The Mre11/Rad50/Nbs1 Complex Plays an Important Role in the Prevention of DNA Rereplication in Mammalian Cells*5

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The Mre11/Nbs1/Rad50 complex (MRN) plays multiple roles in the maintenance of genome stability, including repair of double-stranded breaks (DSBs) and activation of the S-phase checkpoint. Here we demonstrate that MRN is required for the prevention of DNA rereplication in mammalian cells. DNA replication is strictly regulated by licensing control so that the genome is replicated once and only once per cell cycle. Inactivation of Nbs1 or Mre11 leads to a substantial increase of DNA rereplication induced by overexpression of the licensing factor Cdt1. Our studies reveal that multiple mechanisms are likely involved in the MRN-mediated suppression of rereplication. First, both Mre11 and Nbs1 are required for facilitating ATR activation when Cdt1 is overexpressed, which in turn suppresses rereplication. Second, Cdt1 overexpression induces ATR-mediated phosphorylation of Nbs1 at Ser343 and this phosphorylation depends on the FHA and BRCT domains of Nbs1. Mutations at Ser343 or in the FHA and BRCT domains lead to more severe rereplication when Cdt1 is overexpressed. Third, the interaction of the Mre11 complex with RPA is important for the suppression of rereplication. This suggests that modulating RPA activity via a direct interaction of MRN is likely one of the effector mechanisms to suppress rereplication. Moreover, we demonstrate that MRN is also required for preventing the accumulation of DSBs when rereplication is induced. Therefore, our studies suggest new roles of MRN in the maintenance of genome stability through preventing rereplication and rereplication-associated DSBs when licensing control is compromised.

In each cell cycle, the genome has to be precisely replicated and segregated into daughter cells. To ensure the accuracy of DNA replication, it is essential that no segment of chromosome DNA is replicated more than once (1, 2). Re-initiation of DNA replication, even from limited sites of the genome, would inevitably lead to genome instability, which is a common feature of cancer cells (3, 4).

DNA replication is strictly regulated by the licensing mechanism, which allows formation of prereplication complexes (pre-RCs) only in late mitosis and prior to S phase (1, 5). Licensing begins with the recruitment of Cdc6 and Cdt1 to origins by chromatin-bound ORC, which in turn facilitates chromatin loading of the MCM2–7 complexes. Two protein kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase (DDK), are required to activate the licensed origins to initiate DNA replication by stimulating DNA unwinding from the origins. After the onset of replication initiation, the origins are converted to an unlicensed state by disassembling the pre-RCs, which leaves only ORC bound to chromatin.

To avoid a second round of DNA initiation, multiple mechanisms are involved in the prevention of reassembly of pre-RCs within the same cell cycle. Among these mechanisms, control of Cdt1 activity in a cell cycle-regulated manner has been shown to be critical (2, 5). Cdt1 is stable in G1 and is targeted for degradation at the onset of S-phase (6–9). Geminin, expressed after cells enter S-phase, binds to Cdt1 and directly inhibits Cdt1 function at origins (10, 11). Recent studies demonstrated that overexpression of Cdt1 or down-regulation of geminin disrupts the licensing control and induces rereplication, which consequently causes genome instability (12–14). Unbalanced expression of Cdt1 or other replication licensing factors as well as DNA hyperreplication has been observed to be associated with tumorigenesis (3, 4, 15, 16).

DNA rereplication causes accumulation of DNA lesions and triggers DNA damage responses observed in multiple organisms (12, 13, 17–20). The activation of damage checkpoint inhibits the extent of rereplication (19) and arrests cell cycle at the G2/M stages (13, 20). Our recent studies demonstrated that upon Cdt1 overexpression, single-stranded DNA (ssDNA)3 is accumulated prior to the appearance of DSBs, which activates the ATR pathway that effectively prevents further rereplication (75). Therefore, cell cycle checkpoint is not only capable of detecting abnormal DNA structures when the licensing control is compromised, but is also actively involved in inhibiting rereplication.

The Mre11/Rad50/Nbs1 complex (MRN) is a highly conserved protein complex that plays major roles in the maintenance of genome stability (21, 22). Hypomorphic mutations in Nbs1 and Mre11 result in autosomal recessive diseases Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia-like disorder (ATLD), respectively (23, 24). The radioresistant DNA...
The Mre11 Complex Inhibits DNA Rereplication

synthesis (RDS) phenotype of NBS and ATLD cells suggests a critical role of MRN in mediating the intra-S-phase checkpoint (23, 25). MRN binds directly to ATM and stimulates ATM kinase activity to phosphorylate its multiple substrates (26–28). Meanwhile, Nbs1 is also a downstream substrate of ATM, with ATM-dependent phosphorylation of Nbs1 at least at serine residue 343 (Ser343) is required for mediating the intra-S-phase checkpoint (29–32). Recently, our studies demonstrated that a direct interaction of MRN with RPA is required for IR-induced suppression of DNA synthesis, suggesting that RPA is a target of MRN to mediate the intra-S-phase checkpoint (33). Although substantial evidence suggests that MRN plays a key role in the ATM pathway in responses to DSBs, a connection between MRN and ATR was also described recently. MRN was co-purified with ATR (34) and is a direct substrate of ATR under replication stress (35, 36). MRN is required for replication restart during the recovery from fork stalling and G2/M arrest following HU treatment, similar to the requirement of ATR in these damage responses (37–39). It has also been suggested that MRN is involved in facilitating ATR-mediated phosphorylation events, although the mechanism remains unclear (35, 37, 39).

In our previous studies, we demonstrated that Nbs1 inhibits the simian virus 40 (SV40) large T antigen-induced hyperreplication of chromosomal DNA and SV40 origin-containing replicons, suggesting a possible role of Nbs1 in limiting inappropriate DNA rereplication events (40). Here, we demonstrate that in mammalian cells, MRN plays an important role in the prevention of rereplication when the licensing control is disrupted by Cdt1 overexpression. Loss of Mre11 or Nbs1 function does not only increase levels of rereplication in rereplication-susceptible cell lines but also leads to substantial rereplication in the cell lines that are normally resistant to Cdt1 overexpression-induced rereplication. We also showed that a major mechanism by which MRN inhibits rereplication is through regulating the ATR pathway. MRN facilitates ATR activation to phosphorylate Chk1 upon Cdt1 overexpression, an event that appears to be important for inhibiting rereplication (19, 75). Nbs1 phosphorylation at Ser343 by ATR is observed at an early stage after Cdt1 is overexpressed, and this phosphorylation is needed for the inhibition of rereplication. Moreover, the interaction of MRN with RPA that is required for mediating the intra-S-phase checkpoint (33) is also required for the inhibition of rereplication. Collectively, these data suggest that MRN acts through the activation of the S-phase checkpoint in the ATR pathway to suppress DNA rereplication.

EXPERIMENTAL PROCEDURES

Cell Culture, Retroviral Infections, and shRNA—U2OS, T98G, A549, 293T, GM847, or GM847-ATR-KD cells were grown in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and 5% super calf serum. The expression of ATR-KD in the GM847 fibroblasts was induced by the addition of 1 μg/ml of doxycycline to the media for 24 h (41).

Silencing of endogenous Mre11, Nbs1, or ATR in U2OS, T98G, or A549 cells was performed by two rounds of retroviral infection using pMKO vector (42) that expressed two different Mre11, Nbs1, or ATR shRNA target sequences. The shRNA retrovirus plasmids constructed by inserting annealed and phosphorylated shRNA oligos into pMKO retroviral vector. The shRNA target sequences used were Mre11: GAGAGAAACUCUUGGUAAC, and GAGUUAAGAUUAAGCAGAACA; Nbs1: GGAGGAAGAUCAGAAGGAGGAAAGCUGACUAAGGA; ATR: CGAGACCTTTGCGGATTGCAG and AACCTCCGTGATGGTGCTTGA (43).

Adenovirus Construction and Infection—Production of recombinant adenoviruses is conducted by using the AdEasy system method (44). Adenovirus plasmids constructed by inserting, full-length hCdt1, Nbs1 N-terminal fragment (Nbs1 N-1–478), full-length Mre11, and Mre11Δ521–543 into pAd-track-CMV shuttle vector. Then, in vivo recombination was performed by transforming pAd-track-CMV plasmid together with pAd-Easy-1 adenoviral vector into BJ5813 competent cell by electroporation (44). The recombinant adenoviral plasmids were transfected into 293 cells to generate corresponding recombinant adenoviruses. Large scale purification of adenoviruses from 293 cells was accomplished by CsCl density gradient centrifugation. The concentration of purified virus was measured A260 using the equation A260 = 1012 pfu (12).

Immunoblot Analysis and Antibodies—Cells were lysed in NETN (150 mM NaCl, 1 mM EDTA, 20 mM Tris·Cl pH 8.0, 0.5% Nonidet P-40 (v/v)) containing protease and phosphatase inhibitors (1.0 mM sodium orthovanadate, 50 μM sodium fluoride). For immunoblot analysis, proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes (BioRad), incubated overnight in primary antibodies followed by 1 h of incubation in horseradish peroxidase-conjugated secondary antibodies.

Antibodies used in this study are listed as follows: antibodies to Cdt1 (8), Mre11 ((D27; Ref. 40), and Nbs1 (D29; Ref. 29) were described earlier, antibody to phospho-Chk1 (Ser317) was obtained from R & D Systems, phospho-Chk2 (Thr68) and phospho-Nbs1 (Ser343) antibodies were from Cell Signaling, antibodies to Chk1, Chk2, and Ku70 were purchased from Santa Cruz Biotechnology, RPA2 and ATR antibodies were from Oncogene, and Rad50 and γ-H2AX (Ser139) were from Upstate Biotechnology.

Fluorescence-activated Cell Sorting (FACS) Analysis—Cells were rinsed with phosphate-buffered saline, collected by trypsinization and fixed with 70% ethanol overnight at 4 °C. After fixation, cells were stained with propidium iodide solution which containing 38 mM sodium citrate, 10 μg/ml RNase A and 15 μg/ml propidium iodide (Sigma). The labeled cells were analyzed with a Becton-Dickinson flow cytometer using Cellquest software.

Plasmids and Mutagenesis—To generate mutations in Nbs1 (S343A, R28A(FHA), Y176A(BRCT), R28A/Y176A (FHA/BRCT)) and Mre11 (NAAIRS, D543A/D544A), Myc-tagged full-length Nbs1 and Mre11 cloned into mammalian expression vector pcDNA3β was used as template for site-directed mutagenesis (QuikChange, Stratagene). Wild-type and mutant forms of Myc-tagged Nbs1 and Mre11 were cloned into the retroviral vector pBabePuro.

Single Cell Gel Electrophoresis (Comet Assay)—The neutral comet assay was performed as described (45) with minor mod-
were counted as positive for DNA damage. The percentage of nuclei with tails was the number of positive for DNA damage divided by the total number of nuclei counted. Results are presented as range of at least three independent experiments.

RESULTS

Depletion of MRN Leads to Rereplication When Licensing Control Is Disrupted by Cdt1 Overexpression—We previously showed that Nbs1 is involved in limiting SV40 T-mediated DNA hyperrepllication (40). To investigate whether MRN may also play a role in rereplication control other than SV40 T-mediated events, we overexpressed Cdt1 in mammalian cells. After infecting cells with a recombinant adenovirus (Ad-Cdt1), rereplication was monitored by accumulation of cells containing more than 4N DNA content as described (12). Overexpression of Cdt1 is sufficient to induce DNA rereplication in U2OS (Fig. 1A, left panel, top, vector), but not in T98G and A549 cell lines despite higher titers of adenovirus used (Fig. 1A, middle and right panels, top, vector) (75). To inhibit the expression of MRN, Mre11-shRNA and Nbs1-shRNA sequences were introduced by retroviral infection using the MKO vector (42). Silencing Mre11 or Nbs1 by shRNA resulted in a significant reduction in protein levels of Mre11 and Nbs1 in multiple cell lines as compared with the control cells infected with empty vector (Fig. 1A, bottom and data not shown). The expression of Rad50 and Nbs1 was also reduced when the expression of Mre11 was silenced, which is consistent with a reduced expression of Nbs1 and Rad50 in the Mre11-deficient ATLD cells (25). However, silence of Nbs1 expression does not influence the expression of Rad50 and Mre11. This is likely because of a crucial role of Mre11 to stabilize the MRN complex (47). Silencing the expression of Mre11 or Nbs1 by shRNA resulted in a significant reduction in protein levels of Mre11 and Nbs1 in multiple cell lines as compared with the control cells infected with empty vector (Fig. 1A, bottom and data not shown). The expression of Rad50 and Nbs1 was also reduced when the expression of Mre11 was silenced, which is consistent with a reduced expression of Nbs1 and Rad50 in the Mre11-deficient ATLD cells (25). However, silence of Nbs1 expression does not influence the expression of Rad50 and Mre11. This is likely because of a crucial role of Mre11 to stabilize the MRN complex (47). Silencing the expression of Mre11 or Nbs1 led to a significantly higher level of rereplication in U2OS cells when Cdt1 was overexpressed by adenoviral infection (Fig. 1A, left panel, top, vector). Strikingly, robust rereplication was observed in T98G and A549 cells where normally rereplication was not obviously induced by Cdt1 overexpression (Fig. 1A, middle and right panels). Similar results were obtained when Cdt1 was overexpressed by retroviral infection (supplemental Fig. S1 and data not shown). We also examined
whether MRN is required for the suppression of rereplication induced by expressing shRNAs of DDB1 and Cdt2, the components of a Cul4 ubiquitin ligase that are required for Cdt1 ubiquitination and degradation (48, 49). Inhibition of the expression of DDB1 or Cdt2 in U2OS cells led to detectable Cdt1 accumulation and rereplication. Silencing Mre11 by shRNAs prior to the depletion of DDB1 or Cdt2 significantly increased the rereplication levels compared with Mre11-proficient cells (Fig. 1B). These data suggest that MRN is involved in the suppression of rereplication when licensing control is disrupted.

**MRN Is Involved in Facilitating ATR-mediated Checkpoint Activation When Cdt1 Is Overexpressed**—When Cdt1 is overexpressed, multiple ATR substrates are phosphorylated (75). However, when p27 and Cdc7 kinase dead mutant (Cdc7KD) were overexpressed prior to Cdt1 overexpression, both rereplication and the phosphorylation of ATR substrates including Chk1 and Nbs1 were inhibited although Cdt1 overexpression was not altered (supplemental Fig. S2). This suggests that activation of the ATR pathway is not caused by Cdt1 overexpression per se, but by Cdt1-induced rereplication. Under replication stress, MRN is important for facilitating certain ATR-mediated phosphorylation events (35, 37, 39). Because ATR plays a critical role in the inhibition of rereplication (19, 75), we examined the role of MRN in facilitating ATR activation when Cdt1 was overexpressed. To examine whether Nbs1 or Mre11 is involved in the activation of ATR, the expression of Mre11 or Nbs1 was silenced by shRNA as described in Fig. 1A. A reduction in Chk2 phosphorylation following IR in these Mre11- or Nbs1-deficient cells confirmed effective silencing of Mre11 and Nbs1 (Fig. 2A). We monitored the phosphorylation of multiple ATR substrates including Chk1, RPA2, and Rad17 after Ad-GFP or Ad-Cdt1 infection in vector, or Nbs1-shRNA or Mre11-shRNA-expressing cells. Chk1 phosphorylation at serine 317, RPA2 phosphorylation, and Rad17 phosphorylation at serine 645 were all reduced in Nbs1- or Mre11-deficient cells (Fig. 2B), suggesting that MRN is important for activating ATR-mediated phosphorylation events when Cdt1 is overexpressed.

Rereplication generates both ssDNA and DSBs and these two kinds of DNA lesions activate ATR and ATM, respectively (13, 14, 20, 75). MRN plays a critical role in the activation of ATM in response to DSBs (26, 27, 50). Recent studies showed that after ATM activation, MRN is also important for processing the DSBs to generate RPA-bound ssDNA, leading to ATR activa-
Nbs1 phosphorylation at Ser\textsuperscript{343} by ATR Is Required for the Prevention of Rereplication When Cdt1 Is Overexpressed—Nbs1 is phosphorylated at Ser\textsuperscript{343} by ATM in response to IR or by ATR under replication stress (30, 31, 35). Because phosphorylation of Nbs1-S343 is important for mediating the intra-S-phase checkpoint by down-regulation of DNA replication (29–32), we examined whether this regulation might also be used in inhibiting Cdt1 overexpression-induced rereplication. We observed that Nbs1 was phosphorylated at Ser\textsuperscript{343} after Cdt1 overexpression, similar to that after IR and UV treatment (Fig. 3A). Importantly, the phosphorylation of Nbs1 at Ser\textsuperscript{343} occurred at an early stage after Cdt1 overexpression (Fig. 3B), approximately at the same time when Chk1 was phosphorylated (Fig. 2B), indicating that ATR is probably the kinase that phosphorylates Nbs1.

To examine whether Nbs1 phosphorylation at Ser\textsuperscript{343} is indeed dependent upon ATR after Cdt1 overexpression, we inhibited ATR expression by expressing ATR-shRNAs in U2OS cells (Fig. 3C, right). Because ATM may also be activated to phosphorylate Nbs1 at Ser\textsuperscript{343} when DSBs are generated, we examined Nbs1 phosphorylation at Ser\textsuperscript{343} in ATR deficient cells at 16 and 20 hpi after Ad-Cdt1 infection when DSBs were absent as revealed by H2AX phosphorylation (Fig. 2B). We observed that phosphorylation of Nbs1 at Ser\textsuperscript{343} and Chk1 at Ser\textsuperscript{317} was significantly reduced when ATR was silenced (Fig. 3C, left). Similar results were obtained when a fibroblast cell line, GM847-ATR-KD (41), expressing a doxycycline-inducible kinase inactive allele of ATR (ATR-KD) was used (Fig. 3D). Collectively, these results suggest that ATR kinase activity is required for phosphorylation of Nbs1 at Ser\textsuperscript{343}, especially at the initial stage after Cdt1 overexpression in mammalian cell lines.

Because ATM- or ATR-directed Nbs1-S343 phosphorylation is required for mediating IR- or UV-induced down-regulation of DNA replication (30, 31, 35), the early appearance of Nbs1-S343 phosphorylation after Cdt1 overexpression prompted us to examine whether this ATR-dependent phosphorylation event might play a role in mediating the inhibition of rereplication. Myc-tagged wild type Nbs1 or Nbs1-S343A with silent mutations at the shRNA-targeted sites were expressed in U2OS cells by retroviral infection and endogenous Nbs1 was silenced by Nbs1-shRNAs (Fig. 3E, right). Intriguingly, overexpression of Cdt1 in the Nbs1-S343A mutant led to a significant increase of the extent of rereplication than in Nbs1-WT-expressing cells (Fig. 3E, left), suggesting that ATR-mediated Nbs1 phosphorylation at Ser\textsuperscript{343} is important for inhibiting rereplication. These data also suggest that in addition to MRN acting upstream of ATR to regulate the activation of ATR-Chk1, Nbs1 is also a downstream effector of ATR to inhibit rereplication.

The FHA and BRCT Domains of Nbs1 Are Important for Nbs1-mediated Inhibition of Rereplication through Regulating Nbs1 Phosphorylation at Ser\textsuperscript{343}—We previously demonstrated that synthesis of an N-terminal Nbs1 fragment (amino acids 1–478) enhanced SV40 T-mediated hyperreplication and also induced profound DNA rereplication and tetraploidy in NBS-deficient cells in the absence of SV40 T (40). These results suggest that expression of this Nbs1 N-terminal fragment disrupts the cellular mechanism to prevent DNA rereplication, but the mechanism was unknown. To examine whether this Nbs1 N-terminal fragment might influence Cdt1-induced rereplication, we infected U2OS cells with a recombinant adenovirus (Ad-Nbs1-(1–478)) prior to Ad-Cdt1 infection. Overexpression of Nbs1-(1–478) significantly enhanced Cdt1-induced rereplication, while overexpression of this fragment alone did not induce rereplication (Fig. 4A). These studies suggested that overexpression of the Nbs1-(1–478) fragment is not sufficient to override the cellular control to prevent rereplication, but it facilitates Cdt1-induced rereplication.

The Nbs1-(1–478) fragment contains the FHA and BRCT domains and both are important for various functions of Nbs1 including damage-induced Nbs1 phosphorylation at Ser\textsuperscript{343} and foci formation (53, 54). One scenario is that overexpression of the Nbs1-(1–478) fragment may impair the function of the FHA/BRCT domains by a dominant negative effect, thus leading to more severe rereplication after Cdt1 overexpression. Because the FHA/BRCT domains are important for IR-induced Ser\textsuperscript{343} phosphorylation (53, 54), and phosphorylation of Nbs1 at Ser\textsuperscript{343} is important for limiting Cdt1-induced rereplication (Fig. 3E), we examined whether overexpression of the Nbs1-(1–478) fragment may impair the function of the FHA/BRCT domains by a dominant negative effect, thus leading to more severe rereplication after Cdt1 overexpression.
To more directly examine why the FHA and BRCT domains are involved in rereplication control, we used the Nbs1 mutants carrying mutations at the conserved residues Arg28 and Tyr176 in the FHA and BRCT domains, respectively (54). Myc-tagged Nbs1 wild-type, Nbs1-R28A, Nbs1-Y176A, and Nbs1-R28A/Y176A were introduced into U2OS cells by retroviral infection, and the expression levels of Myc-tagged Nbs1 species were similar to endogenous Nbs1 (Fig. 4C). Subsequently, endogenous Nbs1 was inactivated by shRNAs (Fig. 4C). The phosphorylation of Myc-tagged Nbs1 species at Ser343 was monitored by using a Ser343-phosphospecific antibody. While Myc-Nbs1-wildtype was readily phosphorylated at Ser343 after Cdt1 overexpression, phosphorylation of Myc-Nbs1-R28A, Nbs1-Y176A, and Nbs1-R28A/Y176A mutants was significantly reduced, to a similar level as the S343A mutant (Fig. 4C, right). These data suggest that both FHA and BRCT domains are required for the phosphorylation of Nbs1 at Ser343 in response to Cdt1 overexpression. To further investigate the role of FHA and BRCT domains in the prevention of rereplica-
tion, we overexpressed Cdt1 in Nbs1-FHA and/or BRCT mutant cells. Cdt1-induced rereplication was significantly enhanced in the FHA- and/or BRCT-defective mutants, with a more severe effect when mutations were present in both FHA and BRCT domains (Fig. 4D). Taken together, these data suggest that both FHA and BRCT domains are important for Cdt1-induced Nbs1-S343 phosphorylation and thus are required for the prevention of rereplication.

**FIGURE 4.** The FHA and BRCT domains of Nbs1 are required for Nbs1 phosphorylation at Ser343 and for preventing Cdt1-induced rereplication. A, overexpression of an N-terminal fragment of Nbs1 (Nbs1-N-(1–478)) enhances Cdt1-induced rereplication. U2OS cells were infected with Ad-GFP or Ad-Nbs1-N-(1–478) (2 × 10^7 pfu/ml) for 24 h and subsequently infected with Ad-GFP or Ad-Cdt1 (5 × 10^7 pfu/ml) for another 48 h. Infected cells were collected for FACS analysis. B, overexpression of the N-terminal fragment of Nbs1 (Nbs1-N-(1–478)) reduces Nbs1 phosphorylation at Ser343 that is induced by Cdt1 overexpression. U2OS cells were treated as described in Fig. 4A were collected for Western blot analysis using antibodies specific for Nbs1-pS343 and Myc tag. Ku70 was used as a loading control. C, FHA and BRCT domains of Nbs1 are required for Nbs1 phosphorylation at Ser343 after Cdt1 overexpression. U2OS cells were retrovirally introduced with Myc-tagged wild-type Nbs1, the FHA, BRCT mutants (FHA (R28A), BRCT (Y176A), and FHA/BRCT (R28A and Y176A)) or the S343A mutant. Subsequently, the expression of endogenous Nbs1 was silenced by two rounds of retroviral infection with shRNAs against Nbs1. The expression of Myc-tagged Nbs1 and the mutants as well as endogenous Nbs1 was examined by immunoblotting (left). U2OS cells expressing wild-type Nbs1 (WT), the FHA/BRCT mutants (FHA, BRCT, FHA/BRCT mutants), or Ser343A mutant (S343A) with endogenous Nbs1 silenced were infected with Ad-GFP or Ad-Cdt1 (5 × 10^7 pfu/ml) for 48 h. Infected cells were collected for Western blot analysis. Nbs1 phosphorylation at Ser343 was examined by using the antibody specifically recognizing phosphorylated Ser343. Ku70 was used as a loading control. D, mutations in the FHA and BRCT domains of Nbs1 lead to more severe rereplication induced by Cdt1 overexpression. FACS analysis was performed 48 h after Ad-GFP or Ad-Cdt1 infection (5 × 10^7 pfu/ml) using the U2OS cells expressing wild-type Nbs1, the FHA/BRCT mutants, or Nbs1-S343A mutant with the expression of endogenous Nbs1 silenced by shRNAs.
The Mre11 Complex Inhibits DNA Rereplication

A

|         | Vec | WT | NAAIRS | DD |
|---------|-----|----|--------|----|
| Myc-Mre11endo-Mre11 |     |     |        |    |

B

|         | Ad-GFP | Ad-Cdt1 |
|---------|---------|---------|
| Mre11-WT | 3.01%   | 10.1%   |
| Mre11-NAAIRS | 2.68%   | 23.53%  |
| Mre11-DD | 2.73% | 20.47% |

C

|         | WT | NAAIRS | DD |
|---------|----|--------|----|
| Nbs1-pS343 |     |        |    |
| Chk1-pS317 |     |        |    |
| Chk1 |     |        |    |
| Chk2-pT68 |     |        |    |
| Chk2 |     |        |    |
| RPA2 |     |        |    |
| Cdt1 |     |        |    |
| Ku70 |     |        |    |

FIGURE 5. Disruption of the interaction between MRN and RPA enhances Cdt1-induced rereplication without influencing ATR- or ATM-mediated checkpoint activation signaling. A, U2OS cells expressing Myc-Mre11-wildtype (WT), Myc-Mre11-DD (DD), or Myc-Mre11-NAAIRS (NAAIRS) with endogenous Mre11 silenced by shRNAs were generated. U2OS cells infected with vector (Vec) were used as a negative control. The expression of the Myc-Mre11 proteins and the inactivation of endogenous Mre11 in U2OS cells are shown by Western blotting analysis. B, disruption of the interaction between MRN and RPA enhances Cdt1-induced rereplication. FACSc analysis was performed 48 h after Ad-GFP or Ad-Cdt1 infection (1 × 10^7 pfu/ml) using the U2OS cells expressing Myc-Mre11-wildtype (WT), Myc-Mre11-DD (DD), or Myc-Mre11-NAAIRS (NAAIRS) with the expression of endogenous Mre11 silenced by shRNAs. C, interaction between MRN and RPA is not required for activating the ATR/ATM-dependent checkpoint pathways. U2OS cells expressing wild-type Mre11, Mre11-NAAIRS, or Mre11-DD, with the expression of endogenous Mre11 silenced by shRNAs were infected with Ad-GFP or Ad-Cdt1 (2 × 10^7 pfu/ml) for 48 h. Whole cell lysates were immunoblotted with the indicated antibodies. Ku70 was used as a loading control.

The Interaction of MRN with RPA Is Required for the Suppression of Rereplication without Interfering with the S-phase Checkpoint Activation When Cdt1 Is Overexpressed—RPA plays an essential role in the initiation and elongation of DNA replication (55). Interestingly, MRN forms complex with RPA at DNA replication centers and this interaction is required for MRN-mediated down-regulation of DNA synthesis in response to IR (33), suggesting that MRN may regulate RPA activity to inhibit DNA synthesis when the checkpoint is activated. Because RPA must be needed for DNA rereplication, we investigated whether the interaction of MRN and RPA might be involved in the regulation of Cdt1-induced rereplication.

Our previous studies demonstrated that MRN interacts with RPA via a specific site on Mre11 covering residues 540 to 545 on Mre11 (33). Substitution of residues 540 to 545 with a NAAIRS sequence (56) or replacement of the two conserved Asp^543 and Asp^544 with alanines completely abolished the interaction of MRN with RPA (33). To test whether the interaction of MRN and RPA is important for rereplication control, we used U2OS cells expressing Myc-Mre11 wild type or the RPA-binding mutants, Myc-Mre11-NAAIRS (changing amino acids 540–545) and Mre11-DD (D543A and D544A) with endogenous Mre11 silenced by Mre11-shRNAs (Fig. 5A). Overexpression of Cdt1 induced higher levels of rereplication in the Mre11-NAAIRS and Mre11-DD mutants compared with Mre11-wild type (Fig. 5B). These data suggest that the interaction of MRN with RPA is important for inhibiting Cdt1-induced rereplication.

We have shown that MRN facilitates the activation of ATR-mediated S-phase checkpoint, thereby contributing to the inhibition of Cdt1-induced rereplication. In addition to a critical role of RPA in DNA replication, RPA also binds to ssDNA accumulated upon DNA damage and serves as a sensor to activate the ATR-mediated S-phase checkpoint (57). This raised a possibility that the interaction of MRN and RPA may be important for activating ATR when Cdt1 is overexpressed and thus is needed to prevent rereplication. To test this, we examined phosphorylation of both ATR and ATM substrates after infecting the U2OS cell lines with Ad-Cdt1. As shown in Fig. 5C, ATR-mediated phosphorylation of Chk1 at Ser^317 and RPA2, as well as ATM-mediated phosphorylation of Chk2 at Thr^68 induced by Cdt1 overexpression remained at similar levels in the Myc-Mre11-wild-type, Myc-Mre11-NAAIRS, or Myc-Mre11-DD expressing cell lines with endogenous Mre11 silenced. These results suggest that MRN does not require its association with RPA to sense DNA lesions and activate the ATR- or ATM-mediated S-phase checkpoint, thereby contributing to the inhibition of Cdt1-induced rereplication. In addition, we have shown that MRN might suppress Cdt1-induced rereplication. Because Cdt1-induced Nbs1 phosphorylation at Ser^343 was also not affected in the Mre11 mutant cell lines defective in RPA binding (Fig. 5C), MRN/RPA probably acts as a downstream effector to mediate the inhibition of rereplication after Nbs1 Ser^343 is phosphorylated by ATR. It is conceivable that the interaction of MRN with RPA may directly modulate RPA replication activities to inhibit Cdt1-induced rereplication.

Overexpression of MRN Inhibits Cdt1-induced Rereplication through Its Interaction with RPA—Because Nbs1 or Mre11 deficiency led to a significant increase of rereplication induced by Cdt1 overexpression, we tested whether overexpression of MRN might suppress Cdt1-induced rereplication. To do this, we infected U2OS cells with Ad-Mre11 prior to Ad-Cdt1 infection. When Mre11 was overexpressed, the levels of Nbs1 and Rad50 as well as endogenous Mre11 were significantly increased (Fig. 6B) and more MRN complexes were present as demonstrated by co-immunoprecipitation (data not shown). Stabilization of Nbs1 and Rad50 by Mre11 overexpression is probably due to a critical role of Mre11 to stabilize the MRN complex, which is supported by the observation that in Mre11
deficient cells, Nbs1 and Rad50 protein levels are significantly reduced (25). Interestingly, overexpression of Mre11 almost completely inhibited Cdt1-overexpression-induced rereplication (Fig. 6A). These data suggest that MRN is not only required for the prevention of rereplication, but is also a limiting factor to inhibit massive rereplication that is induced by Cdt1.

What is the mechanism that might be involved in suppression of rereplication by overexpression of MRN? The inhibition of Cdt1-induced rereplication by elevated MRN levels is not because of a strengthened checkpoint activation, as phosphorylation of multiple ATR substrates after Cdt1 overexpression remains at similar levels in the presence or absence of Mre11 overexpression (Fig. 6C and data not shown). As described above (Fig. 5B), the interaction of MRN with RPA is important for MRN to prevent Cdt1-induced rereplication. Because rereplication establishes more replication forks, we reasoned that massive rereplication induced by Cdt1 overexpression may require involvement of more replication proteins, including RPA, which exceeds the capacity of endogenous MRN to prevent rereplication through binding RPA. To test this idea, we infected U2OS cells with Ad-Mre11Δ521–543, an RPA-binding mutant of Mre11 prior to Ad-Cdt1 infection. Strikingly, contrary to wild-type Mre11, the Mre11Δ521–543 mutant failed to suppress Cdt1-induced rereplication, suggesting that the suppression of rereplication by Mre11 overexpression is indeed through an interaction with RPA. These studies highlight a critical role of the interaction of MRN with RPA to prevent rereplication and support the idea that MRN directly inhibits RPA activities that are required for rereplication.

DISCUSSION

An intriguing finding was described previously that Nbs1 is important for the suppression of endoreduplication induced by SV40-T and hyperreplication of SV40 origin-containing DNA, which establishes a connection of MRN with DNA replication control in mammalian cells (40). More recently, a genetic approach identified Mre11, Rad50, and Xrs2 (Nbs1 ortholog) as essential genes for the viability of licensing control-compromised yeast cells (17). Further studies demonstrated that Mre11 is important for restraining the extent of rereplication in budding yeast, but the mechanism is not clear (17). Here, we dem-
onstrate that MRN prevents DNA rereplication in mammalian cells when licensing control is compromised by Cdt1 overexpression. Our studies also indicate that multiple mechanisms are involved for MRN to suppress rereplication.

**MRN Plays a Critical Role in the Prevention of Rereplication When Licensing Control Is Compromised in Mammalian Cells**

To more directly investigate the role of MRN in DNA replication control in mammalian cells, we disrupted the cellular licensing control by overexpression of Cdt1 as described previously (12). Depletion of Nbs1 or Mre11 significantly enhanced rereplication levels in multiple cell lines, suggesting that MRN plays a critical role in the prevention of DNA rereplication when cell cycle-regulated replication licensing is compromised. This finding is further supported by the observation that overexpression of MRN restrains the extent of Cdt1 overexpression-induced rereplication, which implies that MRN is not only required for preventing rereplication, but is also a limiting factor that is necessarily required for the suppression of rereplication. These studies demonstrate that the role of MRN in replication control is not limited to the prevention of SV40 T-mediated hyperreplication (40), but is involved in a general regulation against more than one round of DNA replication per cell cycle. Therefore, our studies revealed another critical function of MRN, prevention of DNA rereplication, in the maintenance of genome stability.

**MRN Facilitates ATR Checkpoint Signaling to Limit Rereplication**

DNA rereplication causes accumulation of DSBs and ssDNA, leading to the activation of both ATM- and ATR-mediated checkpoints (12, 13, 20). We showed previously that the ATR-Chk1 pathway is activated prior to the activation of the ATM-Chk2 pathway, and the ATR-Chk1 pathway plays a predominant role in restraining DNA rereplication while the ATM-Chk2 pathway is largely not needed for rereplication control (75). This is consistent with the observation that the deletion of *MEC1* (the ortholog of ATR) and *RAD17* in budding yeast increased the extent of rereplication (17, 62). Our studies demonstrated that multiple substrates of ATR including Chk1, Rad17, and RPA2, are phosphorylated in a manner that is dependent on Mre11 and Nbs1. More importantly, the activation of ATR at the early stage after Cdt1 overexpression, before the activation of ATM, requires MRN function. This suggests that one important function of MRN in rereplication control is to facilitate the activation of ATR, which in turn suppresses DNA rereplication. The activation of ATM after Cdt1 overexpression is also dependent on MRN, but this requirement is likely not involved in rereplication control, because ATM is dispensable for suppressing rereplication (75). As revealed by Chk2 and H2AX phospha-
It has been described that the FHA and BRCT domains of Nbs1 are important for foci formation and Nbs1 phosphorylation in response to DNA damage (35, 53, 54). We demonstrated that these two domains are also important for the prevention of rereplication through participating in the regulation of Nbs1 phosphorylation at Ser^{343}. Currently, the mechanism underlying the involvement of the FHA/BRCT domains in the regulation of checkpoint-induced Nbs1 phosphorylation is not clear. The requirement of these two domains in the interaction of MRN with ATR may contribute to the regulation that facilitates the ATR-mediated Nbs1 phosphorylation at Ser^{343} (35).

**The Interaction of MRN with RPA Is Required for Inhibiting DNA Rereplication Downstream of the Activation of ATR**—MRN forms a complex with replication essential protein RPA and this interaction is important for mediating the intra-S-phase checkpoint (33, 67). Here we demonstrate that this interaction is also required for MRN to inhibit Cdt1-induced rereplication.

In response to DNA damage, RPA binds to ssDNA and activates the ATR pathway (57). One scenario is that RPA recruits MRN to ssDNA via a direct interaction, which places MRN in the vicinity of ATR to facilitate ATR activation. However, our studies showed that the interaction of MRN with RPA is not required for ATR activation when Cdt1 is overexpressed. Thus, the MRN/RPA complex is more likely situated downstream of ATR, serving as an effector to suppress rereplication.

RPA is an essential protein that is required for both replication initiation and elongation (55, 68). We observed that overexpression of MRN suppresses rereplication and this suppression is through the interaction of MRN with RPA. This suggests that the MRN/RPA complex is not only required for MRN to inhibit rereplication, but is also a limiting factor to suppress overt rereplication. One model is that when the S-phase checkpoint is activated by Cdt1 overexpression, MRN suppresses RPA replication activity via the direct interaction, leading to the inhibition of rereplication. Overt rereplication may recruit more RPA to replication centers to support rereplication initiation and elongation, which exceeds the control capacity of MRN by forming a complex with RPA under the normal cell cycle-mediated regulation. Overexpression of MRN may allow more RPA to form a complex with MRN, thus leading to more sufficient inhibition of DNA rereplication. These studies are in support of a downstream effector role of MRN/RPA in the prevention of rereplication.

**MRN Prevents DSBs Accumulation When Cdt1 Is Overexpressed**—DSBs in mitotic cells are the major source for inducing translocations that are highly associated with tumorigenesis (69, 70). It has been demonstrated that DNA rereplication leads to accumulation of DSBs, possibly through head-to-tail collision during the process of rereplication (65). MRN plays an essential role in homologous recombination-mediated repair of DSBs (58, 59), and our studies showed that MRN activity is also required for preventing and/or repairing DSBs that appear during DNA rereplication.

The involvement of MRN in the prevention of DSB formation during rereplication may be multi-faceted. First, MRN directly participates in the DSB repair. Mre11 carries nuclease activities and is possibly needed for processing DNA ends to
The Mre11 Complex Inhibits DNA Rereplication

promote DSB repair (51, 71). Second, MRN may facilitate DSB repair through activation of the ATM pathway when DSBs are accumulated. Activated ATM directly or indirectly phosphorylates multiple proteins involved in homologous recombination such as BRCA1, FANC D2, Rad51, and BRCA2, thereby stimulating cellular repair activities (72–74).

Taken together, our studies suggest that when licensing control is disrupted, MRN is actively involved in the prevention of DNA rereplication as well as the repair of rereplication-associated DSBs, thus revealing new roles of MRN in the maintenance of genome stability.

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