The Werner syndrome and the Nijmegen breakage syndrome are recessive genetic disorders that show increased genomic instability, cancer predisposition, hypersensitivity to mitomycin C and γ-irradiation, shortened telomeres, and cell cycle defects. The protein mutated in the premature aging disease known as the Werner syndrome is designated WRN and is a member of the RecQ helicase family. The Nbs1 protein is mutated in Nijmegen breakage syndrome individuals and is part of the mammalian Mre11 complex together with the Mre11 and Rad50 proteins. Here, we show that WRN associates with the Mre11 complex via binding to Nbs1 in vivo and in vitro. In response to γ-irradiation or mitomycin C, WRN leaves the nucleoli and co-localizes with the Mre11 complex in the nucleoplasm. We detect an increased association between WRN and the Mre11 complex after cellular exposure to γ-irradiation. Small interfering RNA and complementation experiments demonstrated convergence of WRN and Nbs1 in response to γ-irradiation or mitomycin C. Nbs1 is required for the Mre11 complex promotion of WRN helicase activity. Taken together, these results demonstrate a functional link between the two genetic diseases with partially overlapping phenotypes in a pathway that responds to DNA double strand breaks and interstrand cross-links.

DNA damage in the form of double strand breaks (DSBs) or cross-links compromises the integrity of cells. Defects in repairing these lesions are associated with human chromosome fragility syndromes such as Werner syndrome (WS), Nijmegen breakage syndrome (NBS), ataxia telangiectasia (AT), and ataxia telangiectasia-like disorder (ATLD). In contrast to WS, NBS and ataxia telangiectasia-like disorder (with Mre11 mutations) are defective in checkpoint responses and show clinical features of immunodeficiency, hyperpigmentation spots, and ovarian dysgenesis. Noticeably, in addition to the aforementioned cellular similarities, both WS and NBS display graying of the hair, short stature, a “bird-like” face, increased cancer susceptibility, and reduced life span (1–6). Loss of fidelity in repairing DNA DSBs results in chromosomal rearrangements (7), a defect that is prominent in both WS and NBS. The proteins mutated in these syndromes are implicated in the repair of DNA DSBs and interstrand cross-links (ICLs) (2, 4, 5, 8, 9). Vertebrate Nbs1 is essential for repairing DNA DSBs by homologous recombination (10), and WRN participates in this repair pathway by resolving the recombinational intermediates (11). WS cells are more sensitive to DNA cross-linkers than to any other genotoxic drugs and are defective in repairing DNA ICLs in vivo (2, 8). WRN exhibits 3′ → 5′ helicase, 3′ → 5′ exonuclease, and ATPase activities (12, 13). Although Nbs1 has no known enzymatic activity, it is required for optimal exonuclease and endonuclease activities of the Mre11 complex and for the correct nuclear localization of Mre11 and Rad50 proteins (4, 14). The Mre11 complex exhibits clear 3′ → 5′ exonuclease activity but demonstrates only limited DNA unwinding activity (14). Both WRN and the Mre11 complexes promote recombinational repair in vivo (11, 15), and recent advances support the concept that multiple DNA repair proteins and pathways work in a coordinated fashion to resolve DNA DSBs and ICLs (16).

Here, we have detected an interaction between WRN and the Mre11 complex and revealed a novel pathway of DNA damage response. We show that Nbs1 is essential for bridging the interaction between WRN and the Mre11 complex in vivo and in vitro and for the Mre11 complex stimulation of WRN helicase activity. We show co-localization and increased association between WRN and Nbs1 in response to DNA DSBs or ICLs. We also show that cells deficient in WRN and/or Nbs1 respond similarly to these types of DNA damage. Our results provide molecular support for a model in which Nbs1 recruits WRN to deal with DNA DSBs and ICLs and for the observed partially overlapping phenotypes among these chromosome fragility syndromes.

**EXPERIMENTAL PROCEDURES**

*Cell Lines, siRNA, and Transfections—* The primary NBS (GM07168D) and normal (GM00038B, MRC-5/AG105965E) fibroblasts were cultured in minimum Eagle’s medium containing 15% fetal bovine serum. HeLa and the SV-40-transformed NBS and Nbs1-complemented fibroblasts (17) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Transfection of HeLa cells (8 × 10⁵ cells per 60-mm culture dish with cover slips) with the enhanced green fluorescent protein (EGFP)-WRN (18) was conducted by using the PolyFect transfection kit (Qiagen) for 16 h. The SF9 insect cells were co-infected with baculoviruses containing WRN (19) and Mre11, Rad50, or Nbs1 (20). The sequence for the hairpin siRNA (UAGAGGGAAACU-
Interaction of WRN with the Mre11 Complex via Nbs1—To examine whether a linkage exists between WRN and the Mre11 complex, we first conducted co-immunoprecipitation experiments using cell lysates from HeLa cells. Analyses of anti-WRN immunoprecipitates by immunoblotting with antibodies against Nbs1, Mre11, or Rad50 demonstrated associations of WRN with all three components of the Mre11 complex (Fig. 1A). When anti-Nbs1, anti-Mre11, and anti-Rad50 immunoprecipitates were analyzed by immunoblotting with an anti-WRN antibody in reciprocal experiments, the results confirmed the association between WRN and the Mre11 complex (Fig. 1B, top panel). As expected, all three Mre11 components were associated with each other (4) (Fig. 1B, middle and bottom panels). To determine the partner of the Mre11 complex that directly interacts with WRN, we co-infected S99 insect cells with pairwise combinations of baculoviruses for WRN together with Nbs1, Mre11, or Rad50. Each of the three pairs of human proteins was successfully overexpressed (Fig. 1C, lanes 6, 12, and 18). Immunoblotting analyses of anti-WRN immunoprecipitates from the insect cell lysates demonstrated that WRN significantly associated with Nbs1 (Fig. 1C, lane 5), but only very minimally with Mre11 (lane 11) or Rad50 (lane 17). The co-immunoprecipitation between WRN and Nbs1 was not disrupted by ethidium bromide or DNaseI treatment (Fig. 2B, and data not shown), suggesting that this interaction was not due to DNA bridging. These results suggested a direct association between WRN and Nbs1 in vivo. To test this hypothesis, we conducted co-immunoprecipitation experiments using cell lysates from NBS and normal fibroblasts. Analyses of anti-WRN immunoprecipitates by immunoblotting showed that WRN associated with Mre11 and Rad50 only in the normal fibroblasts, but not in the NBS fibroblast (Fig. 1D). The protein levels of WRN, Rad50, and Mre11 were comparable between the two cell lines (Figs. 1, D and E). Next, we compared the protein levels using nuclear and cytoplasmic fractions from NBS (GM07166) and normal fibroblasts (GM00038) before or after γ-irradiation (20 Gy for 8 h). Consistent with an earlier report showing that Mre11 was mis-located to the cytoplasm in the W1799 NBS fibroblasts by immunostaining (4), we detected a significant portion of the Mre11 and Rad50 proteins in the cytoplasmic fraction from the NBS, but not in normal fibroblasts (Fig. 1E). This is concomitant with a decrease in the levels of Mre11 and Rad50 proteins in the nuclear fraction in the NBS fibroblasts, as compared with the normal fibroblasts. The WRN protein level was detected only in the nuclear fraction, and the amount is comparable between these two fibroblasts. Treatment of the cells with γ-irradiation did not affect any of the four protein levels in either of the cells. Therefore, the defect of the WRN association with Mre11 and Rad50 in NBS cells is not due to the lack of Mre11 and Rad50 proteins. Taken together, these data strongly suggest that WRN associates with the Mre11 complex via binding to Nbs1 in vivo.

To confirm a direct protein interaction in vitro, we performed co-immunoprecipitation experiments using purified WRN protein together with a purified Mre11 complex or an Mre11 complex lacking Nbs1. Analyses of anti-WRN immunoprecipitates by immunoblotting showed that the association of WRN with Mre11 and Rad50 did not occur if the Mre11 complex did not contain Nbs1 (Fig. 1F). Therefore, WRN binds directly to the Mre11 complex via Nbs1 in vitro. To investigate how WRN interacts with Nbs1, we fused WRN domains to GST and incubated them with purified Nbs1 in vitro. As shown in Fig. 1G, the WRN helicase (fragment labeled 5) and exonuclease (fragments 2 and 3) domains bound Nbs1 strongly, whereas the C-terminal WRN (fragment 6) bound weakly to Nbs1. Neither GST alone nor the WRN acidic region (Fig. 1G, fragments labeled 1 and 4) bound Nbs1. These data indicate that the Mre11 complex binds WRN via Nbs1 in vitro, and this physical
Cross-talk between WRN and Nbs1

Interaction between WRN and the Mre11 complex via Nbs1

Co-localization and Increased Association between WRN and Nbs1 after DNA Damage—DNA DSBs are introduced into the genome during normal DNA metabolism or by exogenous sources, including ionizing radiation and oxidative stress. Because cells from either WS or NBS individuals are hypersensitive to γ-irradiation (3, 4, 9, 22), and because both WRN and the Mre11 complex form distinct nuclear foci in response to the induction of DNA DSBs (4, 6, 23, 24), we tested whether WRN co-localized with the Mre11 complex by immunofluorescence studies (Fig. 2A). Prior to damage, the majority of WRN was in the nucleolus, whereas Nbs1 was distributed in the nucleoplasm in a scattered form. In response to γ-irradiation (20 Gy), WRN was confined to the nucleoli at 2 h (data not shown) but began to re-localize to the nucleoplasm and formed distinct nuclear foci thereafter, whereas the patterns of Nbs1 immunostaining changed from the smaller dots at 2 h (data not shown) to distinct foci at 8 h. Nbs1 was found to be nucleolus-excluded before and after the γ-irradiation treatment. Kinetic analyses revealed that WRN and Nbs1 did not co-localize at 2 h, but partially co-localized at 4 h and co-localized at distinct foci extensively at 8 h (Fig. 2A). We also treated EGFP-WRN-expressing HeLa cells with γ-irradiation (20 Gy). Consistently, these direct fluorescence results showed that EGFP-WRN re-localized to the nucleoplasm and co-localized with Nbs1 and Mre11 (data not shown) at 8 h after the treatment. Therefore, WRN co-localizes with Nbs1 after γ-irradiation treatment in a time-dependent manner.

Repair of DNA ICLs presents a challenge for cells, partially due to the involvement of both DNA strands and the requirement for several DNA repair pathways, including nucleotide excision and recombinational repair (16). The chemotherapy drug mitomycin C induces DNA ICLs and predisposes WS and NBS cells to death (2, 5). At 16 h after treatment of the MRC-5 primary cells with mitomycin C (0.5 μg/ml), WRN left the nucleolus, and both WRN and Nbs1 formed distinct nuclear foci. As a result, WRN co-localized with Nbs1 to a great extent (Fig. 2A). Taken together, these results demonstrate that WRN leaves the nucleolus and co-localizes with the Nbs1 foci in response to γ-irradiation and the DNA interstrand cross-linker.

To further investigate the nature of the interaction between WRN and the Mre11 complex, we tested for their association before and after cellular stress. Cells were treated with γ-irradiation, and the lysates were used in co-immunoprecipitation experiments to determine whether the association between WRN and the Mre11 complex was altered. We found that treatment of HeLa cells with γ-irradiation (20 Gy; Fig. 2B) substantially increased the amount of Nbs1, Mre11, and Rad50

Mre11 or Rad50, interacts with WRN when they are expressed in the insect Sf9 cells. Lysates from cells co-infected with the indicated baculoviruses were immunoprecipitated with antibodies against WRN or IgG, followed by immunoblotting using the indicated antibodies. D, Mre11 and Rad50 do not associate with WRN in NBS cells (GM07166). Cell lysates were immunoprecipitated, followed by immunoblotting using the indicated antibodies. E, Mre11, Rad50, Nbs1, and WRN protein levels in the nuclear (N) and cytoplasmic (C) fractions from NBS (GM07166) and normal (GM00038) cells with (+) or without (−) γ-irradiation treatment (20 Gy for 8 h). F, WRN associates with the Mre11 complex via Nbs1 in vitro by co-immunoprecipitation using purified WRN protein together with a purified Mre11 complex (M/R/N) or the complex without Nbs1 (M/R). G, Nbs1 interacts strongly with the helicase domain of WRN. Shown is a schematic diagram of human WRN domains, including the exonuclease domain (ED), the acidic region (AR), the helicase domain (HD), the RecQ C-terminal and nucleolar targeting sequence (BQCT/NTS), and the C-terminal helicase-related (HRDC) and nuclear localization sequences (NLS). Nbs1 bound to the GST fragments was analyzed by immunoblotting using the anti-Nbs1 antibody.
in the anti-WRN immunoprecipitates at 8 h. The γ-irradiation treatment had no effect on the level of WRN (Fig. 2B, bottom panel). Incubation of the immunocomplexes with ethidium bromide (Fig. 2B) or DNaseI (data not shown) did not affect the protein associations. Thus, although the yeast Nbs1 ortholog Xrs2 was reported to bind to the sites of DNA DSBs (25), our results demonstrated that the increased interactions observed were not due to DNA bridging. Consistent with the immunofluorescence data, these biochemical results demonstrate that the association between WRN and the Mre11 complex is increased in cells after treatment with γ-irradiation.

**Convergence of the Nbs1 and WRN Pathways in Response to DNA Damage**—To examine a functional role for the interplay between WRN and Nbs1 in vivo, siRNA was used to decrease WRN levels in NBS and Nbs1-complemented NBS cells. Cells were transiently transfected (2–4 days) with either a control or a WRN siRNA-containing vector. Immunoblotting showed that WRN protein levels were almost completely depleted 3 days after transfection (Fig. 3A) in both NBS and Nbs1-complemented cells. Neither WRN siRNA nor Nbs1 complementation affected the control BRCA1 protein levels. Next, siRNA-transfected cells were seeded onto 96-well plates, treated with mitomycin C or irradiated with γ-irradiation, and assessed for their ability to recover by MTT viability assays. Cellular WRN levels began to increase gradually 4 days after the transient transfection (data not shown). Because of the transient nature of the WRN silencing, we were compelled to use a short-term assay like MTT for assessment of cellular resistance. Although a colony formation analysis would have been preferable, the observed effects tend to be underscored in the MTT assay as compared with the long-term (weeks) assays of colony formation. In parallel experiments we have observed that the MTT assay and the colony formation assay give similar results in WRN-depleted human cell lines. Here, we showed that cells deficient in WRN or Nbs1 exhibited hypersensitivity (p < 0.05) to mitomycin C to a similar extent when compared with control cells (Fig. 3B). Cells deficient in both WRN and Nbs1 exhibited

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a similar sensitivity to mitomycin C when compared with cells deficient in either protein alone. These results suggest that WRN and Nbs1 facilitate cellular viability and proliferation similarly in response to mitomycin C and, thus, may participate in the same repair pathway.

We next tested for hypersensitivity to γ-irradiation. Both the Nbs1-deficient cells and the WRN-depleted cells exhibited hypersenstivity (p < 0.05) to the killing effects to γ-irradiation, and the severity was more pronounced in the Nbs1-deficient than in the WRN-depleted cells (Fig. 3C). Cells deficient in both WRN and Nbs1 showed increased sensitivity (p < 0.05) to γ-irradiation when compared with cells deficient in WRN only. Similarly, the WRN/Nbs1 double-deficient cells were slightly more sensitive to γ-irradiation when compared with cells deficient in Nbs1 alone, but this difference was not statistically significant. This suggests that Nbs1 plays a relatively major role in the protection against γ-irradiation and that WRN might assist Nbs1 to facilitate cellular viability in response to γ-irradiation. In consideration of our consistent results from viability and the immunofluorescence analyses using cells with different genetic backgrounds, these findings demonstrate the involvement of WRN and Nbs1 in a common pathway of DNA damage response.

**Functional Interactions between WRN and the Mre11 Complex**—Many members of the RecQ helicase family across species suppress illegitimate or homologous recombination (26, 27), suggesting that WRN also functions in resolving recombination intermediates. DNA helicases promote the separation of complementary strands of the DNA duplex, providing access for proteins to the template during replication, recombination, and repair. We next determined whether WRN helicase activity was affected by its binding to the Mre11 complex. We used a sub-optimal amount of WRN (0.16 nM) to determine whether WRN helicase activity could be stimulated by the Mre11 complex. WRN alone unwound 26% of the substrate (Fig. 4A, lane 4). Pre-incubation of WRN with an increasing amount of the Mre11 complex (up to 0.64 nM, a 4-fold molar ratio) significantly increased (p < 0.05) the percentage of displacement from 26 to 60% (Fig. 4A, lanes 4–7). However, WRN helicase activity was stimulated neither by the heat-inactivated Mre11 complex (Fig. 4A, lane 8) nor by the pre-incubation of WRN with the Mre11 complex without Nbs1 (M/R, Fig. 4A) or with Nbs1 alone (Fig. 4C), demonstrating that the promotion of WRN helicase activity by the Mre11 complex requires Nbs1. Because the Mre11 complex has been reported to exhibit a weak strand dissociation activity (14), we incubated the Mre11 complex with a WRN point mutant (K-WRN) that lacks helicase activity. The results showed that there was no DNA unwinding activity (Fig. 4D) when the K-WRN was pre-incubated with various amounts of the Mre11 complex (up to a 4-fold molar ratio) under these conditions. The 22-bp forked duplex substrate could not be unwound by the Mre11 complex with a WRN point mutant (K-WRN) that lacks helicase activity. The Mre11 complex requires Nbs1, and the severity was more pronounced in the Nbs1-deficient than in the WRN-depleted cells (Fig. 3C). Cells deficient in both WRN and Nbs1 showed increased sensitivity (p < 0.05) to γ-irradiation when compared with cells deficient in WRN only. Similarly, the WRN/Nbs1 double-deficient cells were slightly more sensitive to γ-irradiation when compared with cells deficient in Nbs1 alone, but this difference was not statistically significant. This suggests that Nbs1 plays a relatively major role in the protection against γ-irradiation and that WRN might assist Nbs1 to facilitate cellular viability in response to γ-irradiation. In consideration of our consistent results from viability and the immunofluorescence analyses using cells with different genetic backgrounds, these findings demonstrate the involvement of WRN and Nbs1 in a common pathway of DNA damage response.

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(1.2 nM) to the Mre11 complex (0.6–4.8 nM, Fig. 4, lanes 3–6) did not affect the Mre11 exonuclease activity. To gain potential functional insight into this interaction, the helicase-inactive K-WRN was incubated with the Mre11 complex. The digestion pattern of the Mre11 complex, when incubated together with the K-WRN mutant, was similar to that with the wild-type WRN (data not shown). Thus, WRN and the Mre11 complex additively digest the forked substrate that is independent of the WRN helicase activity.

Because both WRN and Rad50 exhibit ATPase activities, we next determined whether WRN ATPase activity could be affected by its binding to Nbs1. WRN or the Mre11 complex alone hydrolyzes ATP, and pre-incubating WRN with various amount of the Mre11 complex caused a linear increase in the ATP hydrolysis activity in an additive manner (Fig. 5A). However, this effect could not be achieved if WRN was incubated with Nbs1 alone (Fig. 5B). Therefore, WRN and the Mre11 complex hydrolyze ATP in an additive manner.

**Conclusion and Perspective**—Both WRN and the Mre11 complex are implicated in the non-homologous end-joining (NHEJ) and homologous recombination pathways of repairing DNA DSBs. They interact physically via Nbs1, resulting in the stim-
ulation of the WRN helicase and the optimum 3′ → 5′ exonuclease activities, two activities that may contribute to the two major DSB repair pathways. Clearly, WRN and Nbs1 act together in a common pathway. Their cellular co-localization and epistasis in response to mitomycin C treatment suggest a convergent role in repairing DNA ICLs, possibly via the homologous recombination pathway. In response to γ-irradiation, WRN and Nbs1 may act synergistically in repairing DNA DSBs in a manner that is partially dependent on cell cycle and DNA replication and may require additional factors. This is further supported by the observations that WS cells are not as sensitive to mitomycin C as are cells from Fanconi anemia patients or as sensitive to γ-irradiation as are ataxia telangiectasia or NBS cells (2–5, 22, 30). Importantly, this type of assisting role for WRN in a variety of the DNA damage response supports the idea of WRN as a non-essential protein that is, however, capable of slowing down the process of aging. Another possibility is that WRN facilitates the repair of the severe recalcitrant DNA breaks, as evidenced by the late appearance of WRN in the nuclear foci. A recent report suggests that unrepairable DNA DSBs accumulated in aging mouse tissues and senescent cells (31); therefore, it is reasonable to hypothesize that the premature aging phenotypes of WS stems from the lack of WRN for resolving the refractory DNA lesions. As WRN interacts with other proteins responding to DNA damage, it may function as a transducer of various DNA repair pathways likely to be mediated by post-transcriptional modifications of WRN. For example, in vivo phosphorylation of WRN has been demonstrated (22, 32, 33).

A recent model suggests that the Mre11/Rad50 complex tether DNA ends to form a flexible arm up to 1,200 Å, a distance suitable for NHEJ and homologous recombination (20). This topology may be especially important for the latter pathway in the presence of Nbs1, as a recent report clearly shows the essentiality of Nbs1 in DNA repair by homologous recombination but not by NHEJ (10). As described in Fig. 6, γH2AX targets the site of DSBs promptly after the DNA damage (34). Subsequently, either the Mre11 complex or the DNA-dependent protein kinase complex is recruited to the γH2AX foci (17, 34, 35), an action that may determine the fidelity of repair at the broken DNA ends. Nbs1 activation requires its phosphorylation by ATM, and WRN re-localization from the nucleoli can be regulated by the c-Abl tyrosine kinase (33). We have recently found that WRN focus formation was hampered in NBS cells after treatment with γ-irradiation. Thus, BLM may recruit Nbs1 (36), which, in turn may recruit WRN to these damaged sites. The WRN helicase activity is promoted through its interaction with the Mre11 complex via Nbs1, providing a mechanistic explanation for the roles of WRN in resolving the recombinational intermediates (9). In parallel, when bound to the DNA-dependent protein kinase complex, WRN catalytic activities are inactivated by DNA-dependent protein kinase phosphorylation (22, 32), suggesting that this pathway may become the less favored one. It seems likely that WRN may participate in both pathways, i.e. homologous recombination and NHEJ, and may play a role in pathway determination. The functional interaction between WRN and Nbs1 provides a molecular explanation for the similar defects in DNA repair, chromosomal stability, and telomere maintenance by WS, NBS, and ataxia telangiectasia-like disorder cells. Moreover, this interaction provides new insights into the process of aging and the complex relationship between the DNA damage recognition and repair mechanisms for DNA DSBs and ICLs.

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