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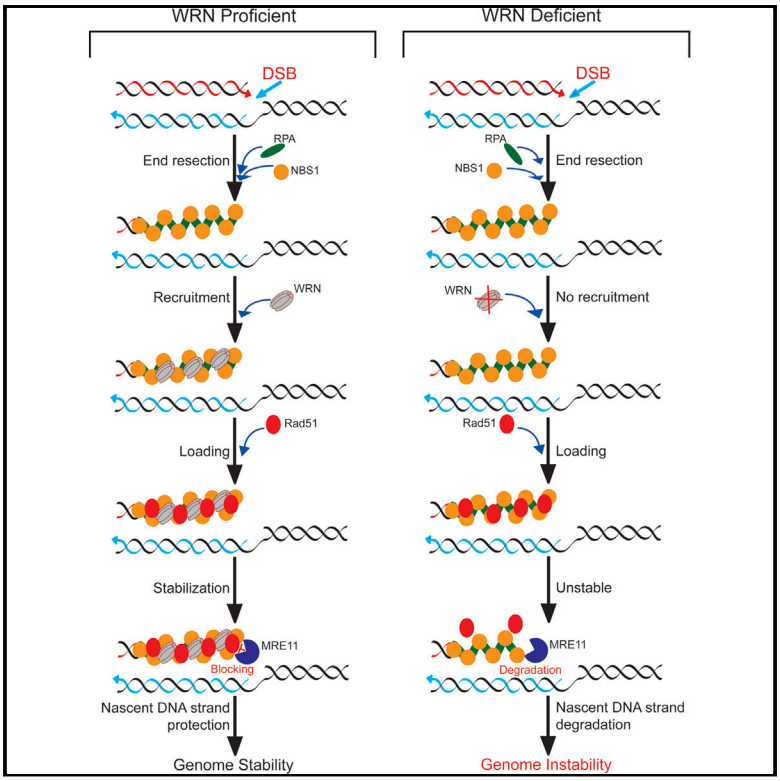
Nonenzymatic Role for WRN in Preserving Nascent DNA Strands after Replication Stress

Graphical Abstract



Highlights

WRN guards nascent DNA strands at collapsed replication forks

MRE11 degrades newly synthesized replication forks in the absence of WRN

NBS1 recruits WRN to limit the exonuclease activity of MRE11 on nascent DNA strands

WRN stabilizes Rad51 at replication-associated DNA double-strand breaks

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In Brief

Su et al. uncover a nonenzymatic function for WRN in DNA replication, giving insight into the molecular origin of genome instability in Werner syndrome individuals. The authors find that WRN recruitment to replication-associated DNA double-strand breaks prevents excessive MRE11-mediated degradation of nascent DNA strands.

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Nonenzymatic Role for WRN in Preserving Nascent DNA Strands after Replication Stress

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SUMMARY

WRN, the protein defective in Werner syndrome (WS), is a multifunctional nuclease involved in DNA damage repair, replication, and genome stability maintenance. It was assumed that the nuclease activities of WRN were critical for these functions. Here, we report a nonenzymatic role for WRN in preserving nascent DNA strands following replication stress. We found that lack of WRN led to shortening of nascent DNA strands after replication stress. Furthermore, we discovered that the exonuclease activity of MRE11 was responsible for the shortening of newly replicated DNA in the absence of WRN. Mechanistically, the N-terminal FHA domain of NBS1 recruits WRN to replication-associated DNA double-stranded breaks to stabilize Rad51 and to limit the nuclease activity of its C-terminal binding partner MRE11. Thus, this previously unrecognized nonenzymatic function of WRN in the stabilization of nascent DNA strands sheds light on the molecular reason for the origin of genome instability in WS individuals.

INTRODUCTION

During DNA replication, moving replication forks may encounter obstacles like DNA lesions, DNA secondary structures, or protein-DNA complexes that can result in prolonged fork stalling and collapse to generate DNA double-strand breaks (DSBs). Alterations in the pathways involved in the recovery of stalled or collapsed replication forks cause genome instability and chromosomal rearrangements that are hallmarks of cancer cells (Bartkova et al., 2005; Petermann and Helleday, 2010). One of the multiple factors involved in DNA replication and repair is WRN, a protein defective in Werner syndrome (WS). WS is a rare autosomal recessive disorder characterized by premature development of features that resemble aging. In addition, WS individuals have an increased cancer predisposition, leading primarily to rare cancers that are mesenchymal in origin (Friedrich et al., 2010; Goto, 1997). Primary cells derived from WS patients exhibit elevated levels of chromosomal translocations, inversions, and deletions of large segments of DNA and have a high spontaneous mutation rate (Fukuchi et al., 1989; Salk et al., 1981). Furthermore, WS cells are hypersensitive to several types of DNA damaging agents including 4-nitroquinoline-1-oxide, crosslinking agents (such as mitomycin C andcisplatin), camptothecin, and hydroxyurea (Pichierri et al., 2001; Poot et al., 1999, 2002). Moreover, WS cells display a prolonged S phase and impaired replication fork progression (Poot et al., 1992; Sidorova et al., 2008). Though these reports suggest that WRN plays crucial roles in one or more genome stability maintenance pathways, the exact contribution of WRN in preventing genome instability is unclear.

WRN belongs to the RecQ DNA helicase family. WRN is unique among known RecQ helicases in having an N-terminal 3′–5′ exonuclease activity (Huang et al., 1998). WRN exonuclease functions on a variety of structured DNA substrates, including bubbles, stem loops, forks, and Holliday junctions, as well as on RNA-DNA duplexes, implying roles for WRN in DNA replication, recombination, and repair (von Kobbe et al., 2003). The 3′–5′ DNA helicase activity (Gray et al., 1997) of WRN shows substrate specificity similar to that for the exonuclease, suggesting that the two enzymatic activities may have coordinated functions. In addition to its nuclease activities, WRN also has nuclease-independent functions during DNA replication and repair (Chen et al., 2003; Kamath-Loeb et al., 2012), although these nonenzymatic activities are not well understood.

WRN forms several dynamic subcomplexes with different factors involved in multiple biological processes. WRN physically interacts with Nijmegen breakage syndrome protein (NBS1) via the forkhead-associated (FHA) domain of NBS1 in response to DSBs, and this interaction is important for the posttranslational modification of WRN (Kobayashi et al., 2010). WRN interacts with MRE11 nuclease via NBS1 (Cheng et al., 2004); MRE11 promotes WRN helicase activity, but WRN does not modulate the nuclease activities of MRE11 (Cheng et al., 2004). WRN interacts directly with Rad51; however, this interaction does not affect the nuclease activities of WRN (Otterlei et al., 2006). Furthermore, WRN directly and functionally associates with XPG, a DNA endonuclease, and this interaction stimulates the helicase activity of WRN (Trego et al., 2011). Furthermore, WRN not only interacts

with NEIL1, but also stimulate its DNA glycosylase activities (Popuri et al., 2010). Importantly, mutations in majority of these genes lead to cancer-prone disorders. However, the contributions of WRN and its interacting partners to the maintenance of genome stability are not well studied.

Though the nuclease and the nonnuclease activities of WRN have been implicated in a multitude of DNA metabolic pathways, how WRN acts at the molecular level to prevent genome instability has not been determined. In this study, we report a nonenzymatic role for WRN in the stabilization of nascent DNA strands at collapsed replication forks. We found that NBS1-mediated recruitment of WRN to the replication-associated DSBs stabilizes Rad51 and prevents the excessive degradation of nascent DNA strands mediated by MRE11. Significantly, stabilization of collapsed replication forks by the coordinated actions of WRN, NBS1, Rad51, and MRE11 prevents chromosome instability. In summary, our study reveals a previously uncharacterized noncatalytic role for WRN in the faithful duplication of the genome and provides insights into the molecular reason for the development of genome instability in WS individuals.

RESULTS

WRN Maintains Nascent DNA Strands in Response to Replication Stress

Faithful and complete replication of genome in human cells is essential for preventing the accumulation of cancer-promoting mutations. WS, a disorder of premature aging manifested in adolescence, is associated with an elevated risk of specific types of cancers. Cells derived from WS patients display elevated levels of chromosome instability. The WRN protein, which is defective in WS, has been implicated in replication fork progression and efficient restart of DNA replication (Ammazzalorso et al., 2010; Sidorova et al., 2008, 2013). To better understand the involvement of WRN in genome stability maintenance in response to replication stress, we used a single-molecule DNA fiber technique (Petermann et al., 2010; Schlacher et al., 2011) (Figure S1A; Tables S1 and S2). In this assay, replicating DNA before and during replication stress induced by camptothecin (CPT) was sequentially labeled by incorporation of the thymidine analogs 5-iodo-2′-deoxyuridine (IdU) and 5-chloro-2′-deoxyuridine (CldU), respectively (Figure S1B). The assay was performed in human telomerase reverse transcriptase (hTERT)-immortalized WS cells (WS) and in WS cells complemented with wild-type WRN (WS+WRN). In CPT-treated WS cells, 39% ± 5.7% of all DNA fibers had both IdU and CldU tracts, whereas 73.2% ± 7.8% fibers contained both IdU and CldU in CPT-treated WS+WRN cells (Figure S1C). These results indicated that, as reported previously (Ammazzalorso et al., 2010), a greater proportion of replication forks fail to restart in WS cells in response to replication stress than in cells that express WRN. Furthermore, we observed significantly lower number of DNA fibers containing only CldU tracts, which represent origins of replication, in WS cells in comparison to WS+WRN cells (28.7% ± 0.09% and 62.6% ± 5.8% in WS and WS+WRN cells, respectively, $p < 0.014$; Figure S1D). Importantly, we observed a significantly higher percentage of DNA fibers that contained only IdU tracts, which represent stalled forks, in CPT-treated WS cells relative to CPT-treated WS+WRN cells (61.02% ± 2.8% and 26.81% ± 3.9%, WS and WS+WRN, respectively, $p < 0.034$; Figure S1E). Our results suggest that a greater proportion of replication forks break in CPT-treated WS cells than in CPT-treated WS+WRN cells.

Interestingly, we noticed that DNA fibers that contained only IdU tracts were significantly shorter in CPT-treated WS cells than in CPT-treated WS+WRN cells (3.5 ± 0.08 and 5.17 ± 0.35 μm, respectively, $p < 0.022$; Figure S1F), implying that the collapsed replication forks are not maintained in WS cells. We further verified these results by labeling WS and WS+WRN cells with IdU for 30 min, after which replication stress was introduced and the IdU-labeled DNA fibers were detected with anti-bromodeoxyuridine (BrdU; mouse monoclonal) antibodies (Figure S1G). Under these conditions, the IdU tract lengths were the same in mock-treated WS and WS+WRN cells (4.92 ± 0.01 and 5.08 ± 0.1 μm, respectively; Figure 1A). Similarly, the IdU tract lengths were comparable between CPT- and mock-treated WS+WRN cells (4.92 ± 0.01 and 4.86 ± 0.03 μm, respectively). In contrast, the IdU tract lengths were significantly shortened in CPT-treated WS cells relative to CPT-treated WS+WRN cells (2.71 ± 0.05 and 4.86 ± 0.03 μm, respectively, $p < 0.0003$; Figure 1A), reflecting a degradation of IdU tracts in WS cells. Thus, in addition to roles in replication fork progression and efficient restart, WRN also plays a role in the maintenance of nascent DNA strands in response to CPT-induced replication stress.

Next, we verified whether the shortening of nascent DNA tracts is unique to cells derived from WS patients or whether shortening also happens in other cell types. We depleted WRN from HeLa cells using *WRN*-specific small hairpin RNA (shRNA) and examined the nascent DNA tract lengths. As in WS cells, nascent DNA tract lengths were significantly shortened in CPT-treated, WRN-depleted HeLa cells compared to CPT-treated HeLa cells transfected with a control shRNA (3.57 ± 0.13 and 5.15 ± 0.11 μm, respectively, $p < 0.0001$; Figure 1B). This result confirmed that WRN is required for the maintenance of nascent DNA strands in non-WS cells.

To further clarify whether the role of WRN in replication fork maintenance is limited to human cells, we performed experiments in mouse embryonic fibroblasts (MEFs) derived from *WRN*-knockout mice (Lombard et al., 2000). As shown in Figure 1C, the nascent DNA tract lengths in CPT-treated $WRN^{-/-}$ MEFs were significantly shorter than in the CPT-treated wild-type MEFs (3.28 ± 0.22 and 5.02 ± 0.12 μm, respectively, $p < 0.0003$). Thus, WRN is involved in the maintenance of nascent DNA strands in response to CPT-induced replication stress in different mammalian cell types.

Evidence indicates that CPT induces both replication stalling and DNA breaks in a concentration-dependent manner. Low doses (25–100 nM) of CPT induce replication fork slowing and reversal without inducing detectable levels of DSBs, and at higher concentrations (>100 nM) CPT induces DNA breaks (Berti et al., 2013; Ray Chaudhuri et al., 2012). To discriminate whether WRN is required for the stability of nascent DNA strands in response to replication fork stalling or replication-associated DSBs, we measured IdU tract lengths in cells treated with low doses of CPT. As shown in Figure 1D, IdU tract lengths in WS

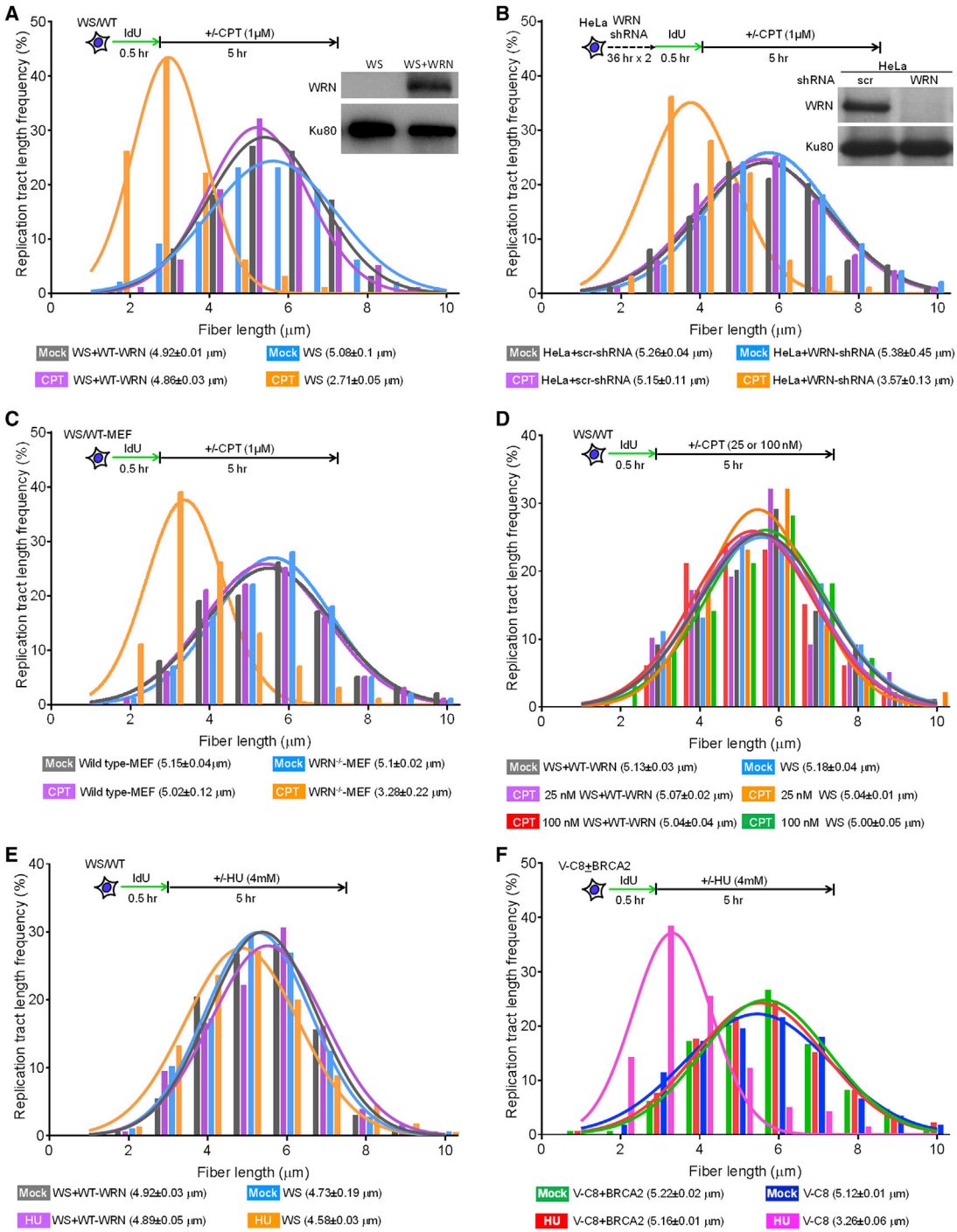

Figure 1. WRN Stabilizes Nascent DNA Strands in Response to Replication Stress

(A) IdU-tract-length distribution in hTERT-immortalized WS and WS cells complemented with wild-type WRN (WS+WRN), treated with or without 1 μM CPT for 5 hr. See also Figure S1 and Tables S1 and S2.

(B) Nascent DNA-tract-length distribution in HeLa cells transfected with either *WRN* or control shRNA, treated with or without 1 μM CPT for 5 hr. Inset, western blot for WRN expression in HeLa cells transfected with *WRN* shRNA and control shRNA.

(C) IdU-tract-length distribution in WRN-deficient mouse embryonic fibroblasts (MEFs), treated with or without 1 μM CPT for 5 hr.

(D) Nascent DNA-tract-length distribution in hTERT-immortalized WS and WS cells complemented with wild-type WRN (WS+WRN), treated with 25 or 100 nM CPT for 5 hr.

(E) IdU-tract-length distribution in WS and WS+WRN cells treated with 4 mM HU for 5 hr.

(F) Nascent DNA-tract-length distribution in BRCA2-deficient V-C8 and V-C8+BRCA2 cells, treated with 4 mM HU for 5 hr.

cells treated with 25 and 100 nM CPT (5.04 ± 0.01 and 5.00 ± 0.05 μm, respectively) were similar to lengths in WS+WRN cells treated with 25 and 100 nM CPT (5.07 ± 0.02 and 5.04 ± 0.04 μm, respectively). These results suggest that WRN is not involved in the maintenance of nascent DNA tracts in response to replication fork stalling.

Treatment of cells for a short time with hydroxyurea (HU) causes replication fork stalling, but it does not lead to DSBs formation (Petermann et al., 2010). To further rule out the possibility that WRN is not involved in the maintenance of nascent DNA strands in response to replication fork stalling, we exposed WS and WS+WRN cells to 4 mM HU for 5 hr and then quantified the nascent DNA tract lengths. Similar to low-dose CPT treatment, the nascent DNA tract lengths in HU-treated WS cells were comparable to those in HU-treated WS+WRN cells (4.58 ± 0.03 and 4.89 ± 0.05 μm, respectively; Figure 1E). However, using similar HU treatment conditions, nascent DNA strands were significantly shortened in HU-treated BRCA2-defective V-C8 cells relative to V-C8 cells complemented with BRCA2 (3.26 ± 0.06 and 5.16 ± 0.01 μm, respectively, $p < 0.02$; Figure 1F), confirming a previous finding (Schlacher et al., 2011). Taken together, these results clearly reveal that, unlike BRCA2, WRN is not important for the stabilization of nascent DNA strands in response to replication fork stalling.

To further evaluate whether WRN is particularly important for the maintenance of nascent DNA strand in response to replication breaks or important only for replication breaks induced by CPT, we examined IdU tract lengths after treatment of cells with HU for 24 hr; under these conditions, the stalled replication forks are broken (Petermann et al., 2010). As shown in Figure 2A, IdU track lengths in HU-treated WS+WRN cells were comparable to those in mock-treated WS+WRN cells (5.00 ± 0.04 and 5.09 ± 0.03 μm, respectively). In contrast, IdU track lengths were significantly shortened in HU-treated WS cells relative to HU-treated WS+WRN cells (3.53 ± 0.15 and 5.0 ± 0.04 μm, respectively, $p < 0.03$; Figure 2A). These results suggest that WRN is critical for the maintenance of nascent DNA strands in response to replication-associated DSBs and is not specific only to CPT-induced replication breaks.

To further confirm the contribution of WRN to nascent DNA strand maintenance in response to replication breaks, we inhibited the formation of CPT-induced replication-mediated DSBs in cells using the DNA polymerase inhibitor aphidicolin (APH; Figure 2B, top) (Pommier et al., 2010; Takemura et al., 2006). Treatment of WS cells with APH entirely prevented the CPT-dependent shortening of nascent DNA strands in WS cells (Figure 2B, bottom). The nascent DNA tract lengths in APH+CPT-treated WS cells were comparable to those in APH+CPT-treated WS+WRN cells (4.93 ± 0.3 and 5.14 ± 0.05 μm, respectively; Figure 2B, bottom). Collectively, these results reveal that WRN is required for the stabilization of nascent DNA strands in response to replication breaks.

WRN Is Recruited to the Sites of Replication-Associated DSBs and It Colocalizes with RPA2, Rad51, NBS1, and MRE11

Because WRN maintains newly synthesized DNA strands after replication stress, we next verified that WRN is recruited to the sites replication in response to CPT-induced replication stress. As shown in Figure S2A, WRN clearly juxtaposed with the 5-ethynyl-2′-deoxyuridine-labeled replication sites in CPT-treated early-, mid-, and late-S phase cells, indicating that WRN is indeed recruited to the sites of replication in all S phase cells in response to replication stress. Further, as reported previously (Cheng et al., 2005; Kobayashi et al., 2010), we noticed that most WRN foci (82% ± 8%) clearly overlapped with γH2AX foci in CPT-treated but not in mock-treated cells (Figure S2B), confirming that WRN is recruited to the sites of replication-associated DSBs. As with previous reports (Futami et al., 2007; Patro et al., 2011; Sakamoto et al., 2001), we observed colocalization of 70% ± 6% of WRN foci with RPA2 and Rad51 foci in CPT-treated cells (Figures S2C and S2D). In addition, as reported previously (Cheng et al., 2004), we also observed colocalization of WRN and NBS1 foci (71% ± 9%) in CPT-treated cells (Figure S2E). Furthermore, 53% ± 6% of WRN foci overlapped with MRE11 foci in CPT-treated cells (Figure S2F), confirming a previous report (Pichierri and Franchitto, 2004). Taken together, these data demonstrate that WRN colocalizes with many of its known interacting partners at replication-associated DSBs.

Nuclease Activities of WRN Are Not Required for the Stabilization of Nascent DNA Strands in Response to Replication-Associated DSBs

WRN contains multiple protein-protein interaction domains and has DNA binding motifs in the C-terminal domain (Lan et al., 2005; von Kobbe et al., 2003). It also has exonuclease (Huang et al., 1998) and helicase activities (Gray et al., 1997). We set out to identify the domain and the enzymatic activity that are involved in the maintenance of nascent DNA strands in response to replication breaks. We stably expressed Flag+EGFP tagged 1–366 ($WRN_{1–366}$), 250–366 ($WRN_{250–366}$), and 940–1,432 ($WRN_{940–1432}$) amino acid regions of WRN and the exonuclease defective (WRN_{E84A}) and the helicase defective (WRN_{K577A}) full-length WRN constructs in WS cells (Figures 3A, S3A, and S3B). Subsequently, we used these cell lines to identify the WRN domain that is recruited to replication-associated DSBs. As shown in Figure 3B, $WRN_{1–366}$, but not the $WRN_{250–366}$ and $WRN_{940–1432}$ domains, formed distinct foci in response to CPT-induced replication stress. Further, indirect immunostaining with 2 μm anti-WRN revealed that both exonuclease (WRN_{E84A}) and helicase (WRN_{K577A}) defective WRNs formed distinct foci in response to CPT (Figure 3B). In response to CPT treatment, 55% ± 7.7% of $WRN_{1–366}$, 71% ± 5.4% of WRN_{E84A}, and 82% ± 6.1% of WRN_{K577A} foci overlapped with γH2AX foci. Thus, $WRN_{1–366}$, exonuclease-defective and helicase-defective WRN, but not $WRN_{250–366}$ and $WRN_{940–1432}$, were recruited to the sites of replication-associated DSBs.

To verify that the recruitment of $WRN_{1–366}$ to the sites of replication breaks functioned in the maintenance of nascent DNA strands, we evaluated lengths of IdU-labeled fragments in WS cells stably expressing $WRN_{1–366}$. The IdU tract lengths in CPT-treated WS+$WRN_{1–366}$ cells were comparable to those in mock-treated WS+$WRN_{1–366}$ cells (4.82 ± 0.02 and 5.02 ± 0.08 μm, respectively; Figure 3C). Therefore, the $WRN_{1–366}$ fragment is required for the maintenance of nascent DNA strands in response to replication stress.

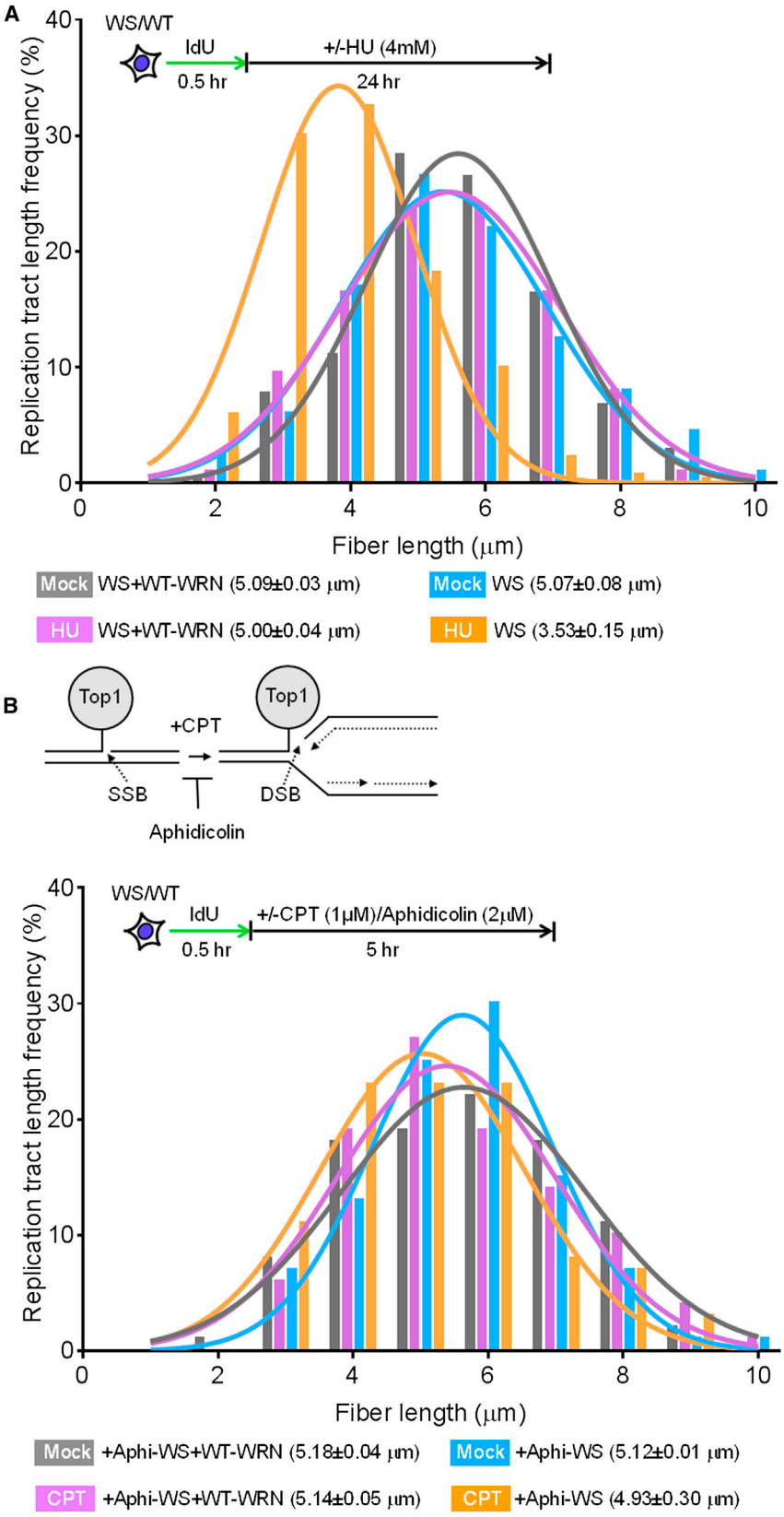

Figure 2. WRN Preserves Nascent DNA Strands in Response to Replication Breaks

(A) IdU-tract-length distribution in WS and WS+WRN cells treated with 4 mM HU for 24 hr.

(B) Nascent DNA-tract-length distribution in WS and WS+WRN cells, exposed to 2 μM aphidicolin/1 μM CPT or CPT only for 5 hr.

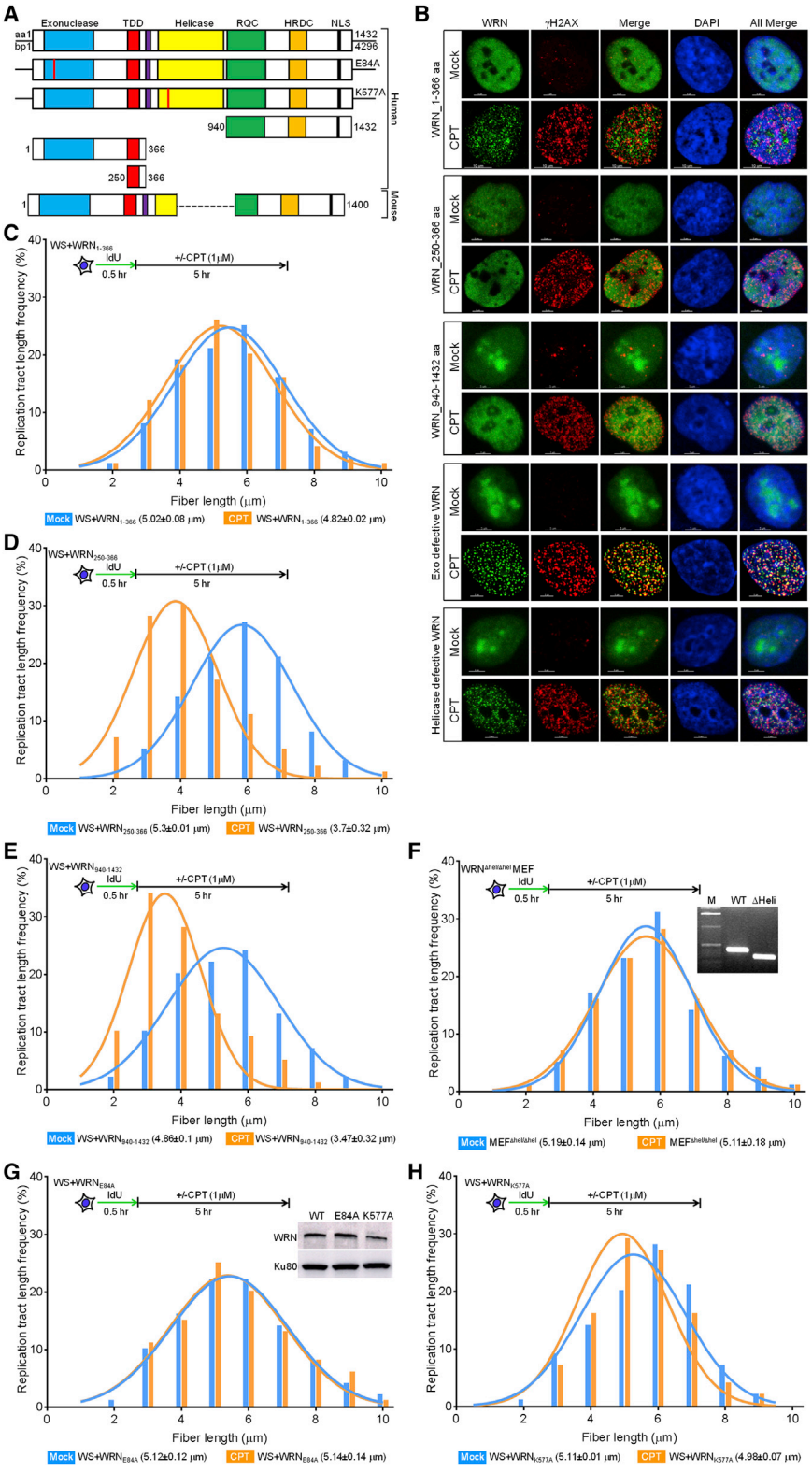

Figure 3. N-Terminal Region of $WRN_{1–366}$ Is Sufficient to Stabilize Nascent DNA Strands in Response to Replication-Associated DSBs

(A) Schematics show different functional regions of human and mouse WRN, and the various WRN domains used in the study. TDD, WRN-WRN interaction domain; HRDC, helicase and RNase D C-terminal domain; RQC, RecQ conserved domain; NLS, nucleolar localization signal; E84A, exonuclease mutant; K577A, helicase mutant. See also Figure S3.

(B) WS cells stably expressing 1–366 aa, 250–366 aa, 940–1,432 aa, exonuclease-defective (E84A), and helicase-defective (K577A) WRN were treated with 1 μM CPT for 1 hr. After 5 hr, cells were immunostained with anti-γH2AX and anti-WRN. Representative confocal microscope images are shown. Scale bars, 5 and 10 μm.

(C–E) IdU-tract-length distribution in WS cells stably expressing $WRN_{1–366}$ (C), $WRN_{250–366}$ (D), and the C-terminal region containing HRDC and DNA binding motifs (940–1,432 aa) (E), treated with or without 1 μM CPT for 5 hr.

(F) Nascent DNA-tract-length distribution in mouse embryonic fibroblasts (MEFs) derived from wild-type (WT) and helicase domain deficient WRN ($WRN^{\Delta hel/\Delta hel}$) mice, treated with or without 1 μM CPT for 5 hr. Inset, ethidium-bromide-stained agarose gel picture for WT and $WRN^{\Delta hel/\Delta hel}$ mice genotyping.

(G and H) IdU-tract-length distribution in WS cells stably expressing exonuclease-defective (E84A; G) and helicase-defective (K577A; H) WRN, treated with or without 1 μM CPT for 5 hr. Inset, western blot analyses for WT, E84A, and K577A WRN expression in WS cells.

Because the $WRN_{1–366}$ fragment contains both exonuclease (1–250 aa) and WRN-WRN interaction (250–366 aa) domains (Perry et al., 2006, 2010), we set out to further narrow down the region of WRN required for the maintenance of nascent DNA strands. The IdU tract lengths were significantly shortened in CPT-treated WS cells stably expressing $WRN_{250–366}$ relative to mock-treated WS+$WRN_{250–366}$ cells (3.7 ± 0.32 and 5.3 ± 0.01 μm, respectively, $p < 0.002$; Figure 3D). Thus, the WRN-WRN interaction domain is not involved in the maintenance of nascent DNA strands.

We then examined the involvement of the C-terminal domain (940–1,432 aa), which contains the DNA binding motifs (Lan et al., 2005; von Kobbe et al., 2003), and the HRDC domain in nascent DNA strand maintenance. We observed that the IdU tract lengths were significantly reduced in CPT-treated WS+$WRN_{940–1432}$ cells as compared with the mock-treated WS+$WRN_{940–1432}$ cells (3.47 ± 0.32 and 4.86 ± 0.1 μm, respectively, $p < 0.002$; Figure 3E). Thus, the C-terminal domain (940–1,432 aa) containing the DNA binding motifs and the HRDC domain of WRN is not required for the maintenance of nascent DNA strands.

To rule out the possibility that the lack of nascent DNA strand maintenance in CPT-treated WS+$WRN_{250–366}$ and WS+$WRN_{940–1432}$ cells could be due to improper folding, we examined the interaction of $WRN_{250–366}$ with full-length WRN and $WRN_{940–1432}$ with the Ku70/80 heterodimer. As previously demonstrated (Perry et al., 2010), the $WRN_{250–366}$ domain interacted with full-length WRN in vivo (Figure S3C). Similarly, the $WRN_{940–1432}$ domain clearly interacted with Ku70/80 heterodimer in vivo (Figure S3D), confirming a previous study (Cooper et al., 2000). These results clearly suggest that the lack of complementation of nascent DNA strand maintenance by $WRN_{250–366}$ and $WRN_{940–1432}$ domains is not due to the improper folding of these constructs.

To determine the contribution of helicase domain, we used MEFs derived from WRN helicase domain-deficient mouse ($WRN^{\Delta hel/\Delta hel}$) (Lebel and Leder, 1998). As shown in Figure 3F, the IdU tract lengths were similar in CPT-treated and mock-treated $WRN^{\Delta hel/\Delta hel}$ MEFs (5.11 ± 0.18 and 5.19 ± 0.14 μm, respectively), suggesting that the central helicase domain of WRN does not contribute to the stabilization of nascent DNA strands in response to replication stress. Next, we examined the involvement of exonuclease and helicase activities in the stabilization of replication forks. The nascent DNA strand lengths in CPT-treated WS cells expressing WRN_{E84A} were similar to those in mock-treated WS+WRN_{E84A} cells (5.12 ± 0.12 and 5.14 ± 0.14 μm, respectively; Figure 3G). Similarly, the IdU tract lengths in CPT-treated WS cells expressing WRN_{K577A} were comparable to those in mock-treated WS+WRN_{K577A} cells (4.98 ± 0.07 and 5.11 ± 0.01 μm, respectively; Figure 3H). Thus, the nuclease activities of WRN are not important for the stabilization of newly replicated genome.

Subsequently, we verified whether the physical presence of WRN at replication breaks is critical for the maintenance of nascent DNA strands. For this purpose, we disrupted the recruitment of WRN to replication breaks in HeLa cells by stably expressing the WRN-WRN interaction domain (Perry et al., 2010). As shown in Figure S4A, recruitment of endogenous WRN to the sites of replication breaks was attenuated in CPT-treated HeLa cells stably expressing $WRN_{250–366}$. Further, we noticed that the nascent DNA strands were significantly shortened in CPT-treated HeLa+$WRN_{250–366}$ cells relative to mock-treated HeLa+$WRN_{250–366}$ cells (3.46 ± 0.1 and 5.21 ± 0.07 μm, respectively, $p < 0.0001$; Figure S4B). Therefore, it is not the nuclease activities of WRN, but the physical presence of WRN at the sites of replication breaks is critical for the maintenance of nascent DNA strands.

NBS1 Recruits WRN to Stabilize Collapsed Replication Forks

Though the $WRN_{1–366}$ fragment lacks any known DNA binding motif, it is sufficient to maintain nascent DNA strands. Therefore, it is possible that this function of WRN is mediated indirectly via one of its binding partners. The FHA domain of NBS1 is known to recruit WRN to the sites of DSBs (Cheng et al., 2004; Kobayashi et al., 2010), and the WRN-NBS1 interaction is mediated through the N-terminal region of WRN (Cheng et al., 2004) (Figure 4A). As shown in Figure 4B, WRN is recruited to replication-associated DSBs in CPT-treated NBS+WT NBS1 cells but not in NBS or NBS+ΔFHA-NBS1 cells. Further, DNA-fiber-length measurements revealed no substantial differences in the IdU tract lengths between CPT- and mock-treated NBS cells expressing full-length NBS1 (NBS+WT; 5.19 ± 0.09 and 5.27 ± 0.23 μm, respectively; Figure 4C). In contrast, nascent DNA strand lengths were significantly shortened in CPT-treated NBS cells expressing ΔFHA-NBS1 relative to mock-treated NBS+ΔFHA-NBS1 cells (3.32 ± 0.07 and 5.15 ± 0.02 μm, respectively, $p < 0.00001$; Figure 4D). Thus, the FHA domain of NBS1 recruits WRN to replication-associated DSBs, and this interaction is critical for the stabilization of newly replicated DNA.

MRE11 Degrades Nascent DNA Strands in the Absence of WRN

When we examined the nascent DNA strand lengths in CPT-treated NBS cells, the IdU tract lengths were similar to those in mock-treated NBS cells (5.04 ± 0.1 and 5.24 ± 0.07 μm, respectively; Figure 5A), implying that the newly replicated DNA is maintained in the absence of NBS1. Evidence suggests that the recruitment of MRE11 to the nucleus and damaged DNA sites is NBS1 dependent (Sakamoto et al., 2007). In the absence of full-length NBS1, MRE11 cannot be recruited to the replication-associated DSBs; hence, the nascent DNA strands are intact in NBS cells. In the NBS+ΔFHA-NBS1 cells, MRE11 is recruited to replication-associated DSBs, but WRN is not; therefore, the nascent DNA tracts are shortened in NBS+ΔFHA-NBS1 cells. Further, a number of studies have found that MRE11 degrades nascent DNA strands in the absence of BRCA2, Fanconi anemia (FA) factors, and Rad51 in response to replication stalling (Hashimoto et al., 2010; Schlacher et al., 2011, 2012). For these reasons, we hypothesize that MRE11 is the nuclease that degrades nascent DNA strands in the absence of WRN. To verify this hypothesis, we first determined the rate of nascent DNA strand degradation in WS cells. As shown in Figure 5B, exposure of WS cells to CPT for different times resulted in a gradual shortening of nascent DNA strands (5.1 ± 0.16, 4.53 ± 0.4, 3.7 ± 0.12, and 2.71 ± 0.05 μm, in mock-treated cells and in CPT-treated

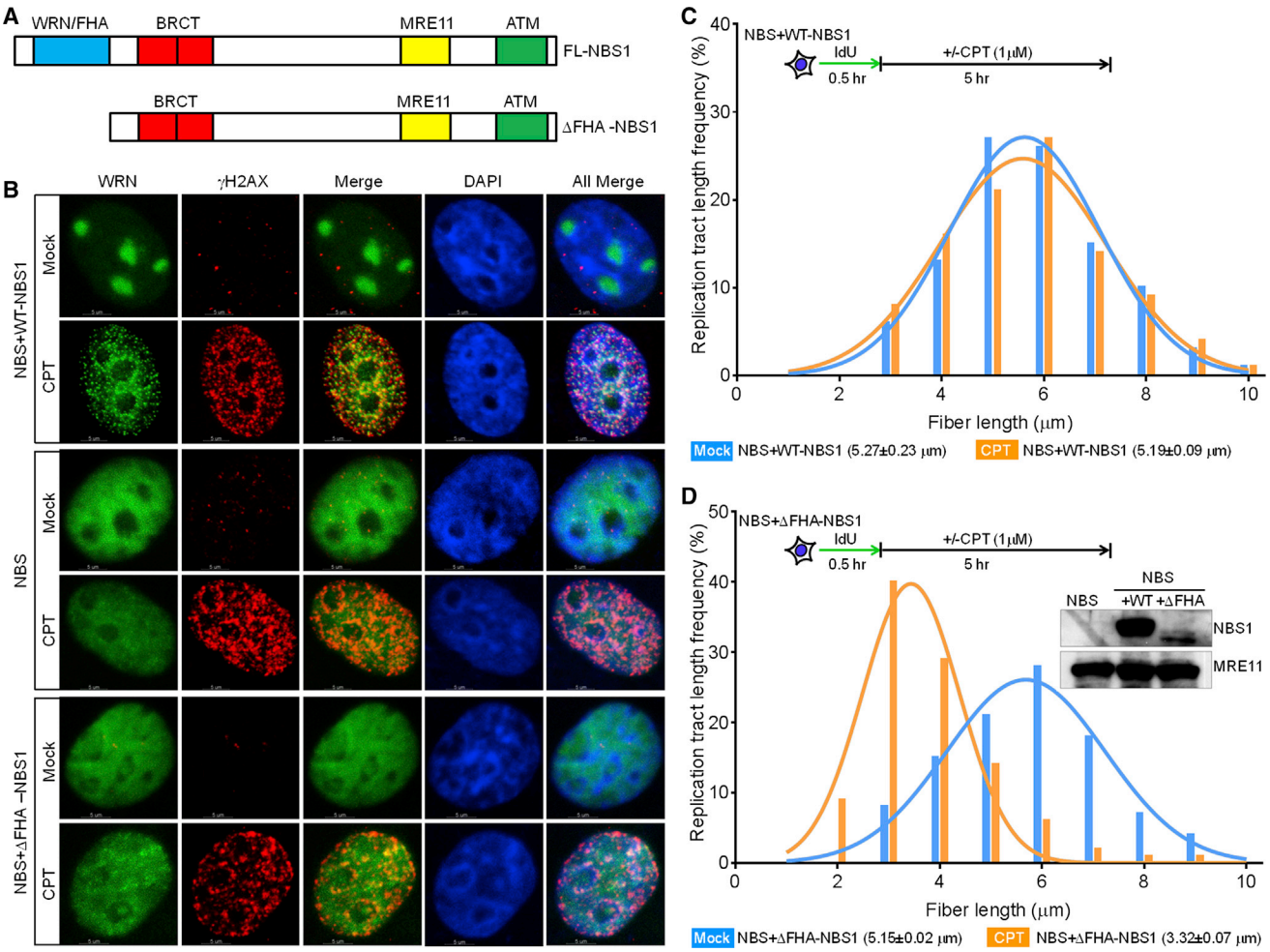

Figure 4. Nascent DNA Strand Maintenance Function of WRN Is NBS1 Dependent

(A) Diagram shows different functional protein-protein interaction domains of NBS1. FHA, Forkhead-associated domain; BRCT, BRCA1 C terminus domain; WRN, ATM, and MRE11, WRN, ATM, and MRE11 interaction domains, respectively.

(B) Representative confocal images show recruitment of WRN to the sites of replication-associated DSBs in NBS, NBS+WT NBS1, and NBS+ΔFHA-NBS1 cells treated with or without 1 μM CPT for 5 hr. Cells were immunostained with anti-WRN and anti-γH2AX. Scale bars, 5 μm.

(C and D) Nascent DNA-tract-length distribution in NBS cells stably expressing full-length (C) and ΔFHA (D) NBS1, treated with or without 1 μM CPT for 5 hr. Inset, western blot analysis for full-length and ΔFHA NBS1 expression in NBS cells.

cells at 1, 2.5, and 5 hr, respectively). The calculated rate of nascent DNA strand degradation was 0.54 ± 0.05 μm/hr. The slow kinetics of nascent DNA strand degradation and lack of replication fork degradation in NBS cells together reveal that the nuclease activities of MRE11 are responsible for the degradation of nascent DNA strands in the absence of WRN.

Subsequently, we examined the direction of nascent DNA strand degradation in WS cells by sequentially labeling the replicating DNA first with CldU and then with IdU. The IdU:CldU ratio was significantly smaller in CPT-treated *WRN*-depleted HeLa cells than in mock-treated *WRN*-depleted HeLa cells (0.62 ± 0.09 and 1 ± 0.01, respectively, $p < 0.0097$; Figure 5C). Importantly, we observed the shortening of very recently replicated DNA (i.e., IdU-labeled DNA) in CPT-treated *WRN*-depleted HeLa cells. MRE11 has 3′–5′ exonuclease and endonuclease activities (Shibata et al., 2014), and the nascent DNA strands were degraded from 3′–5′ direction, supporting our hypothesis that the degradation of nascent DNA strands in WRN-defective cells is mediated by the exonuclease activity of MRE11.

When the exonuclease activity of MRE11 was inhibited with mirin (Dupré et al., 2008), the IdU tract lengths were not shortened in CPT-treated cells, and the DNA fiber lengths in CPT+mirin-treated WS cells were comparable to that of mock+mirin-treated WS cells (4.87 ± 0.01 and 5.37 ± 0.42 μm, respectively; Figure 5D). To rule out the possibility of nonspecific inhibitory activities of mirin, we depleted MRE11 in WS cells using *MRE11* shRNA and then measured the nascent DNA tract lengths. As shown in Figure 5E, the DNA fiber lengths were similar in CPT-treated *MRE11*-depleted WS cells and in mock-treated WS+MRE11-shRNA cells (5.14 ± 0.03 and 5.17 ± 0.01 μm, respectively). To further validate these findings, we depleted WRN in MRE11-defective (ataxia telangiectasia-like

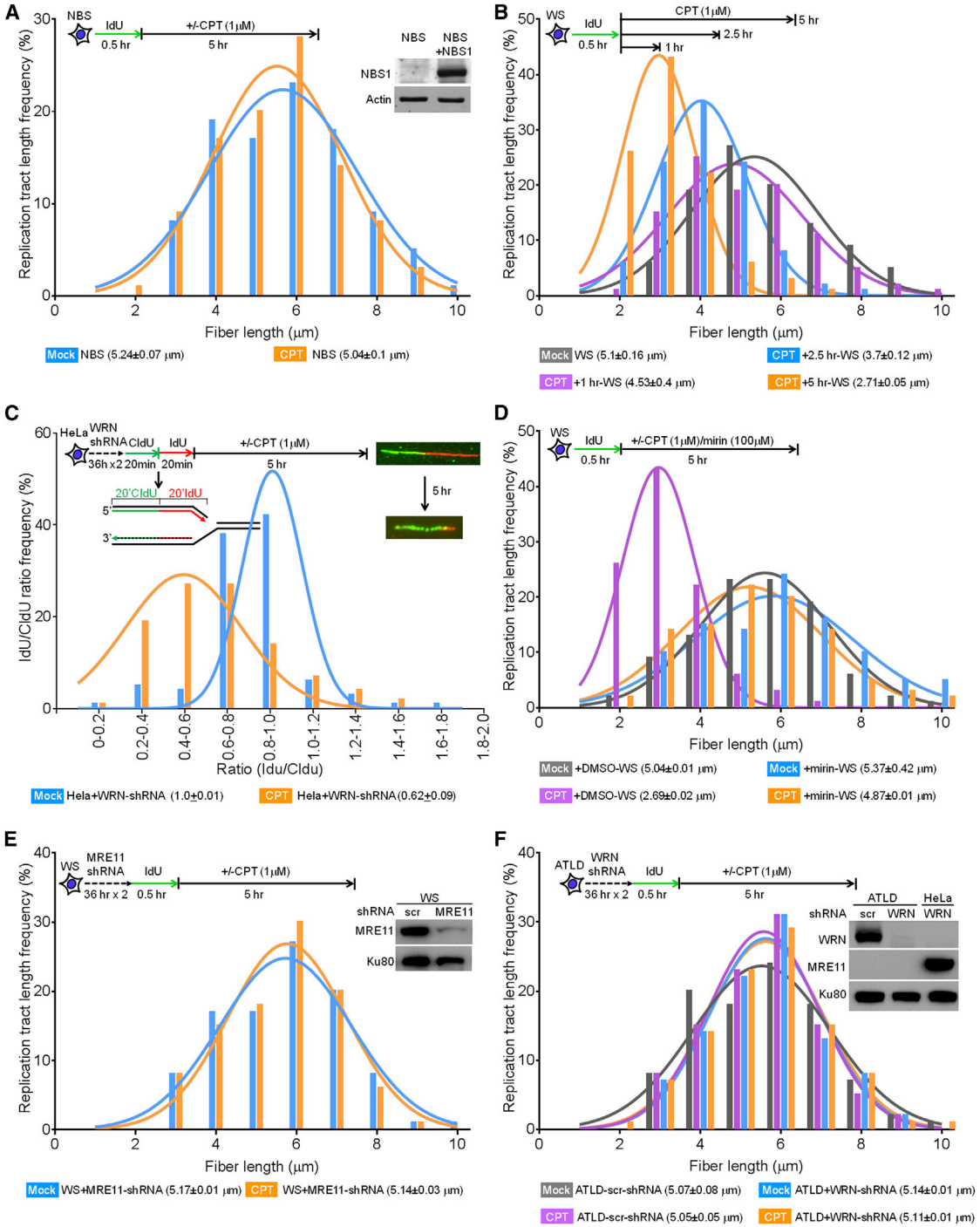

Figure 5. MRE11 Degrades Nascent DNA Strands in Response to Collapsed Replication Forks in the Absence of WRN

(A) IdU-tract-length distribution in NBS1-deficient cells, treated with or without 1 μM CPT for 5 hr. Inset, western blot analysis for NBS1 expression in NBS and NBS+NBS1 cells.

(B) Nascent DNA-tract-length distribution in WS cells, treated with or without 1 μM CPT for 1, 2.5, and 5 hr.

(C) Distribution curves of the ratio of IdU/CldU tract lengths in HeLa cells transfected with either *WRN* or control shRNA treated with or without 1 μM CPT for 5 hr.

(D) IdU-tract-length distribution in WS cells exposed to 100 μM mirin/1 μM CPT or DMSO/1 μM CPT for 5 hr.

(E) Nascent DNA-tract-length distribution in WS cells transfected with either *MRE11* or control shRNA, treated with or without 1 μM CPT for 5 hr. Inset, western blot for MRE11 expression in WS cells transfected with *MRE11* shRNA and control shRNA.

(F) IdU-tract-length distribution in MRE11-defective ATLD cells transfected with either control or *WRN* shRNA, treated with or without 1 μM CPT for 5 hr. Inset, western blots for WRN and MRE11 expression in ATLD cells.

disorder [ATLD]) cells and found that the nascent DNA tract lengths were comparable in CPT-treated and in mock-treated WRN-depleted ATLD cells (5.11 ± 0.01 and 5.14 ± 0.01 μm, respectively; Figure 5F). Collectively, these data indicate that MRE11 is the main nuclease that degrades nascent DNA strands after replication breaks in the absence of WRN.

WRN and Rad51 Function Additively to Block MRE11-Mediated Degradation of Nascent DNA Strands

How does WRN limit the exonuclease activity of MRE11 on the nascent DNA strands at replication-associated DSBs? WRN may modulate either the nuclease activity of MRE11 or the stability of the interaction of Rad51 with replication breaks. Because WRN neither stimulates nor inhibits the nuclease activities of MRE11 (Cheng et al., 2004), we excluded the possibility of WRN-mediated regulation of the nuclease activity of MRE11. Because WRN directly interacts with Rad51 (Otterlei et al., 2006) and colocalizes with Rad51 at the replication-associated DSBs (Figure S2D), it is possible that WRN and Rad51 function together in the stabilization of the broken replication forks. To determine whether WRN and Rad51 function cooperatively to protect nascent DNA strands, we first evaluated the involvement of Rad51 in the maintenance of nascent DNA strands using HT1080 cells that stably express tetracycline-inducible *Rad51* shRNA. The DNA fiber lengths were significantly shortened in CPT-treated *Rad51*-depleted HT1080 cells relative to CPT-treated control HT1080 cells (3.36 ± 0.11 and 5.09 ± 0.01 μm, respectively, $p < 0.002$; Figure 6A), confirming a previous report (Hashimoto et al., 2010). Further, we validated these results using a small-molecule B02 that prevents the formation of Rad51 nucleofilaments (Huang et al., 2012). As shown in Figure S5A, pretreatment of WS and WS+WRN cells with B02 prevented the formation of Rad51 foci in 77% ± 11% of the cells. In cells pretreated with B02, nascent DNA strands were shorter in CPT-treated than in mock-treated WS+WRN cells (3.4 ± 0.26 and 5.1 ± 0.02 μm, respectively, $p < 0.01$; Figure 6B). Moreover, similar to a previous finding (Hashimoto et al., 2010), treatment of WS+WRN cells with mirin, B02, and CPT completely prevented the degradation of IdU tracts. The nascent DNA tract lengths were comparable to those of mock+B02-treated WS+WRN cells (4.7 ± 0.2 and 4.8 ± 0.05 μm, respectively; Figure 6B), implying that MRE11 degrades nascent DNA tracts in the absence of Rad51. Like WRN, Rad51 blocks the MRE11-meditated degradation of nascent DNA strands in response to CPT-induced replication stress.

Recent reports indicate that BRCA2/FA factors and Rad51 act epistatically to protect the nascent DNA strands (Schlacher et al., 2011, 2012). Further, we noticed that the extent of nascent DNA strand degradation was comparable between WRN- and Rad51-deficient cells. To determine the relative contribution of WRN and Rad51 to the stability of replication forks, we downregulated the expression of WRN and Rad51 in the same cell (Figure 6C, inset). Surprisingly, we found that the nascent DNA tracts in CPT-treated *WRN* shRNA/*Rad51* shRNA cells (2.6 ± 0.3 and 5.03 ± 0.1 μm, CPT and mock, respectively, $p < 0.0002$) were shorter than in CPT-treated cells that expressed only one of the shRNAs (Figure 6C). Additionally, we observed similar results when WS cells were first treated with B02 and then exposed to CPT (2.73 ± 0.21 and 4.78 ± 0.06 μm, CPT and mock treatment, respectively, $p < 0.0001$; Figure 6D). Further, addition of mirin prevented the degradation of nascent DNA strands in CPT-treated HT1080 cells that expressed both *WRN* and *Rad51* shRNAs (5.03 ± 0.1 and 4.9 ± 0.15 μm, mock and CPT treatment, respectively; Figure 6C) and in CPT-treated WS+B02 cells (4.78 ± 0.06 and 4.58 ± 0.19 μm, mock and mirin, respectively; Figure 6D). Collectively, these results demonstrate that, unlike BRCA2/FA factors and Rad51, WRN and Rad51 function additively to protect nascent DNA strands after replication stress.

Additive effects of WRN and Rad51 in the protection of replication forks suggest that these proteins are functioning in independent pathways. If this is the case, then how does WRN protect nascent DNA strands in response to replication breaks? Evidence suggests that WRN directly interacts with Rad51 (Otterlei et al., 2006). Further, it has recently been shown that stabilization of Rad51 at stalled replication forks in BRCA2 and FA-pathway-defective cells blocks MRE11-mediated degradation (Schlacher et al., 2011, 2012). Furthermore, the level of Rad51 expression in WS cells was similar to that of WS+WRN cells (Figure S5B). Therefore, it is possible that WRN might stabilize Rad51 at collapsed replications forks to block the MRE11-mediated degradation of nascent DNA strands. To determine whether WRN is involved in the stabilization of Rad51 in response to replication stress, first we enumerated the number of Rad51 foci in WS and WS+WRN cells. We noticed that the number of Rad51 foci remain unchanged in WS+WRN cells exposed to CPT for 1, 2.5, and 5 hr (86.1 ± 11.4, 79.8 ± 7.1, and 83.8 ± 12.4 foci per cell, respectively; Figures S5C and S5D). On the contrary, similar to a previous report (Pichierri et al., 2001), the number of Rad51 foci was reduced significantly in WS cells (24.1 ± 9.3 foci per cell) relative to WS+WRN cells exposed to CPT for 5 hr (Figures S5C and S5D). Subsequently, we verified these results by a chromatin fractionation assay. As shown in Figure S5E, the level of chromatin-bound Rad51 was lower in CPT-treated WS cells relative to CPT-treated WS+WRN cells. Overall, these results suggest that WRN is somehow involved in the stabilization of Rad51 at replication-associated DSBs.

To test whether stabilization of Rad51 at replication breaks prevents MRE11-mediated degradation of nascent DNA strands, we expressed a Rad51 mutant (K133R) in WS cells (Figure S5F). ATP hydrolysis by Rad51 is required for efficient dissociation of Rad51 from the DNA (van Mameren et al., 2009). The Rad51 K133R mutant lacks ATPase activity, and it forms stable DNA-Rad51 complexes and promotes strand exchange in vitro (Morrison et al., 1999). In WS cells overexpressing the Rad51 K133R mutant, the nascent DNA tract lengths were not shortened in CPT-treated cells compared to mock-treated cells (4.38 ± 0.04 and 4.46 ± 0.01 μm, respectively; Figure 6E). Furthermore, Rad51 levels are often elevated in tumor cells (Brown and Holt, 2009), and the formation of stable Rad51 filaments are observed in these cells (Raderschall et al., 2002). We tested whether overexpression of Rad51 in WS cells stabilizes Rad51 filaments and therefore protects replication forks (Figure S5F). As shown in Figure 6F, overexpression of wild-type Rad51 partially protected the nascent DNA tracts in CPT-treated WS cells (3.99 ± 0.12 and 4.52 ± 0.02 μm, CPT

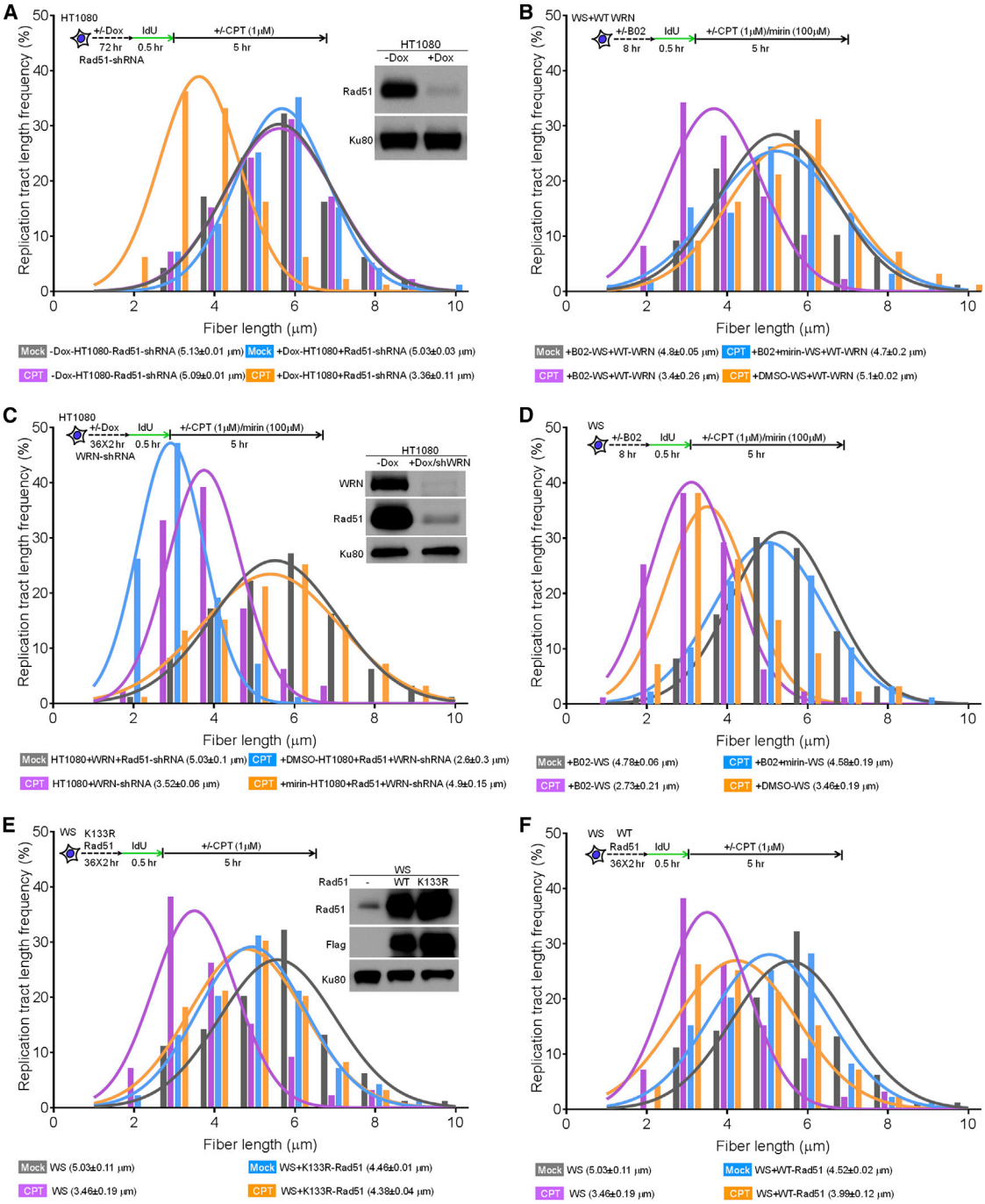

Figure 6. WRN and Rad51 Additively Protect Nascent DNA Strands from MRE11-Mediated Degradation

(A) IdU-tract-length distribution in HT1080 cells stably expressing tetracycline-inducible *Rad51* shRNA, treated with or without 1 μM CPT for 5 hr. Inset, analysis of Rad51 expression in HT1080 cells with or without doxycycline treatment for 72 hr.

(B) Nascent DNA-tract-length distribution in WS+WRN cells exposed to 100 μM B02/1 μM CPT or 1 μM CPT only for 5 hr. See also Figure S5.

(C) IdU-tract-length distribution in HT1080 cells stably expressing tetracycline-inducible *Rad51* shRNA transfected with *WRN* shRNA, exposed to 100 μM mirin/1 μM CPT or 1 μM CPT only for 5 hr. Inset, western blots for Rad51 and WRN expression in HT1080 cells.

(D) Nascent DNA-tract-length distribution in WS cells exposed to 100 μM B02/1 μM CPT or 100 μM B02/100 μM mirin/1 μM CPT or 1 μM CPT only for 5 hr. See also Figure S5.

(E and F) IdU-tract-length distribution in WS cells transfected with K133R mutant (E) and wild-type (F) Rad51, treated with or without 1 μM CPT for 5 hr. Inset shows overexpression of wild-type and K133R mutant Rad51 in WS cells. See also Figure S5.

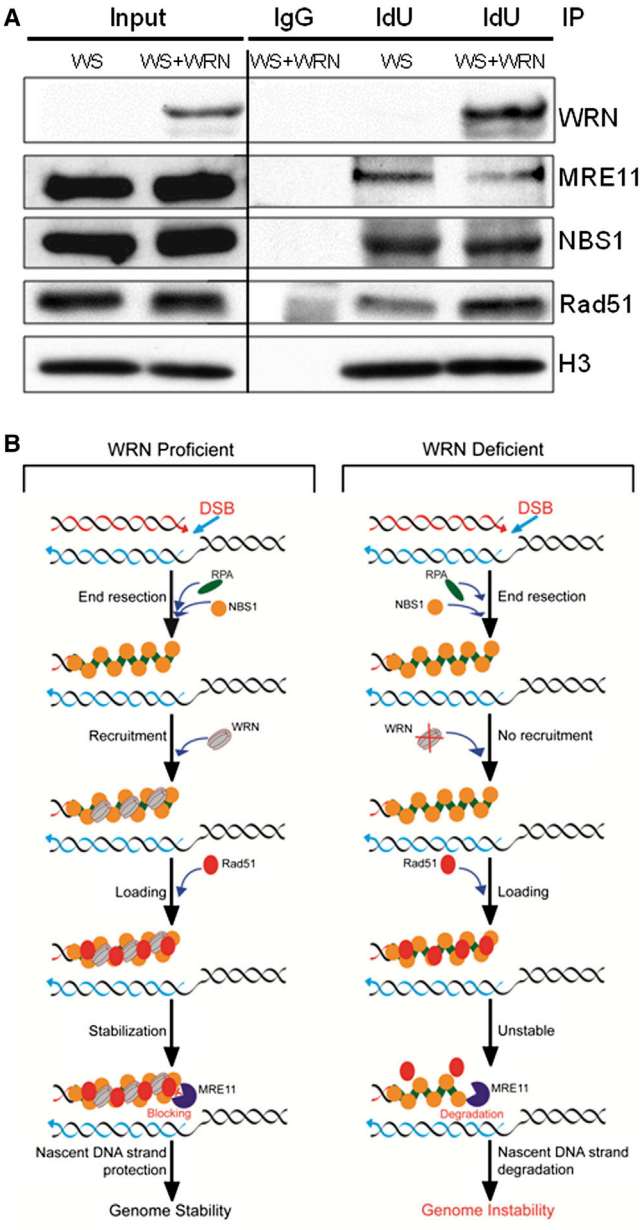

Figure 7. WRN, NBS1, Rad51, and MRE11 Assemble onto Nascent DNA Strands to Maintain Genome Stability in Response to Replication Stress

(A) WRN, NBS1, Rad51, and MRE11 associate with nascent DNA strands. WS and WS+WRN cells were labeled with IdU for 60 min and then treated with 1 μM CPT for 5 hr. Cells were crosslinked with paraformaldehyde and the chromatin fraction (input) was subjected to coimmunoprecipitation using anti-BrdU (IdU) antibodies. Western blots were probed with anti-WRN, anti-MRE11, anti-NBS1, anti-Rad51, and anti-Histone 3 (H3).

(B) A model depicting the choreography of WRN, NBS1, Rad51, and MRE11 action in the maintenance of nascent DNA strands in response to replication-associated DSBs. See also Figure S6.

and mock, respectively). Thus, the stabilization Rad51 at replication breaks in WRN-defective cells blocks MRE11-mediated degradation of nascent DNA strands.

WRN Is Critical for Chromosome Stability in Response to CPT Treatment

To investigate the contribution of WRN to maintenance of genome stability in response to replication stress, we evaluated gross chromosomal aberrations in metaphase cells derived from WS and WS+WRN cells exposed to CPT for 5 hr. Classical chromosome analysis of metaphase spreads revealed that exposure of WS cells to CPT significantly elevated the number of chromosomal aberrations per mitotic cell relative to the number per mitotic WS+WRN cell ($p = 0.0006$; Figure S6A). The average number of aberrations per WS cell treated with CPT was 2.29 ± 0.02, but it was only 0.56 ± 0.03 per WS+WRN cell exposed to CPT. Further, the percentage of metaphases with gaps, breaks, and radials was significantly higher in WS cells relative to WS+WRN cells (Figure S6B). Number of gaps, breaks, triradial, chromosomal breaks, and end-end fusions were also significantly elevated in CPT-treated WS cells compared to CPT-treated WS+WRN cells (Figure S6C). Thus, WRN plays an important role in the suppression of chromosome instability in response to replication stress.

WRN, NBS1, Rad51, and MRE11 Assemble onto Nascent DNA Strands

To verify whether WRN, NBS1, Rad51, and MRE11 assemble onto nascent DNA strands, we carried out chromatin immunoprecipitation (ChIP) assay (Petermann et al., 2010). As shown in Figure 7A, WRN, NBS1, Rad51, and MRE11 clearly interacted with the nascent DNA strands in WS+WRN cells. Interestingly, we noticed that association of Rad51 with nascent DNA strands was reduced in CPT-treated WS cells relative to WS+WRN cells (Figure 7A), further supporting our observation on the reduced number of Rad51 foci and chromatin binding of Rad51 in WS cells. Evidences indicate that WRN directly interacts with NBS1 (Cheng et al., 2004) and Rad51 (Otterlei et al., 2006), and its interaction with MRE11 is mediated via NBS1 (Cheng et al., 2004). Therefore, it is possible that WRN, NBS1, Rad51, and MRE11 assemble onto nascent DNA strands not as a complex but via pairwise interactions.

DISCUSSION

Here, we report a previously uncharacterized nonenzymatic role for WRN in the stabilization of nascent DNA strands in response to replication stress. We found that the nuclease activities of WRN were not required for the maintenance of nascent DNA strands in response to replication breaks. The N-terminal FHA domain of NBS1 recruits WRN to the replication-associated DSBs to limit the nuclease activity of its C-terminal binding partner, MRE11, on the newly replicated genome. Notably, WRN functions together with Rad51 to block the MRE11-mediated degradation of nascent DNA strands. Overall, our findings reveal that WRN, NBS1, and Rad51 cooperatively protect collapsed replication forks to maintain genome stability (Figure 7B). See also the Supplemental Information.

A unique finding of our study is that the physical presence of WRN at replication-associated DSBs, rather than its enzymatic activities, is critical for the protection of nascent DNA strands. This is evident from our observation that the nascent DNA

strands are not degraded in WS cells expressing exonuclease- or helicase-defective WRNs (Figures 3G and 3H). The nascent DNA strands were degraded when recruitment of WRN to the replication-associated DSBs was blocked in wild-type cells by expression of the WRN-WRN interaction domain (Figure S4B). In support of our findings, a previous report indicated that WRN plays a structural role during DSB repair by protecting DSB ends from enzymatic degradation (Chen et al., 2003), and another study found that WRN forms complexes with a range of DNA structures independently of its catalytic activities (Kamath-Loeb et al., 2012). Our identification of the nonenzymatic contribution of WRN in the stabilization of nascent DNA tracts clarifies why WS patients are symptomatic even though most of the *WRN* mutations identified result in premature termination of WRN protein leading to a loss of WRN nuclear localization rather than to mutations that eliminate exonuclease or helicase activity.

Our results revealed that nascent DNA strands are shortened more in CPT-treated cells deficient in *WRN* and *Rad51* than in cells treated with CPT and either *WRN* or *Rad51* shRNA alone (Figures 6C and 6D), suggesting that WRN and Rad51 function additively to maintain newly replicated DNA. This is in contrast to replication fork protection functions of BRCA2 and FA factors. BRCA2 and FA factors functions are epistatic with Rad51 function (Schlacher et al., 2011, 2012). There are a number of potential explanations for this puzzling observation. Evidence indicates that CPT can induce both replication fork stalling and breaks. In the absence of WRN, Rad51 may collaborate with BRCA2 and FA factors to maintain the nascent DNA strands in response to CPT-induced stalling of replication forks. In support of this possibility, replication fork stalling caused by low-dose CPT or short-term HU treatments did not shorten the nascent DNA strands in WS cells (Figures 1D and 1E). On the other hand, in the absence of Rad51, WRN can bind to replication breaks and may be sufficient to block the MRE11-mediated degradation of nascent DNA strands. Alternatively, WRN may interact with Rad52 via its C-terminal domain (Baynton et al., 2003) to protect newly synthesized DNA from MRE11. These possibilities can be tested by identifying and mutating the Rad51/Rad52 interaction domain in WRN and by examining the ability of WRN mutants to protect nascent DNA strands. Furthermore, in the absence of both WRN and Rad51, it is probable that both stalled and broken replication forks are degraded by the MRE11 and, therefore, more shortening of nascent DNA strands in CPT-treated *WRN*- and *Rad51*-deficient cells was observed than in cells deficient in either Rad51 or WRN. Despite unknowns, our study, together with other reports, clearly indicates that Rad51 together with BRCA2/FA factors or with WRN blocks MRE11-mediated degradation of both stalled and broken replication forks, respectively.

We found that WRN is somehow involved in the stabilization of Rad51 at replication-associated DSBs (Figures S5C–S5E). This result was totally unexpected, because there is no evidence for the WRN-mediated stabilization of Rad51 at replication breaks. However, the mechanism by which WRN directly or indirectly stabilizes Rad51 at replication breaks is unclear. WRN is shown to directly interact with Rad51 (Otterlei et al., 2006), and this interaction may facilitate the stability of Rad51 at replication breaks. In the absence of WRN, Rad51 loads onto the replication breaks but might immediately be unloaded. This can happen either passively due to lack of WRN at the breaks or actively dislodged by other proteins. For example, Bloom syndrome protein and/or RecQL5 might disrupt Rad51 filaments (Bugreev et al., 2007; Hu et al., 2007) in the absence of WRN; however, further experiments are required to support this notion. Nevertheless, our study together with other studies (Schlacher et al., 2011, 2012) suggest that Rad51 prevents MRE11-mediated degradation of nascent DNA strands through two parallel, yet cooperative, pathways: (1) Rad51 cooperates with BRCA2 and FA factors to stabilize nascent DNA strands in response to stalled replication forks (Schlacher et al., 2011, 2012) and (2) Rad51 collaborates with WRN to maintain nascent DNA strands in response to replication breaks.

Mutations in *WRN*, *NBS1*, and *MRE11* genes lead to WS, Nijmegen breakage syndrome (NBS), and Ataxia telangiectasia-like disorder (ATLD), respectively. Individuals with these syndromes are all at risk for development of cancer. What is the link between unprotected replication forks and genome instability? The presence of a large number of chromosomal aberrations in CPT-treated WRN-defective cells suggests that maintenance of nascent DNA strands is critical for the prevention of chromosomal instability. Though exposure of WS cells to CPT compromises cell survival (Poot et al., 1999), some cells with chromosomal aberrations enter mitosis. Every subsequent round of replication is expected to increase the overall mutation level in surviving cells. Therefore, the biological significance of unprotected replication forks is high, because replication of a damaged genome can provide the opportunity for genomic rearrangements and can increase genomic instability leading to genetic changes required for progression from an initiated cell to a metastatic tumor cell.

Our data suggest a model for the interplay of WRN, NBS1, MRE11, and Rad51 in preserving genome integrity during replication (Figure 7B). NBS1 recruits WRN through the N-terminal FHA domain and MRE11 via its C-terminal domain to the replication-associated DSBs. The physical presence of WRN at collapsed replication forks somehow stabilizes the interaction of Rad51 with replication breaks and that limits the excessive exonuclease activity of MRE11 on the newly replicated genome. Thus, the WRN, NBS1, and Rad51 cooperatively protect nascent DNA strands to maintain genome integrity during replication.

In summary, we have deciphered the molecular choreography of WRN, NBS1, MRE11, and Rad51 that occurs at nascent DNA strands to maintain chromosomal integrity during replication. The coordinated action of these factors prevents the accumulation of cancer-promoting mutations in humans. Our experiments revealed a nonenzymatic role for WRN in DNA replication and provide insight into the molecular origin of genome instability in WS individuals.

EXPERIMENTAL PROCEDURES

Cell Lines

The simian virus 40 transformed control (AG07217A) and Werner syndrome (AG11395) fibroblasts, HT1080 cells, and HeLa cells were obtained from the ATCC. hTERT-immortalized WS and WS cells complemented with wild-type

(WT), exonuclease defective (E84A), or helicase-defective (K577A) WRN were described previously (Perry et al., 2006). NBS, NBS cells complemented with full-length or FHA-deleted NBS1, and ATLD cells were described previously (Kobayashi et al., 2010). Chinese hamster ovary cells defective in BRCA2 (V-C8) and V-C8 cells complemented with WT BRCA2 were described previously (Nagasawa et al., 2008).

Drugs

Aphidicolin, MRE11 inhibitor [5-(4-hydroxybenzylidene)-2-iminothiazolidin-4-one, (mirin)], IdU (I7125), CldU (C6891), hydroxyurea, and camptothecin were obtained from Sigma. Rad51 inhibitor [(E)-3-benzyl-2(2-(pyridine-3-yl)vinyl)quinazolin-4(3H)-one, (B02)] was purchased from Calbiochem.

DNA Fiber Assay

DNA fiber technique was performed as previously described (Petermann et al., 2010; Schlacher et al., 2011). Briefly, 2.5 × 10^5 cells were labeled with IdU (150 μM) for 30 min, washed four times with warm PBS, and exposed to 1 μM CPT for 1–5 hr. After three washes with warm PBS, both labeled and unlabeled cells were trypsinized and mixed at 1:15 ratio (labeled:unlabeled) and lysed on a clean glass slide in 20 μl of lysis buffer (0.5% SDS, 50 mM EDTA, and 200 mM Tris-HCl [pH 7.4]) for 8 min, and slides were tilted slightly (~15° angle) to help DNA spread slowly. Subsequently, slides were immunostained with anti-BrdU antibodies, and the DNA fiber lengths were measured using Axiovision Software. See also Supplemental Information.

Statistical Analysis

The Student's t test was performed to calculate the level of significance and a value of $p < 0.05$ was considered statistically significant. GraphPad Prism (version 6.0) was used to calculate DNA-fiber-length distribution and for making the graphs.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.025.

AUTHOR CONTRIBUTIONS

F.S. and A.A. conceived the study, designed and performed the experiments, analyzed data, and made the figures. S.M., Y.Y., E.M., S.B., S.M.Y., and J.K. performed experiments. D.J.C. provided reagents, materials, and analysis tools. A.A. wrote the paper.

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