirally infected with vector alone or with two shRNAs directed against either Nbs1 or Mre11 were lysed and immunoblotted as indicated (bottom).
FIGURE 2. Chk1 and SMC1 phosphorylation depends on Mre11 and Nbs1 after low dose of UV radiation or HU treatment. A, Nbs1-deficient cell line (GM07166, left panel) and the Mre11-deficient cell line (ATLD, right panel), both immortalized by the expression of hTERT, were retrovirally infected with vector or with wild-type Nbs1 and Mre11, respectively. Whole cell lysates were immunoblotted as indicated. B, Nbs1-deficient cell line (GM07166) reconstituted with vector (Vec) or Nbs1 and the Mre11-deficient cell line (ATLD) reconstituted with vector or Mre11 were mock-treated (−), exposed to a low dose (2 J/m²), a relatively high dose (10 J/m²), or an intermediate dose of UV light (5 J/m²) and then lysed 2 h after treatment. Lysates were immunoblotted with a phospho-specific antibody to serine 317 of Chk1 (Chk1-S317p), a phospho-specific antibody to serine 966 of SMC1 (Smc1-S966p), and antibodies to total cellular Chk1 and Ku70 as loading controls. The relative intensities of the bands for Chk1-S317p and SMC1-S966p in the GM07166 and ATLD derivative cell lines with and without UV or HU treatment were quantified by densitometry using Chk1 and Ku70 as loading controls. At the low UV dose (2 J/m²), the addition of Nbs1 to the GM07166 cell line (top left panel) resulted in a 2.3- and 2.0-fold increase in the phosphorylation of Chk1-S317 and SMC1-S966, respectively (top, left, marked as Nbs1/Vec). In the ATLD cell line reconstituted with Mre11 (top right panel), a 2.7- and a 4.3-fold increase is seen for Chk1-S317p and Smc1-S966p, respectively, compared with adding vector alone (top, right, marked as Mre11/Vec). At a higher dose (10 J/m²) of UV light (bottom panel), Chk1 phosphorylation at serine 317 is independent of Nbs1, whereas partial dependence on Nbs1 was observed at an intermediate dose (5 J/m²). C, U2OS cells retrovirally infected twice with vector alone, with two shRNAs directed against Mre11, or with two shRNAs directed against Nbs1 were mock-treated (−), exposed to a low dose of UV light (2 J/m², 2 h after, top left), treated with a low concentration of HU (0.1 mM, 2 h after, top right) or to higher doses of UV light (10, and 30 J/m², 2 h after, bottom). Two hours after treatment, cells were lysed, and whole cell lysates were immunoblotted as indicated. The relative intensities of the bands for Chk1-S317p and Smc1-S966p were quantified by densitometry as described above. Upon low dose UV (2 J/m²) and HU (0.1 mM) treatment, silencing the expression of Mre11 or Nbs1 in U2OS cells by shRNAs resulted in a decrease in Chk1-S317 and SMC1-S966 phosphorylation, and the relative decrease is indicated.
the shRNAs used to silence Mre11 and Nbs1 were acting effectively. The rate of DNA synthesis following exposure to UV radiation was determined in these cells, with the vector-infected cells serving as a control. Although the rate of DNA synthesis was down-regulated following UV treatment in the cells infected with vector alone, silencing endogenous Mre11 or Nbs1 inhibited this down-regulation (Fig. 1, top). This result suggests that MRN is required for mediating the replication checkpoint in response to UV-induced DNA damage. These data are also consistent with the finding that inhibition of Rad50 expression impairs the down-regulation of DNA synthesis in response to a low dose of UV treatment (30).

**Upon UV and HU Treatment, Chk1 and SMC1 Phosphorylation Depends on Mre11 and Nbs1 Only at Low Doses, whereas RPA2 Phosphorylation Requires Mre11 and Nbs1 Even at High Doses**—MRN is required to activate ATM in response to IR (10, 13–15, 46) and is also required downstream of ATM to mediate the S-phase checkpoint response (17, 18). Recently, some reports suggest that MRN is also required for activating ATR to phosphorylate its substrates (29, 30, 32), although other reports demonstrate that MRN is dispensable for ATR activation (31, 33). Because silencing Mre11 or Nbs1 resulted in a defect in the inhibition of DNA synthesis in response to UV radiation, we investigated in detail whether MRN is required to activate ATR following UV- or HU-induced DNA damage, and thereby involved in the regulation of the S-phase checkpoint.

Nbs1-deficient cells (GM07166) or Mre11-deficient cells (ATLD), immortalized by the expression of hTERT, were reconstituted with Nbs1 or Mre11, respectively, or with vector alone (Fig. 2A). To examine the effect of Nbs1 or Mre11 deficiency on the activation of ATR, phosphorylation of Chk1 and SMC1 was monitored after mock-treatment (−) or exposure to UV light, or treatment with HU. Chk1 phosphorylation at serine 317 and SMC1 phosphorylation at serine 966 were both reduced in the Nbs1- and the Mre11-deficient cells after exposure to a low dose of UV radiation (2 J/m²; Fig. 2B, top panels) in comparison with Nbs1 and Mre11 reconstituted cell lines. However, at a higher dose of UV radiation (10 J/m²), the absence of Nbs1 did not have any obvious effect on either of these phosphorylation events, whereas partial dependence on Nbs1 was observed at an intermediate dose (5 J/m²) (Fig. 2B, bottom). Similar results were obtained when the expression of Mre11 or Nbs1 was silenced in U2OS cells by shRNAs. Phosphorylation of Chk1 and SMC1 significantly decreased after low doses of UV (2 J/m²) and HU (0.1 mM for 2 h) treatment, when compared with the vector-infected control cells (Fig. 2C, top panels). However, there was no significant difference when higher doses of UV light (10 or 30 J/m²) or HU (1 mM for 24 h) were used (Fig. 2C, bottom, and data not shown). These data suggest that the requirement of MRN to facilitate ATR-mediated Chk1 and SMC1 phosphorylation is dependent upon the dose of UV light or HU. At higher doses of UV light and HU, redundant pathways may be activated, and MRN becomes dispensable for ATR-mediated phosphorylation of Chk1 and SMC1.

When replication forks are stalled, RPA2 is phosphorylated on chromatin by ATR (40). Interestingly, contrary to Chk1 and SMC1 phosphorylation, optimal phosphorylation of RPA2 by ATR requires MRN even at high doses of UV light and HU. RPA2 phosphorylation was impaired in the Nbs1-deficient cell line (GM07166) after a high dose of UV treatment (30 J/m²), and the defect was corrected when Nbs1 was reconstituted (Fig. 3A). Inhibition of Nbs1 or Mre11 expression by shRNAs in U2OS cells also inhibited ATR-dependent RPA2 phosphorylation after high doses of UV (10 J/m²) and HU treatment (1 mM for 24 h; Fig. 3B and data not shown). These data demonstrate that the requirement of MRN to promote ATR activation is different when Chk1 and SMC1 or RPA2 is phosphorylated by ATR, which suggests that MRN likely serves as a mediator for ATR activation. Depending on cellular context, MRN may play different roles to facilitate ATR-mediated phosphorylation toward its different substrates.

**Both RPA2 Phosphorylation and Nbs1 Phosphorylation at Ser-343 by ATR Are Required for Mediating the S-phase Checkpoint following UV Treatment**—MRN is required for optimal phosphorylation of multiple ATR substrates when replication forks are stalled after low dose of either UV or HU treatment. However, at the UV dose (10 J/m²) we used to examine DNA...
RPA2 phosphorylation in response to UV light is required for the inhibition of DNA synthesis following UV light (40), the dependence of RPA2 phosphorylation on MRN may explain why Mre11 and Nbs1 deficiency results in a defect in the UV-induced S-phase checkpoint response. On the other hand, mutating major ATR phosphorylation sites on RPA2 only led to a partial UVDS phenotype, whereas inhibition of Nbs1 expression had a stronger effect (Fig. 4A, compare Vector and RPA2-pMut) (40). This suggests that redundant pathways may exist, and the requirement of MRN for ATR-mediated phosphorylation of RPA2 is important in one pathway for MRN to mediate the S-phase checkpoint.

It has been shown that in response to IR when DSBs are generated, Nbs1 is phosphorylated at Ser-343, and this phosphorylation plays a critical role in down-regulating DNA synthesis following IR (17, 18). Nbs1 phosphorylation at Ser-343 was also observed after UV and HU treatment (Fig. 4B) (47). We examined whether phosphorylation of Ser-343 after UV treatment is important for MRN to mediate the S-phase checkpoint. Myc-tagged Nbs1 wild-type or the Nbs1-S343A mutant carrying silent mutations at the shRNA-targeting sites was expressed in U2OS cells after retroviral infection, and the expression of endogenous Nbs1 was suppressed by shRNAs (Fig. 4A, right). Mutating Ser-343 of Nbs1 led to a partial UVDS phenotype (Fig. 4A, left), suggesting that phosphorylation of Ser-343 is also important for mediating the S-phase checkpoint following UV radiation. Therefore, both RPA2 phosphorylation and Nbs1 phosphorylation at Ser-343 contribute to the inhibition of DNA synthesis following UV treatment.

To examine whether Nbs1 phosphorylation at Ser-343 was dependent upon ATR kinase activity after UV and HU treatment, an SV40-transformed fibroblast cell line, GM847 or GM847 cells expressing a doxycycline-inducible kinase inactive allele of ATR (GM847-KD; see Ref. 34), was either mock-treated (−) or
treated with UV light or HU. Lysates from the cells overexpressing the kinase-inactive allele of ATR (GM847-KD) showed a substantial decrease in Nbs1 phosphorylation at serine 343 following UV and HU treatment, compared with lysates from the GM847 cells in which ATR kinase activity was normal (Fig. 4C). Overexpression of the kinase-inactive allele of ATR also led to a decrease in UV-induced Chk1 phosphorylation and RPA2 phosphorylation, indicating that ATR kinase activity was being inhibited when the kinase-inactive allele was induced in the GM847-KD cells (40). This result suggests that ATR kinase activity is required for phosphorylation of Nbs1 at serine 343 in response to UV and HU treatment of cells.

To determine whether ATR could phosphorylate serine 343 of Nbs1 directly in vitro, a fragment of Nbs1 consisting of amino acids 332–363 with a GST tag at its amino-terminal end or this same fragment carrying S343A was expressed and purified from E. coli and used as substrates for an ATR in vitro kinase assay. Although GST Nbs1-(332–363) was phosphorylated by ATR in vitro, mutation of S343A in this fragment completely abolished ATR-mediated phosphorylation (Fig. 4D), demonstrating that ATR can directly phosphorylate Nbs1 at serine 343 in vitro.

Our results suggest that both ATR-mediated phosphorylation of RPA2 and Nbs1 at Ser-343 contribute to the down-regulation of DNA synthesis following UV treatment. As it was described that ATM-mediated phosphorylation of Ser-343 in turn facilitated ATM to phosphorylate Chk2 (10, 46), we determined whether phosphorylation of RPA2 and Nbs1 at Ser–343 was truly two independent events after UV treatment. RPA2 phosphorylation was examined after mock (–) or UV treatment in the U2OS cells expressing Myc-tagged Nbs1 wild type or the Nbs1–S343A mutant with endogenous Nbs1 silenced by shRNAs. UV- induced RPA2 phosphorylation was not changed in the Nbs1–S343A mutant compared with that in Nbs1 wild-type cells (Fig. 4E, left). Similarly, Nbs1 phosphorylation at Ser-343 in response to UV light was also not altered by a defect in RPA2 phosphorylation (Fig. 4E, right). Collectively, these results suggest that ATR-mediated phosphorylation of RPA2 and Nbs1 are two independent pathways required to mediate the S-phase checkpoint following treatment of cells with UV light. Both of these two pathways are involved to activate the S-phase checkpoint in an MRN-dependent manner.

**Nbs1 Interacts with ATR/ATRIP through Its FHA/BRCT Domains**—To investigate further how MRN participates in the ATR pathway to regulate the S-phase checkpoint, we studied the interaction of ATR with MRN. It was described that MRN was co-purified with ATR by using chromatography (31). To examine the interaction of MRN with ATR, we overexpressed Myc-tagged Nbs1 with FLAG-tagged ATR. FLAG–ATR was co-immunoprecipitated with Myc-Nbs1, consistent with the previous finding that ATR interacts with MRN (31) (Fig. 5A). A weak association of endogenous ATR with MRN was observed when T98G cells were synchronized in S-phase but not in G1 (Fig. 5B, left), and HU treatment enhanced the endogenous interaction of ATR with MRN (Fig. 5B, right). These data suggest that a transient association of MRN with ATR may be induced when replication is initiated or when replication forks are stalled.

To map the domain on MRN that mediates its interaction with ATR, a series of Myc-tagged overlapping fragments of Nbs1 covering the entire open reading frame were transiently transfected into 293T cells, along with FLAG-tagged ATR. Myc-tagged Nbs1 or the fragments of Nbs1 were immunoprecipitated using an anti-Myc antibody. In addition to wild-type Nbs1, two fragments (amino acids 1–116 and 92–246) at the amino terminus of Nbs1 interacted with ATR (Fig. 5C). At the amino terminus, Nbs1 contains an FHA domain (residues 24–100) and two tandem BRCT domains (BRCT1-(114–182) and BRCT2-(221–330), Fig. 5C) (48–51). The two fragments that interact with ATR contained either the FHA (amino acid 1–116) or the first BRCT domain (amino acid 92–246) of Nbs1. Unlike ATR, endogenous Mre11 and Rad50 interacted with the carboxyl-terminal fragment of Nbs1 consisting of amino acids 613–754 (Fig. 5C). Consistently, a larger amino-terminal fragment of Nbs1 (amino acid 1–476) containing both FHA and BRCT domains bound ATR, whereas the carboxyl-terminal fragment of Nbs1 did not (amino acids 476–754, Fig. 5D). These results suggest that the FHA and BRCT domains at the amino terminus of Nbs1 may be involved in mediating the interaction of Nbs1 with ATR and that Nbs1 can interact with ATR independent of its interaction with Mre11 and Rad50.

To further examine whether the FHA and BRCT domains are important for mediating the interaction of MRN with ATR, deletion mutants of Nbs1 lacking the FHA domain (amino acids 116–754), the first BRCT domain (Δ92–209 amino acids), or both the FHA and two BRCT domains (amino acids 300–754) were examined. Deletion of the FHA or BRCT domain significantly reduced the interaction of Nbs1 with ATR, and deletion of both FHA and BRCT domains abolished the interaction of Nbs1 and ATR (Fig. 5E). We also mutated conserved amino acids in the FHA and BRCT domains and examined the interaction of Nbs1 with ATR. Serine to alanine substitutions at conserved residues R28A, Y176A, and E309A (49, 52) were introduced into the FHA and the two BRCT domains, respectively. As shown in Fig. 5F, the interaction of ATR with Nbs1-R28A (FHA) was reduced in comparison with wild-type Nbs1. The binding between ATR and the Nbs1 mutants Y176A (BRCT) and R28A/Y176A/E309A (FHA/BRCT) was reduced more significantly (Fig. 5F). These results suggest that both the FHA and the BRCT domains in the amino-terminal end of Nbs1 are important for mediating its interaction with ATR.

**Nbs1 Interacts with ATRIP Mutants That Are Defective in ATR or RPA Binding**—It has been shown that MRN interacts with RPA when replication forks are stalled (53). Because ATR associates with its regulatory partner, ATRIP (27), and ATRIP binds RPA (28), one possibility is that the interaction we observed between MRN and ATR is mediated through RPA. To test this possibility, we used two mutant alleles of ATRIP. The first allele (ATRIp-(108–791)) is missing the first 108 amino acids at the amino-terminal end of the ATRIP protein and disrupts the in vivo binding between ATRIP and RPA (39). The second mutant allele of ATRIP-(Δ656–684ATRIP) contains an internal deletion of amino acids 656–684 and disrupts the binding between ATRIP and ATR (39). Characterization of these two ATRIP mutant alleles confirmed that Myc-ATRIP-
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A

B

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D

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F

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whether the interaction of MRN and ATR/ATRIP is important for optimal phosphorylation of multi-proteins (54). The phosphorylation modification that is mediated by RPA. Because ATRIP can bind to MRN independent of its association with ATR, these results also suggest that either ATRIP mediates the association of MRN with ATR/ATRIP or both ATRIP and ATR interact with MRN.

To determine whether the interaction between Nbs1 and ATRIP is direct, we examined the interaction between Nbs1 and ATRIP in vitro using baculovirus-expressed proteins purified from insect cells. Using this method, we failed to detect direct binding between ATRIP and Nbs1 (data not shown). These results suggest the possibility that another protein that interacts with the FHA/BRCT domains of Nbs1 may mediate the in vivo association between MRN and ATR/ATRIP. Alternatively, the interaction of MRN with ATR/ATRIP is regulated through in vivo phosphorylation events, because both the FHA and BRCT domains generally interact with phosphorylated proteins (54). The phosphorylation modification that is required for the interaction is likely missing in the in vitro purified proteins.

The FHA and BRCT Domains of Nbs1 Are Important for Mediating Nbs1 Phosphorylation at Ser-343 and the S-phase Checkpoint When Replication Forks Are Stalled—We showed that MRN is required for optimal phosphorylation of multiple ATR substrates after UV and HU treatment. To test whether the interaction of MRN and ATR/ATRIP is important for MRN to modulate ATR activities, we examined phosphorylation of ATR substrates when the association of Nbs1 with ATR is disrupted by mutations in the Nbs1 FHA/BRCT domains.

Because Nbs1 itself is an ATR substrate, we first examined whether loss of interaction with ATR influenced Nbs1 phosphorylation. Myc-tagged Nbs1 wild type, the FHA mutant (R28A), the BRCT mutant (Y176A), or the FHA/BRCT double mutant (R28A/Y176A) with silent mutations at the shRNA sites were introduced into U2OS cells by retroviral infection. Expression of Myc-tagged Nbs1 cDNAs was similar at levels as endogenous Nbs1 (Fig. 6A, left). Subsequently, the expression of endogenous Nbs1 was silenced by shRNAs (Fig. 6A, right). Nbs1 phosphorylation at Ser-343 was monitored by using a phospho-specific antibody recognizing phosphorylated Ser-343. Although the mutation in FHA domain (R28A) reduced phosphorylation of Ser-343 after UV treatment, the mutations in BRCT domain (Y176A) and in both FHA/BRCT domains (R28A/Y176A) more dramatically impaired the UV-induced Nbs1 phosphorylation at Ser-343 (Fig. 6B). These data suggest that both FHA and BRCT domains are important for mediating Nbs1 phosphorylation when replication forks are stalled. Because the reduction of Nbs1 phosphorylation at Ser-343 correlates with the extent of impairment of ATR association, abnormal Nbs1 phosphorylation in the FHA and BRCT mutants is most likely caused by an impaired interaction of MRN with ATR.

We also examined whether the FHA/BRCT domains of Nbs1 are important for ATR to phosphorylate other substrates. U2OS cells expressing Nbs1 wild type or the FHA/BRCT mutant (R28A/Y176A) with endogenous Nbs1 silenced by shRNAs were lysed after mock treatment (−) or different doses of UV treatment. Mutations in the FHA/BRCT domains did not reduce ATR-mediated phosphorylation of Chk1 and SMC1 at either the low or the high doses of UV or HU treatment, and in fact, a slight increase was observed (Fig. 6C, left, and data not shown). Interestingly, RPA2 phosphorylation in the FHA/BRCT domains of Nbs1 was also reduced after UV treatment. U2OS cells expressing Nbs1 wild type or the FHA/BRCT mutant (R28A/Y176A) with endogenous Nbs1 silenced by shRNAs were lysed after mock treatment (−) or different doses of UV treatment. Mutations in the FHA/BRCT domains did not reduce ATR-mediated phosphorylation of Chk1 and SMC1 at either the low or the high doses of UV or HU treatment, and in fact, a slight increase was observed (Fig. 6C, left, and data not shown). Interestingly, RPA2 phosphorylation in the FHA/BRCT domains of Nbs1 was also reduced after UV treatment.
BRCT mutant significantly increased in comparison with Nbs1 wild-type expressing cells (Fig. 6C, right). These data suggest that the FHA and BRCT domains of Nbs1 are dispensable for MRN to promote ATR phosphorylation of its substrates such as Chk1, SMC1, and RPA2, although these domains are required for Nbs1 phosphorylation itself. Increased phosphorylation of ATR substrates, especially RPA2, could be due to a repair defect associated with mutations in the FHA/BRCT domains (see below).

We showed that impairment of Nbs1 phosphorylation at Ser-343 led to a partial UVDS phenotype, and ATR-mediated Nbs1 phosphorylation was largely dependent on the FHA/BRCT domains of Nbs1. Consistently, the Nbs1 FHA/BRCT mutant was defective in the S-phase checkpoint and led to a partial UVDS phenotype (Fig. 6D). These data suggest the idea that the ATR-mediated Nbs1 phosphorylation is important for mediating the S-phase checkpoint.

The FHA and BRCT Domains of Nbs1 Are Important for the Prevention of DSB Accumulation after UV Treatment—We showed that although the FHA and BRCT domains are required for Nbs1 phosphorylation, they are not required for ATR activation to phosphorylate several other substrates when replication forks are stalled. Intriguingly, RPA2 phosphorylation actually increased when the FHA/BRCT domains of Nbs1 were mutated. In response to IR, the FHA/BRCT domains are needed for IR-induced foci formation (52, 55, 56), suggesting that these two domains may be important for efficient DNA repair. To test this possibility, we checked H2AX phosphorylation in the U2OS cell lines expressing wild-type Nbs1 or the FHA/BRCT mutant, whereas the expression of endogenous Nbs1 was inhibited by shRNAs. H2AX phosphorylation indeed increased in the FHA/BRCT mutant in comparison with the Nbs1 wild-type cell line (Fig. 7A), suggesting that DSBs accumulated when the function of the FHA/BRCT domain of Nbs1 was impaired. Therefore, the FHA/BRCT domains are involved in the prevention of DSB accumulation when replication forks are stalled after UV treatment.

To test whether the FHA/BRCT domains are required for damage-induced foci formation after UV treatment similar to what is observed in response to IR (52, 55, 56), we performed immunostaining experiments. We introduced wild-type Nbs1 or the FHA/BRCT mutant (R28A/Y176A) into mouse NIH3T3 cell line by retroviral infection. The Nbs1 polyclonal antibody we used only detected the expression of human Nbs1 in NIH3T3 cells but not the endogenous mouse Nbs1 (Fig. 7B, top). Immuno-
staining was performed after UV or mock treatment. Although wild-type Nbs1 formed clear UV-induced damage foci, the FHA/BRCT domain mutant failed to do so (Fig. 7B), suggesting that the FHA/BRCT domains of Nbs1 are required for UV-induced foci formation. One scenario is that DSBs are generated when stalled replication forks collapse after UV treatment, and the FHA/BRCT domains that are required for damage foci formation are important for preventing DSB formation and/or repairing DSBs. Persistent presence of DSBs in the FHA/BRCT mutant leads to stronger phosphorylation of RPA2.

DISCUSSION

Our studies demonstrate that MRN regulates the S-phase checkpoint following UV treatment by at least two mechanisms. One is that MRN acts upstream of ATR to facilitate ATR-dependent phosphorylation of RPA2. The other is that Nbs1 is a direct substrate of ATR and acts downstream of ATR to suppress DNA replication following UV treatment. These studies establish further biological links of ATR and MRN when replication forks are stalled and clarify the pathways leading to S-phase checkpoint activation through the functional interaction of MRN with ATR.

MRN Regulates the S-phase Checkpoint following UV Treatment by at Least Two Mechanisms—The involvement of MRN in the S-phase checkpoint following treatment of cells with DNA cross-link agents or with a low dose of UV treatment has been described previously (30, 32). The interaction of Fanconi anemia proteins and MRN is suggested to play a role in the DNA cross-link-induced S-phase checkpoint (32). The mechanisms underlying MRN-dependent S-phase checkpoint activation following UV treatment is not clear. We showed that when relatively high doses of UV light are used, the S-phase checkpoint activation to down-regulate DNA synthesis still depends on MRN. Although under such conditions ATR-mediated Chk1 and SMC1 phosphorylation largely does not depend on MRN, RPA2 phosphorylation still requires MRN. We demonstrated previously that ATR-mediated RPA2 phosphorylation prevents the migration of RPA to replication centers, thereby contributing to the suppression of DNA replication following UV treatment (40). Therefore, MRN in part regulates the S-phase checkpoint by facilitating ATR to phosphorylate RPA2. These studies also suggest that the ATR/Chk1 and MRN/ATR pathways work in parallel to control the S-phase checkpoint following relatively high doses of UV treatment.

In response to IR, ATM directly phosphorylate Nbs1 at Ser-343, and this phosphorylation is important for down-regulating
DNA replication after DSBs are generated (17, 18). We demonstrated that when replication forks are stalled, phosphorylation of Nbs1 at Ser-343 is also induced and is directly mediated by ATR. This phosphorylation significantly contributes to S-phase checkpoint control following UV treatment. These results suggest that both ATM and ATR pathways utilize Nbs1 as a downstream effector to mediate the S-phase checkpoint. Although phosphorylation of Nbs1 at Ser-343 appears as an important switch to initiate the down-regulation of DNA replication when the S-phase checkpoint is activated, the mechanism is not clear. Our recent studies showed that a specific interaction of MRN with RPA is likely involved in the damage-induced suppression of DNA replication. At this point, it is tempting to speculate that through the interaction of MRN with RPA, ATM/ATR-mediated phosphorylation of Nbs1 at Ser-343 may regulate RPA replication function, leading to replication inhibition. Although RPA2 phosphorylation requires MRN, this phosphorylation and ATM-mediated Nbs1 phosphorylation are two independent events. Preventing RPA2 phosphorylation does not influence Nbs1 phosphorylation at Ser-343 by ATR following UV treatment, and vice versa. Mutating RPA2 phosphorylation sites or Ser-343 on Nbs1 each significantly leads to UVD5 but to a lesser extent than when the expression of MRN is suppressed. These data suggest that ATR-mediated RPA2 phosphorylation and Nbs1 phosphorylation are two independent pathways required to suppress DNA replication. MRN acts upstream of ATR to modulate RPA2 phosphorylation and also serves as a downstream effector to mediate the S-phase checkpoint.

**MRN Facilitates ATR-mediated Phosphorylation in Both a Substrate- and Dose-dependent Manner When Replication Forks Are Stalled**—MRN facilitates ATR-mediated phosphorylation of certain substrates in a manner that is dependent upon the dose of UV light or HU used. H2AX phosphorylation after HU and UV treatment does not depend on MRN (data not shown and see Refs. 29, 30). Phosphorylation of Chk1 and SMC1 requires MRN only after low dose of UV treatment. When a relatively high dose of UV light or HU is used, MRN becomes dispensable for Chk1 and SMC1 phosphorylation, whereas RPA2 phosphorylation is still dependent on MRN. These data suggest that MRN is not required for ATR activation per se, which is different from ATM activation. When DSBs are generated, MRN recruits ATM to the DSB sites and activates ATM as a damage sensor (10, 11, 57). MRN apparently plays a different role in ATR activation when replication forks are stalled.

ATR is recruited to sites of stalled replication forks. In the Xenopus cell-free system, ATR is recruited to chromatin under replication stress (58-61). Studies in mammalian cells demonstrated that ATR binds to chromatin preferentially in S-phase and forms nuclear foci, co-localized with RPA, when replication forks are stalled (62, 63). These observations suggest that ATR plays a central role in monitoring DNA replication on chromatin and detecting the signals when replication forks are stalled. Interestingly, MRN also binds to chromatin in an S-phase-specific manner, and this binding is enhanced by replication fork stalling (56, 64). Similar to ATR, MRN forms nuclear foci after UV and HU treatment and localizes to the sites of stalled replication forks (47, 64, 65). These observations suggest that MRN is recruited to the sites adjacent to where ATR binds on chromatin when forks are stalled. Accumulating evidence suggests that ATR phosphorylates its substrates on chromatin. Chk1 phosphorylation requires its association with chromatin, and after ATR-mediated phosphorylation, Chk1 disassociates from chromatin, a mechanism that is important for spreading the signals (66). It has also been shown that ATR-mediated phosphorylation of RPA2 and Rad17 occurs on chromatin (40, 67). MRN may act as a mediator to correctly position ATR and its substrates on chromatin, thereby facilitating ATR-mediated phosphorylation. Alternatively, MRN may be important for the stable association of ATR with chromatin, thus serving to amplify the ATR signals. It was described that the retention of ATR at damage sites depends on the presence of functional Nbs1 (29).

ATR-mediated phosphorylation of Chk1 and SMC1 is dependent upon MRN only at low doses of DNA damage-inducing agents. This is probably because of the existence of other mediator proteins, such as Claspin and TOPBP1 (68, 69). At relatively high doses, multiple pathways in addition to the MRN pathway are activated, and these additional pathways may play more predominant roles. In contrast to Chk1 and SMC1, ATR-mediated RPA2 phosphorylation depends on MRN even at relatively high doses. In this respect, an interaction of MRN and RPA was described after UV and HU treatment, and phosphorylated RPA co-localizes with MRN at replication forks (29, 53). This physical interaction of RPA and MRN may be important for ATR to recognize RPA2 as a substrate on chromatin and subsequently to phosphorylate RPA2, which makes RPA2 unique from other ATR substrates.

**The MRN-ATR Interaction Is Dependent on the FHA/BRCT Domains of Nbs1**—An interaction between MRN and ATR was detected (see Ref. 31 and this work). The interaction occurs in S-phase and is enhanced when replication forks are stalled. Because both MRN and ATR bind to chromatin preferentially in S-phase (56, 63, 64), it is possible that MRN and ATR interact on chromatin after both are recruited to chromatin at sites where replication forks are stalled. Alternatively, their interaction may be induced in S-phase, and subsequently as a complex, MRN and ATR are loaded onto chromatin. However, the interaction between MRN and ATR is weak and cannot be detected in vitro, suggesting that the interaction may be a transient event that is regulated by the cell cycle and/or damage signals.

The interaction of MRN with ATR requires the FHA and BRCT domains of Nbs1. Because both the FHA and the BRCT domains are implicated in mediating phosphorylation-dependent protein-protein interactions (54), the association of MRN and ATR is likely achieved by cell cycle and/or damage-regulated phosphorylation events. If the interaction is direct, we would speculate that through the FHA/BRCT domains, Nbs1 binds to ATRIP and/or ATR after they are phosphorylated. In this respect, phosphorylation of ATRIP by ATR after replication stress was reported (70). If the interaction is indirect, the protein(s) bridging the interaction of MRN with ATR might be specifically phosphorylated in S-phase and/or when replication forks are stalled and then subsequently recruits ATR/ATRIP to MRN through a specific interaction with the FHA/BRCT domain.
domains of Nbs1. If this is the case, identifying the proteins bridging the interaction of MRN with ATR would significantly help understand how the interaction of MRN and ATR and chromatin loading of these proteins are regulated.

The interaction of MRN with ATR is not required for the general function of MRN as a mediator to facilitate the activation of ATR. Mutations in the FHA/BRCT domains significantly impair the interaction of MRN with ATR, but they do not reduce ATR-mediated phosphorylation of Chk1, SMC1, or RPA2. However, the phosphorylation of Nbs1 at Ser-343 is dramatically reduced in the FHA/BRCT mutants. The phosphorylation levels of Ser-343 in these mutants are proportional to the ability of these mutants to interact with ATR, suggesting that the interaction of MRN with ATR is likely required for Nbs1 phosphorylation by ATR. One model is that when cells enter S-phase, a period of the cell cycle that is most susceptible to damage, or when replication forks are stalled, the interaction of MRN with ATR is induced through phosphorylation events. Nbs1 is thus presented to ATR on chromatin, and as soon as ATR is activated by replication stress or a failure to repair DSBs when forks are collapsed.

Specific involvement of the FHA/BRCT domains in Nbs1 phosphorylation is consistent with the idea that ATR activation, which is facilitated by MRN, and ATR-mediated Nbs1 phosphorylation are two independent events. Therefore, modulation of RPA phosphorylation by MRN and phosphorylation of Nbs1 by ATR are likely two parallel pathways to mediate the S-phase checkpoint when replication forks are stalled.

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**Mre11 Complex Mediates UV-induced S-phase Checkpoint Activation**