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Accumulation of Werner protein at DNA double-strand breaks in human cells

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Summary

Werner syndrome is an autosomal recessive accelerated-aging disorder caused by a defect in the *WRN* gene, which encodes a member of the RecQ family of DNA helicases with an exonuclease activity. In vitro experiments have suggested that WRN functions in several DNA repair processes, but the actual functions of WRN in living cells remain unknown. Here, we analyzed the kinetics of the intranuclear mobilization of WRN protein in response to a variety of types of DNA damage produced locally in the nucleus of human cells. A striking accumulation of WRN was observed at laser-induced double-strand breaks, but not at single-strand breaks or oxidative base damage. The accumulation of WRN at double-strand breaks was rapid, persisted for many hours, and occurred in the absence of several known interacting proteins including polymerase β, poly(ADP-ribose) polymerase 1 (PARP1), Ku80, DNA-dependent protein kinase (DNA-PKcs), NBS1 and histone H2AX. Abolition of helicase activity or deletion of the exonuclease domain had no effect on accumulation, whereas the presence of the HRDC (helicase and RNaseD C-terminal) domain was necessary and sufficient for the accumulation. Our data suggest that WRN functions mainly at DNA double-strand breaks and structures resembling double-strand breaks in living cells, and that an autonomous accumulation through the HRDC domain is the initial response of WRN to the double-strand breaks.

Supplementary material available online at
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Key words: Werner protein, Double-strand breaks, Laser irradiation, Damage accumulation, HRDC domain

Introduction

People with Werner syndrome display several clinical signs and symptoms associated with early aging, including graying of hair, cataracts, osteoporosis, atherosclerosis, type II diabetes mellitus and a high incidence of malignant neoplasms (Chen and Oshima, 2002). The gene defective in Werner syndrome, *WRN*, encodes a protein of the RecQ family of DNA helicases (Hickson, 2003; Opresko et al., 2003; Yu et al., 1996), which possesses DNA-dependent ATPase, 3′→5′ helicase activities at the central region and the 3′→5′ exonuclease activity at its amino (N) terminus. The substrate specificity of WRN helicase and exonuclease activities has been determined by in vitro studies and includes a variety of intermediates produced during DNA replication, recombination and repair (Shen et al., 1998; Xue et al., 2002). The carboxyl (C) terminal region of WRN protein contains the conserved RQC (RecQ conserved) domain, including a nucleolar targeting sequence (NTS) (Marciniak et al., 1998; von Kobbe and Bohr, 2002) and an HRDC (helicase and RNaseD C-terminal) domain, whose function is still unclear but has been shown to play a role in DNA binding in the *Saccharomyces cerevisiae* homologue Sgs1 (Liu et al., 1999). The nuclear targeting of the WRN protein is due to the presence of a classical nuclear localization signal (NLS) in the C-terminal region of the protein (Matsumoto et al., 1997). Full-length WRN binds DNA with low efficiency in vitro (Orren et al., 1999; Shen and Loeb, 2000), and the exonuclease, RQC, and HRDC regions of WRN have been shown to be three distinct, structure-specific, but not sequence-specific, DNA binding domains (von Kobbe et al., 2003b).

It has been reported that many proteins physically and functionally interact with WRN. The interacting proteins appear to function at various levels in the mechanisms for maintaining the integrity of the genome and in the DNA damage response, suggesting that WRN plays one or more roles in DNA repair. WRN has been shown to interact with Ku and DNA-PKcs (Karmakar et al., 2002a; Karmakar et al., 2002b; Li and Comai, 2000; Orren et al., 2001), and a recent report suggests that WRN may participate in non-homologous end-joining (Li and Comai, 2002). Werner syndrome fibroblasts transformed with Simian Virus-40 (SV40) T antigen or immortalized by expressing human telomerase reverse transcriptase (hTERT) display a mild but distinct sensitivity to ionizing radiation when compared with appropriate control fibroblasts and Werner syndrome fibroblasts expressing exogenous WRN (Cheng et al., 2004; Yannone et al., 2001). This suggests that WRN may be involved

in processing ionizing radiation-induced double-strand breaks. WRN also interacts physically with the Mre11-Rad50-NBS1 complex, which functions in homologous recombination for double-strand break processing (Cheng et al., 2004). Other reports suggest that WRN may play a role in base excision repair because of a physical interaction between WRN and polymerase β (POL β) involved in base excision repair and the repair of single-strand breaks (Harrigan et al., 2003). Furthermore, p53 has been shown to interact with the C-terminus of WRN and to inhibit WRN exonuclease activity in vitro (Blander et al., 1999; Brosh et al., 2001). Consistent with possible roles of WRN in DNA repair and genome stability, Werner syndrome cells show an attenuated p53-mediated apoptosis (Spillare et al., 1999) and display extensive deletions at non-homologous joined ends as well as non-homologous chromosome exchanges (Oshima et al., 2002). With regard to the localization of WRN in cells, various distribution patterns of the protein have been reported. WRN foci have been observed as diffuse nuclear, nucleolar or nuclear foci depending on the stage of the cell cycle (Gray et al., 1998; Opresko et al., 2003). Although the number of WRN-containing nuclear foci increases after replication fork arrest and upon DNA damage (Sakamoto et al., 2001; Szekely et al., 2000), the significance of the formation of these foci remains to be elucidated. Furthermore, in spite of many reports describing that WRN modified the enzymatic activity of the interaction partners and vice versa, it has not be shown whether WRN responds to DNA damage and whether WRN is involved in base excision repair, repair of single-strand breaks or double-strand breaks in living cells.

We recently established a laser micro-irradiation system for the localized production of single-strand breaks, double-strand breaks and oxidative base damage in a cell nucleus (Lan et al., 2004). Using this system we have analyzed the accumulation of endogenous and green-fluorescent-protein (GFP)-tagged WRN at various types of DNA damage and found that WRN accumulated via its HRDC domain at double-strand breaks within 1-2 minutes after irradiation and remained for a longer period at the site. Our results showed, for the first time, an immediate accumulation of WRN at double-strand breaks and suggest important roles for WRN in genome stability of living cells.

Materials and Methods

Plasmid construction for GFP-fused genes

Oligonucleotides containing *Xho*I, *Sma*I and *Not*I sites were introduced into the cloning sites of pEGFP-N1 or C1 vectors (Clontech), and cDNA for *WRN* and *WRN* containing a helicase mutation (K577M), *NBS1, BRCA1* were introduced into the vectors in frame. Deletion fragments of *WRN* (a.a. 152-1432) and (a.a. 1012-1432) were obtained by digestion of the whole *WRN* cDNA by *Eco*RV and *Not*I, *Pvu*II and *Not*I, respectively, and the each fragment was then introduced into the *Sma*I and *Not*I sites of the vector. *WRN* (a.a. 1-1150) and (a.a. 1229-1432) fragments were constructed by PCR, and *WRN* (a.a. 1229-1432) was introduced into the *Xho*I and *Not*I site of pEGFP vector, while *WRN* (a.a. 1-1150) was cloned into a vector harboring an extra nuclear localization signal.

Cell lines and transfection

Cell lines of HeLa, *Parp1*- (a cell line from mouse embryonic fibroblasts, a generous gift of Mitsuko Masutani), Susa/T-n cells (p53+/+, telomerase expressed, a generous gift of Kanji Ishizaki), 1022QVA (NBS1-deficient Nijmegen patient cells), CHO9 (WT cells), XR-1 (XRCC4 deficient cell line), XR-C1 (DNA-PKcs-deficient cell line), XRV15B (Ku80-deficient cell line), and H2AX-deficient MEF (Celeste et al., 2003) were used. All the above cell lines were propagated in D-MEM (Nissui) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. 38Δ (mouse *Polβ* –/– cell line) and MB36.3 (mouse *Polβ* –/–cell transfected with wild-type human *POLβ*), generous gifts from Samuel H. Wilson, were grown at 34°C and 10% CO_2. Cells were plated on glass bottom dishes (Matsunami Glass) at 50% confluence 24 hours before the transfection (Fugene-6, Life Technology) and irradiated by laser under a microscope 48 hours after transfection.

Microscopy and laser irradiation

Fluorescence images were obtained and processed using a FV-500 confocal scanning laser microscopy system (Olympus). A 365 nm pulse laser micro-irradiation apparatus combined with the confocal microscope was used as previously described (Lan et al., 2004). We used two irradiation doses, a lower dose (0.75 μJ) or a higher dose (2.5 μJ), which were obtained by passing lasers through either an F20 or an F25 filter, respectively, in front of the lens. By using this system, various types of DNA damage, such as single-strand breaks (produced by lower dose and higher dose irradiation), double-strand breaks and oxidative base damage (produced by higher dose irradiation), were produced at restricted nuclear regions of mammalian cells. We also used a 405 nm pulse laser system (Olympus) for irradiation of cells in the epi-fluorescence path of the microscope system. The power of the laser scan can be controlled by the number of scans used or by laser dose. One scan of the laser light at full power delivers energy of around 1600 nW. We used only a full power scan from the 405 nm laser in this study and regulated the dose by changing the number of scans. Both 365 nm and 405 nm lasers were focused through a 40× objective lens. Cells were incubated with Opti-medium (Gibco) in glass-bottom dishes, which were placed in chambers to prevent evaporation, on a 37°C hot plate. The energy of fluorescent light at the irradiated site was measured with a laser power/energy monitor (Orion, Ophir Optronics, Israel). The mean intensity of each focus was obtained after subtraction of the background intensity in the irradiated cell. Each experiment was performed at least three times and the data presented here are mean values obtained in a given experiment. Local UVC-light irradiation were performed as previously described (Okano et al., 2003).

Cell-cycle synchronization

Cell synchronization was performed by the double thymidine block method. In all, 5×10^4 cells were seeded in a 3.5 cm dish and grown for 2 days. Thymidine was then added to 2.5 mM final concentration and cells were further incubated for 22-24 hours. Thymidine-containing medium was removed and cells were washed three times with Hank's buffer and fresh medium was added. After 10 hours, cells were treated with hydroxyurea at 1 mM final concentration and incubated for 14-16 hours. Under these conditions, cells accumulate at the G1/S border. Cells were then washed three times with Hank's buffer. Synchronization of the cell cycle was analyzed by a FACSCalibur (Becton Dickinson). Cells were incubated for 3 hours, 8 hours and 16 hours in fresh medium to obtain S-phase, G2-M-phase and G1-phase cells, respectively.

Immunocytochemistry and chemicals

Cells were irradiated and stained with antibodies raised against human γH2AX, WRN and XRCC1. Cells were fixed 15 minutes after irradiation. Immunofluorescence was performed as described previously (Okano et al., 2003). Anti-phosphorylated H2AX

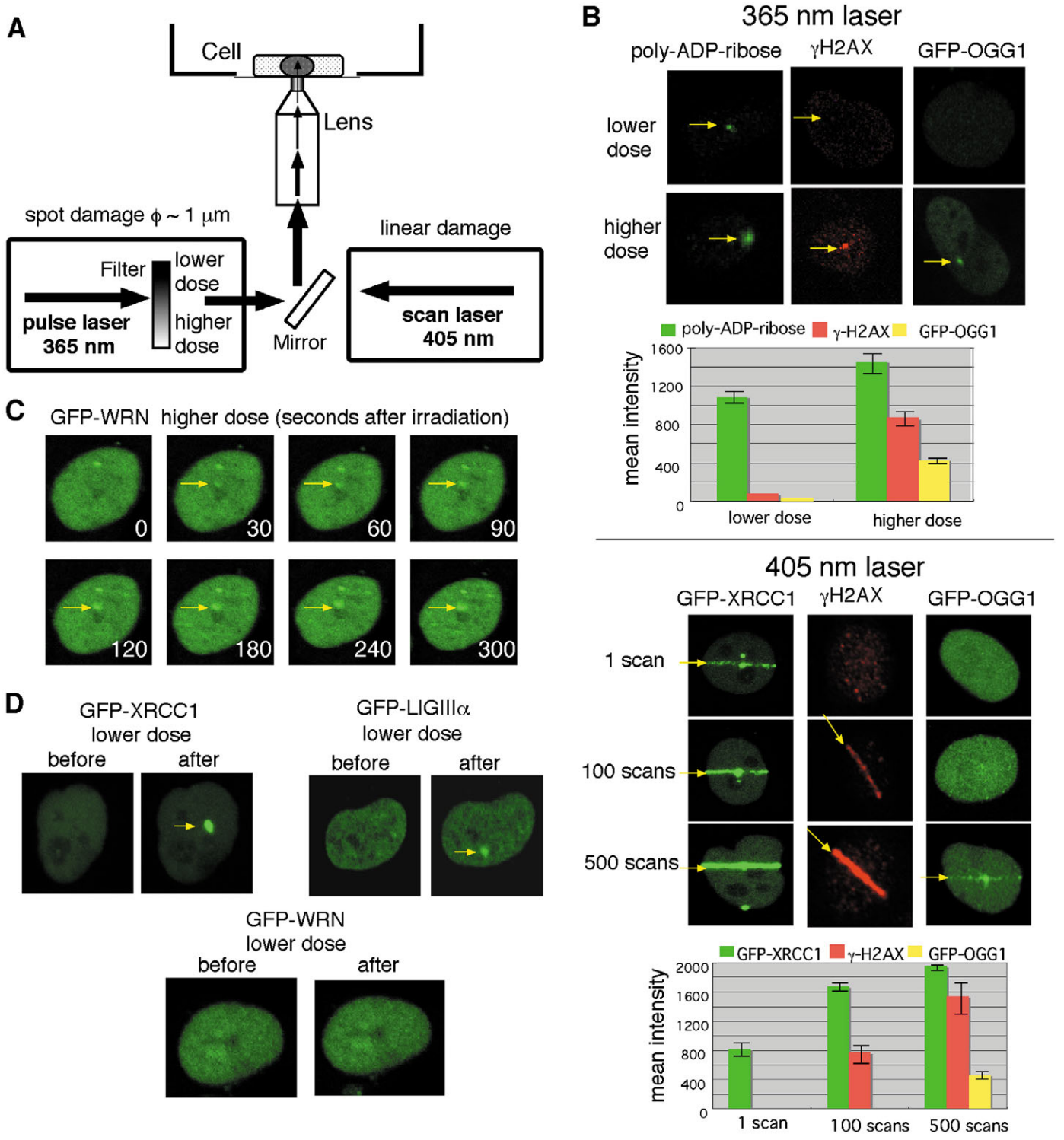

Fig. 1. Laser irradiation systems and accumulation of WRN. (A) Laser irradiation systems. The left column shows the 365 nm pulse laser irradiation system, producing the lower dose and the higher dose irradiation, which are regulated by the filter in front of the mirror. The right column shows the 405 nm laser system. (B) Three types of damage induced by 365 nm and 405 nm laser irradiation. HeLa cells irradiated with the lower dose or the higher dose of 365 nm laser, or with 405 nm laser of different scan times, were stained with anti-poly(ADP)ribose (left, for single-strand breaks) or by γH2AX antibody (middle, for double-strand breaks), respectively, or accumulation of GFP-tagged OGG1 at irradiated sites (right, for base damage) is shown. The amounts of accumulated molecules for poly(ADP)ribose, γH2AX and GFP-OGG1 after lower and higher dose irradiation with the 365 nm or 405 nm laser were quantified in the graphs. (C) Time-dependent accumulation of GFP-tagged WRN after higher dose irradiation with the 365 nm laser in HeLa cells. (D) Accumulation of GFP-tagged XRCC1 and LIGIIIα (upper panels) and no accumulation of GFP-tagged WRN (lower panels) after lower dose irradiation with the 365 nm laser in HeLa cells. Arrows indicate the sites of irradiation.

(γH2AX) (1:200 dilution; Upstate Biotechnology), anti-WRN (1:20) and anti-XRCC1 (1:200 dilution, Abcam) were used. For photosensitization treatment of cells, Ro-19-8022 (Roche) at a final concentration of 250 nM was added into the medium and cells were incubated at 37°C for 5 minutes. For 1,5-dihydroxyisoquinoline (DIQ) treatment, cells were incubated with DIQ (Sigma) in the medium at a final concentration of 500 μM for 1 hour before irradiation. Camptothecin (Sigma) at a final concentration of 1 μM was added to the medium and cells were incubated at 37°C for 6 hours. 5-Bromo-2′-deoxyurine (BrdU; Roche) at a final concentration of 10 μM was added to the medium 8 hours before laser irradiation.

Results

Accumulation of WRN at sites of DNA damage induced by laser micro-irradiation.

To analyze whether WRN responds to DNA damage in vivo, we used a laser micro-irradiation apparatus combined with a confocal microscope (Lan et al., 2004). A single pulse of 365 nm laser micro-irradiation in the nucleus of a human cell at the lower dose (0.75 μJ) or one scan with 405 nm laser produced hardly any detectable DNA lesions other than single-strand breaks (detected with antibody against poly-ADP-ribose, Fig. 1B), whereas irradiation at the higher dose (2.5 μJ) or 100 and 500 scans with 405 nm laser produced double-strand breaks (detected by antibody against phosphorylated H2AX; γH2AX) and base damage (detected by accumulated GFP-OGG1 for 8-oxoGuanine) in addition to single-strand breaks at the irradiated sites (Fig. 1B) (Lan et al., 2004). For the analysis of WRN accumulation at the irradiated sites we used GFP-tagged WRN, which is functional in cells as previously reported (Opresko et al., 2003), or antibody raised against human WRN (see below). Using this system, we found that WRN accumulated at the site irradiated with the higher dose of laser in a time-dependent manner (Fig. 1C). Thirty seconds after irradiation the accumulated GFP-WRN was barely visible, but 3 minutes after irradiation the intensity of the fluorescence reached its maximum (Fig. 1C). GFP-WRN did not accumulate at irradiated cytoplasm or regions in mitotic cells without DNA (Fig. S1 in supplementary material), indicating that the accumulation of WRN is DNA damage-specific. We also analyzed the accumulation of WRN in cells irradiated with the low dose of laser, which produced almost single-strand breaks alone (Fig. 1B). Even though XRCC1 and LIGIIIα, which are involved in the repair of single-strand breaks, efficiently accumulated at the site irradiated with the lower dose, WRN did not accumulate at all at the site irradiated with the lower dose (Fig. 1D), suggesting that the substrate for the accumulation of WRN was not single-strand breaks. To confirm this, we examined the accumulation of WRN at single-strand breaks produced in a nucleotide excision repair-deficient xeroderma pigmentosum group A (XP-A) cell line expressing *Neurospora crassa* UV damage endonuclease (UVDE), which introduces a nick 5′ to UVC-light induced lesions, and thereby produces 5′-blocked single-strand breaks (Okano et al., 2003). WRN did not accumulate at all at the UVDE-induced single-strand breaks, whereas XRCC1 accumulated at the single-strand breaks very efficiently (Fig. S2 in supplementary material). These results provide further indications that the substrate for the accumulation of WRN is not single-strand breaks.

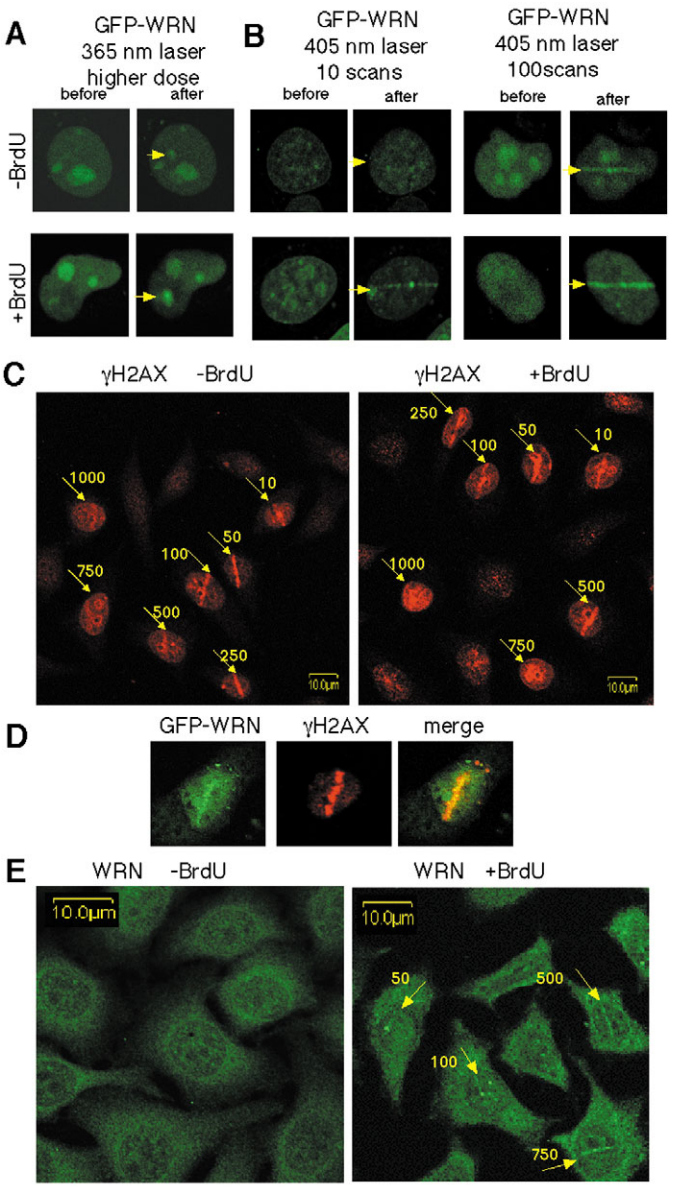

Fig. 2. Enhanced accumulation of WRN by pre-treatment of cells with BrdU. (A) Accumulation of GFP-tagged WRN indicated by yellow arrows after higher dose irradiation with the 365 nm laser in HeLa cells with or without pre-treatment with BrdU. (B) Accumulation of GFP-tagged WRN after 10 and 100 scans with the 405 nm laser with or without pre-treatment with BrdU in HeLa cells. (C) Immunochemical detection of γH2AX after laser irradiation in HeLa cells with or without pre-treatment with BrdU. The number of scans with the 405 nm laser are indicated in yellow. (D) Colocalization of GFP-tagged WRN and γH2AX at irradiated sites in HeLa cells after 100 scans with the 405 nm laser. (E) Immunochemical detection of endogenous WRN after 405 nm laser irradiation with or without pre-treatment with BrdU in HeLa cells. The number of scans with the 405 nm laser are indicated in yellow. Arrows indicate the sites of irradiation.

Accumulation of WRN at the irradiated sites was significantly enhanced by BrdU treatment

Incorporation of BrdU in DNA has been shown to enhance the production of double-strand breaks induced by UVA light and

laser irradiation (Kim et al., 2002; Limoli and Ward, 1993; Xiao et al., 2004). To determine whether the accumulation of WRN with the higher dose laser was at double-strand breaks, we analyzed the effect of BrdU on the accumulation. We found that the intensity of accumulated GFP-WRN at the site irradiated with a higher dose 365 nm laser pulse was significantly enhanced in cells pre-treated with BrdU compared with the accumulation without BrdU treatment (Fig. 2A). To confirm this result and to distinguish fluorescence at the irradiated sites from the spotted distribution of GFP-WRN before irradiation, we introduced the 405 nm laser producing linear irradiation sites in the nucleus (Fig. 1A, right). Accumulation of WRN at 405 nm-laser-irradiated sites was significantly enhanced by BrdU pre-treatment after scanning 10 as well as 100 times (Fig. 2B). The intensity of the fluorescence of GFP-WRN at the irradiated site increased more than threefold compared with the intensity without BrdU, suggesting that GFP-WRN accumulated at double-strand breaks produced by the addition of BrdU. To detect the production of double-strand breaks, γH2AX was immunostained after irradiation with the 405 nm laser. γH2AX was detected as clear lines in irradiated cells following up to 500 scans, whereas scanning 750 and 1000 times produced dispersed signals of γH2AX in the whole nucleus (Fig. 2C). This result corresponds with the previous report using 337 nm laser irradiation (Lukas et al., 2003) and may be explained by the transmission of strong γH2AX signals to other parts of the nucleus. γH2AX was stained much more brightly after irradiation with the 405 nm laser following pre-treatment with BrdU than without pre-treatment (Fig. 2C), showing that increased numbers of double-strand breaks were produced in the presence of BrdU compared with those in the absence of BrdU. As expected, the lines of γH2AX were merged with accumulated GFP-tagged WRN, even though the staining of γH2AX was much stronger and wider than the distribution of GFP-WRN (Fig. 2D), indicating that the accumulation of WRN is much more limited at the irradiated sites than the distribution of γH2AX.

To exclude the possibility that the accumulation of WRN is an artifact due to overexpression of WRN tagged with GFP, we examined the accumulation of native WRN protein using antibody against WRN. Accumulation of WRN was not detected at irradiated sites in cells not pre-treated with BrdU (Fig. 2E left), but was detected 15 minutes after various numbers of scans using 405 nm laser irradiation following pre-treatment with BrdU in HeLa cells at the narrow irradiated lines (Fig. 2E right). GFP-tagged WRN accumulated at the irradiated site even without pre-treatment with BrdU, which may be explained by the fact that the antibody recognizes the endogenous human WRN with a very low affinity. Accumulation of WRN was observed by the antibody only at the irradiated sites in cells, further suggesting that WRN accumulated only at the region where double-strand breaks are present.

Accumulation kinetics of GFP-tagged WRN at the sites of double-strand breaks

Having established the accumulation of WRN at the site of laser-induced double-strand breaks, we characterized the kinetics of the accumulation of WRN and compared it with those of other repair proteins. Fig. 3 depicts the accumulation kinetics of GFP-tagged WRN and other proteins after the higher dose of 365 nm laser irradiation. WRN accumulated rapidly at the irradiated sites and the fluorescence at the sites reached a plateau 3 minutes after irradiation. NBS1, an early response protein to double-strand breaks (Celeste et al., 2003), accumulated at irradiated sites with very similar kinetics as for WRN, whereas BRCA1 accumulated much more slowly. Once they had accumulated at the irradiated sites, all these proteins remained there up to 4 hours after irradiation. In contrast to these proteins, LIGIIIα, which functions in a final step in the repair of single-strand breaks, dissociated from the irradiated site around 1 hour after irradiation (Fig. 3). Like NBS1 and BRCA1, which are involved in the repair of double-strand breaks via homologous recombination, WRN remained at the irradiated site for a long time, suggesting that the response of WRN might also be an early event in homologous recombination repair.

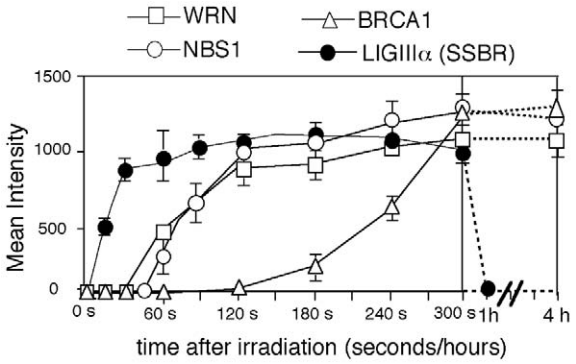

Fig. 3. Comparison of accumulation kinetics of GFP-tagged WRN, NBS1, BRCA1 and LigIIIα after laser irradiation of HeLa cells. HeLa cells transfected with the various GFP-tagged genes were irradiated with high-dose laser irradiation, and accumulation and dissociation kinetics of the proteins were quantified.

Accumulation of WRN at irradiated sites is not enhanced by photosensitization, which increases the production of oxidative base damage

As WRN has been reported to enhance the repair of oxidative base damage in vitro (Harrigan et al., 2003), we wanted to examine the accumulation of WRN in cells pre-treated with RO-19-8022, a photosensitizer, which increases the production of oxidative base damage by absorption of light around 400 nm (Will et al., 1999). We have previously shown that treatment of cells with RO-19-8022 before laser irradiation enhanced the accumulation of various glycosylases for oxidized bases, such as NTH1, OGG1, NEIL1 and NEIL2, as well as other repair proteins, such as POL β and PCNA, involved in base excision repair at irradiated sites (Lan et al., 2004). This indicates that the amount of various types of produced base damage is increased by the photosensitization. However, in contrast to the glycosylases and the other proteins, the accumulation of WRN was not enhanced at all in cells pre-treated with RO-19-8022 (Fig. 4A) under the same irradiation condition as that of GFP-NTH1 shown here (Fig. 4B), suggesting that the observed accumulation of WRN is different from that of the glycosylases and that WRN is not directly involved in the repair of oxidative base damage.

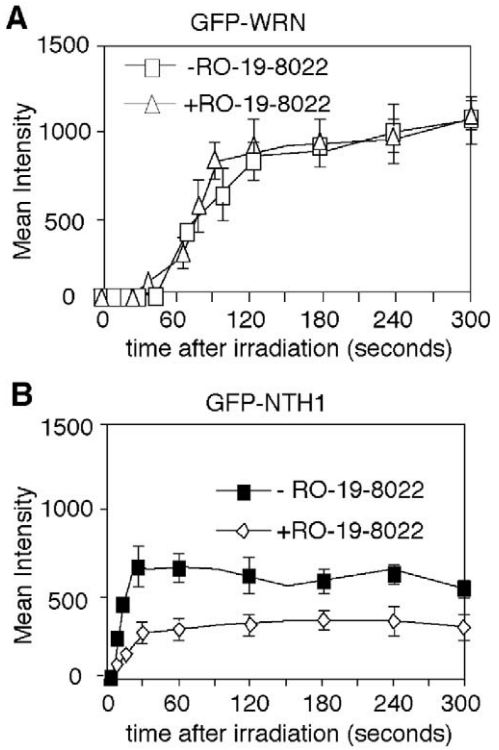

Fig. 4. Influence of RO-19-8022 on accumulation of GFP-WRN in HeLa cells. After higher dose irradiation with the 365 nm laser in HeLa cells, accumulation of WRN was not affected (A), whereas that of GFP-tagged NTH1 was affected (B) by pre-treatment with RO-19-8022.

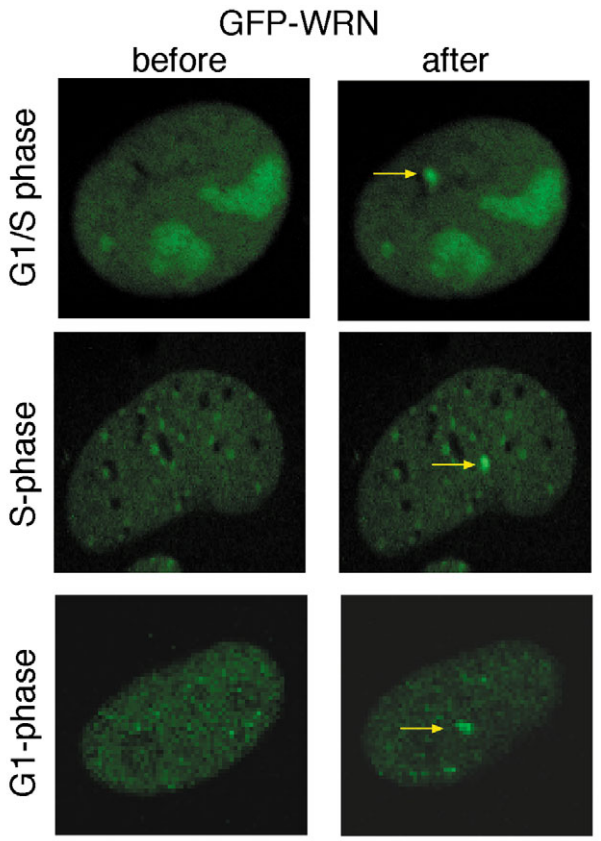

Fig. 5. Replication-independent accumulation of WRN at irradiated site.Accumulation of GFP-tagged WRN in G1/S, S and G1 phase HeLa cells after higher dose irradiation with the 365 nm laser is shown. At least ten cells from each cell-cycle phases were irradiated, and representative data are shown. Arrows indicate the sites of irradiation.

The accumulation of WRN at irradiated sites is independent of DNA replication

A previous study showed that, 6 hours after treatment of cells with camptothecin, WRN formed distinct foci, which partially colocalized with RPA and RAD51 (Sakamoto et al., 2001). We therefore investigated the dependence of the accumulation of WRN at sites of laser irradiation on DNA replication. HeLa cells were synchronized at the G1/S border by the double thymidine block method and released by removing HU-containing medium; they were then irradiated with the 365 nm laser in each phase of the cell cycle. WRN accumulated at the laser-induced damage site in all the cells of different cell-cycle phases shown in Fig. 5. There was no significant difference in the kinetics of accumulation of WRN at different cell-cycle phases (not shown). These data suggest that accumulation of WRN at sites of DNA damage is independent of DNA replication.

WRN accumulated at double-strand breaks via its HRDC domain

WRN contains an exonuclease domain, a DEAH (single-letter amino acid code) helicase domain, a conserved RecQ family C-terminal domain (RQC) with NTS, a HRDC domain and a NLS (Fig. 6A). To determine which domain is responsible for the accumulation of WRN at laser-irradiated sites, we examined the accumulation of deletion mutants of WRN. We found that a GFP-tagged helicase mutant of WRN (K577M) without the helicase activity (Chen et al., 2003) and WRN with the deletion of the N-terminal exonuclease domain (a.a. 152-1432) accumulated at the irradiated site (Fig. 6B). To our surprise, the C-terminus HRDC domain (a.a. 1021-1432) lacking exonuclease and helicase domains accumulated at the irradiated site (Fig. 6B; see also Fig. S3 in supplementary material), indicating the importance of the C-terminal region for the damage response. To further address the importance of the HRDC domain for the damage response, we examined the accumulation of the GFP-tagged N-terminus WRN (a.a. 1-1150), which lacks the HRDC domain but has an additional NLS attached at the C-terminus, and a C-terminal WRN-NLS fragment without the HRDC domain (a.a. 1229-1432). Both deletion fragments failed to accumulate at sites of laser-induced double-strand breaks (Fig. 6B; see also Fig. S3 in supplementary material). Thus, the minimum region necessary for the accumulation contains the HRDC domain with the C-terminal NLS domain. Using the GFP-HRDC (a.a. 1021-1432) we analyzed its accumulation kinetics. As shown in Fig. 6C, the accumulation kinetics of the HRDC domain was exactly the same as that of the full-length WRN, and the HRDC domain remained at the irradiated site for a long period. Thus, the HRDC domain is the domain responsible for the accumulation of WRN at sites of double-strand breaks.

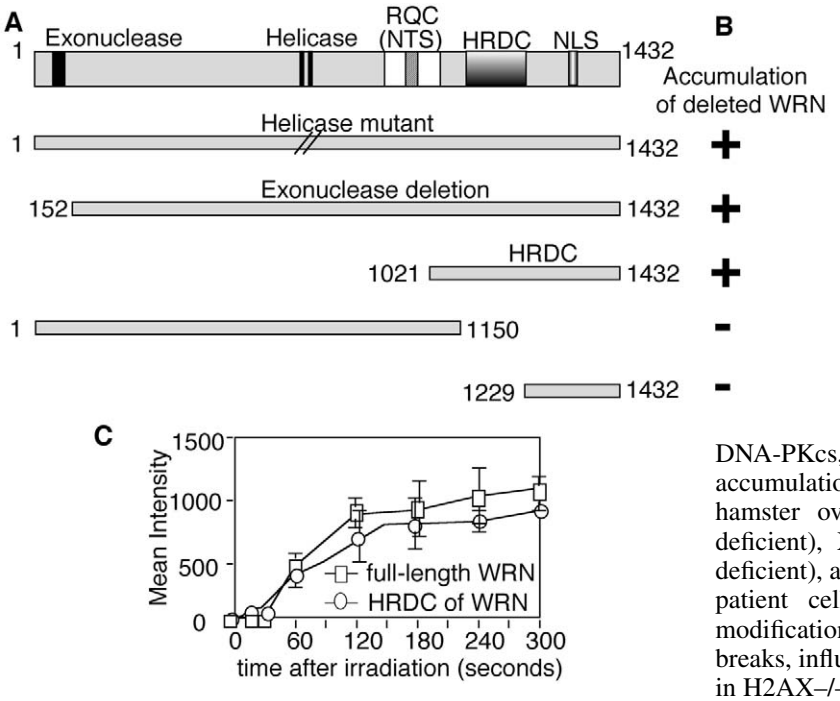

Fig. 6. WRN accumulates at double-strand breaks via its HRDC domain in vivo. (A) Domains and mutations introduced in GFP-WRN. (B) Results of accumulation of the mutants of GFP-WRN at double-strand breaks. + means positive and – means negative accumulation. (C) Accumulation kinetics of GFP-tagged full-length WRN (WRN) and the HRDC domain (HRDC) after laser irradiation of HeLa cells. Standard deviations derived from at least three independent data are indicated.

Interacting proteins do not influence WRN accumulation at double-strand breaks

Many proteins involved in DNA repair interact with WRN. To identify whether the accumulation of WRN at sites of double-strand breaks is dependent on the double-strand breaks-repair proteins that have been reported to interact with WRN, we first examined the accumulation of WRN in cells deficient in the proteins. Because WRN has been reported to interact with Ku, DNA-PKcs, and NBS1 (Cheng et al., 2004), we checked the accumulation of WRN in cells derived from the Chinese hamster ovary (CHO) cell lines of XR-C1 (DNA-PKcs-deficient), XR-V15B (Ku80-deficient) and XR-1 (XRCC4-deficient), as well as in a human cell line 1022QVA (Nijmegen patient cells, NBS1-deficient). To examine whether the modification of H2AX, an initial signal of double-strand breaks, influences the accumulation of WRN, its accumulation in H2AX–/– MEF cells was also analyzed. WRN accumulated in all of the cell lines listed above in the same way as it accumulated in their corresponding wild-type cells after a higher dose of irradiation with the 365 nm laser (Fig. 7), which indicates that the accumulation of WRN at double-strand breaks is independent of these proteins. WRN is phosphorylated in an ATM/ATR-dependent manner on production of stalled replication fork (Pichierri et al., 2003). Because pre-treatment of cells with caffeine has been reported to interfere efficiently with ATM-dependent events such as H2AX phosphorylation, we also examined the accumulation of WRN in cells treated with caffeine. Accumulation of WRN was not affected by caffeine (not shown), supporting the above result of H2AX-independent WRN accumulation.

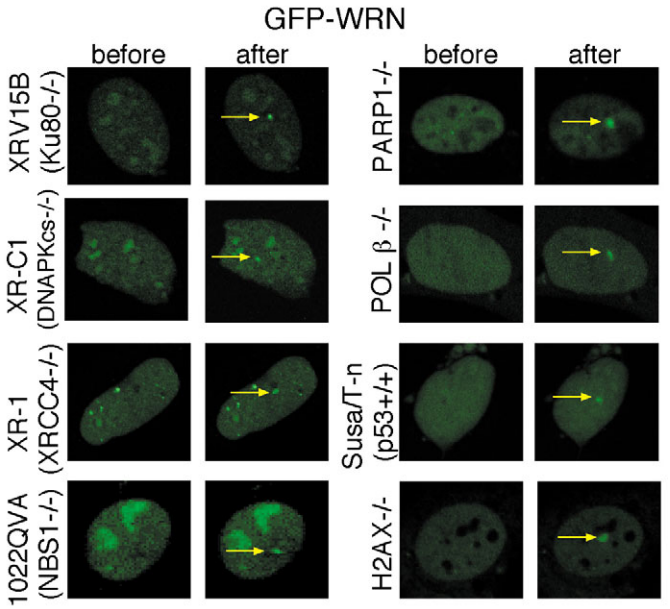

Fig. 7. Accumulation of WRN after higher dose laser irradiation at double-strand breaks in the cell lines defective in various putative WRN-interacting proteins. Accumulation of GFP-tagged WRN indicated by yellow arrows 3 minutes after higher dose irradiation with the 365 nm laser in corresponding cell lines. Arrows indicate the sites of irradiation.

Although we showed that WRN did not accumulate at single-strand breaks and base damage, WRN has been reported to interact with poly(ADP)ribose polymerase-1 (PARP1) and POL β (von Kobbe et al., 2003a), which play important roles in single-strand breaks and base excision repair. Because these proteins may also influence the accumulation of WRN at double-strand breaks, we tested the accumulation of WRN in PARP1$^{-/-}$ or POL β$^{-/-}$ MEF cells and found that accumulation of WRN at the damage site in POL β$^{-/-}$ MEF cells and PARP1$^{-/-}$ MEF cells was the same as that in the parental cells after the higher dose of 365 nm laser irradiation (Fig. 7A). Because PARP2 is known to act as a backup for PARP1, a potent inhibitor for both PARPs, 1,5-dihydroxyisoquinoline (DIQ) was used in HeLa cells. DIQ treatment significantly suppressed the accumulation of XRCC1 at the irradiated sites (Lan et al., 2004) but did not influence the accumulation of WRN (not shown). Thus, PARP1, PARP activation and POL β did not influence the accumulation of WRN at the irradiated sites. It has also been reported that an interaction between WRN and p53 may be involved in cellular responses to DNA damage (Blander et al., 1999; Brosh et al., 2001). Because the presence of functional p53 may influence the accumulation in WRN at the irradiated site, we investigated the accumulation of WRN at laser-induced double-strand breaks in hTERT-

immortalized human fibroblast cells (Susa/T-n) with functional p53 (Nakamura et al., 2002). WRN accumulated at double-strand breaks in Susa/T-n cells as well as in other cell lines, suggesting that the functional p53 does not influence the accumulation of WRN at double-strand breaks (Fig. 7).

Discussion

Werner syndrome patients display various clinical symptoms and signs of early aging. The gene responsible for the disease encodes WRN, which has helicase and exonuclease activities. Several proteins that interact with WRN have been identified, most of which are involved in various types of DNA repair. However, how WRN functions in cells remains unanswered. In this report we showed for the first time that WRN accumulates at sites of double-strand breaks locally produced by laser micro-irradiation in living human cells. A recent report indicated that the level of phosphorylation of H2AX at a double-strand break, measured by a chromatin IP assay, is high on each side of the double-strand breaks, extending up to around 60 kb on each side, but is low in the region immediately adjacent to the double-strand break (Unal et al., 2004). As shown in Figs 2 and 3, γH2AX is dispersed over the whole nucleus after high-dose irradiation (over 500 scans), whereas only a restricted accumulation of WRN at sites of double-strand breaks was observed after pre-treatment with BrdU under high-dose irradiation (Fig. 2). Furthermore, WRN accumulated at double-strand breaks even in an H2AX-deficient mutant cell line as shown in Fig. 7. These results indicate that WRN accumulates only at sites of double-strand breaks independently of γH2AX.

By comparing the accumulation kinetics of WRN with other proteins, we found that WRN showed similar kinetics to NBS1, a protein exhibiting an immediate response to double-strand breaks and playing a central role in the repair of double-strand breaks. These results suggest that the response of WRN and NBS might cooperate with each other in related repair pathways. Although the interaction between WRN and double-strand breaks repair-related Ku proteins has been reported (Cooper et al., 2000; Li and Comai, 2001), our results showed that deletion of the Ku-interaction domain of WRN (N-terminal of WRN) did not prevent its accumulation at double-strand breaks and that WRN accumulated at double-strand breaks even in Ku86-deficient cells, suggesting that the accumulation of WRN at double-strand breaks is not dependent on Ku molecules. A recent study showed that TRF2 accumulated at laser-induced double-strand breaks independently of the presence of Ku70, DNA-PKcs, MRE11/RAD50/NBS1 complex and WRN as an early response to DNA damage (Bradshaw et al., 2005). The accumulation of WRN at double-strand breaks is quite similar to that of TRF2, as the accumulation of WRN at sites of double-strand breaks is also independent of the above proteins (Fig. 7) and WRN responds to double-strand breaks as early as TRF2 does. Both proteins associate with telomeres as well as with double-strand breaks, indicating that they may join and cooperate in the repair of double-strand breaks as well as in the processes for protecting telomere ends.

Domain analysis indicated that the HRDC domain is essential and sufficient for the accumulation of WRN at sites of double-strand breaks. The function of the HRDC domain in mammalian WRN has not been elucidated. The threedimensional structure of the HRDC domain has been determined for the *Saccharomyces cerevisiae* RecQ helicase Sgs1p, the yeast homologue of WRN, by nuclear magnetic resonance (NMR) spectroscopy (Liu et al., 1999). Structural similarities of Sgs1p to bacterial DNA helicases suggest that the HRDC domain of Sgs1p may function as an auxiliary DNA-binding domain. However, most of the amino acids in the basic patch of the HRDC domain in Sgs1p are not conserved in the HRDC domains of other helicases including human WRN, and a structural model of the HRDC domain of human WRN shows different surface properties when compared with that of Sgs1p (Liu et al., 1999). A previous study indicated that the HRDC domain of WRN (a.a. 1072-1432) binds to the forked duplex and Holliday junction with high affinity but to the 5′-overhang duplex with lower affinity, whereas exonuclease and helicase domain of WRN also contain these binding activities in vitro (von Kobbe et al., 2003b). In addition, full-length WRN showed a very low binding affinity to DNA in a non-sequence-specific, structure-dependent manner, and the HRDC domain is not a specific DNA-binding domain in vitro. However, our study showed very clearly that only the HRDC domain provides the protein with the ability to accumulate at DNA damage. Although the exonuclease and helicase domains showed binding activities to specific DNA structure in vitro (von Kobbe et al., 2003b), these two domains did not respond to double-strand breaks in our assay. This may suggest a difference in the DNA damage response between in vivo and in vitro. Our data showed that the HRDC domain of WRN is able to assemble at double-strand breaks and may function in guiding the whole WRN to DNA damage in cells. Because the HRDC domain was shown to be an independently folded structural domain, the HRDC domain may either bind directly to double-strand breaks or interact with other protein(s) present at double-strand breaks, which remain to be identified.

All the mutations identified so far in patients with Werner syndrome result in a truncated WRN protein that lacks the C-terminus and the NLS. The inability of WRN to be transported into the nucleus has been thought to be crucial for the pathogenesis of WRN. Although Werner syndrome has been associated with mutations in the HRDC domain, the deleted WRN protein in the patients lost both the complete HRDC domain and NLS (Moser et al., 2000; Moser et al., 1999; Oshima, 2000). Therefore, the importance of HRDC might have been concealed because of the simultaneous loss of NLS, and it is possible that the response to double-strand breaks via the HRDC domain of WRN is actually important for the functions of WRN in cells.

Because camptothecin-induced WRN foci formation was inhibited by aphidicolin, the formation of foci is thought to be related to replication (Sakamoto et al., 2001). We have tested the ability of deletion mutants of WRN to form foci. Although the helicase mutant formed foci as efficiently as the full-length WRN in cells treated with camptothecin, all the other deletion mutants including HRDC domain alone failed to form foci (Fig. S4 in supplementary material). From previous reports, MRE11-RAD50-NBS1 complex seems to be a key factor for repair of DNA double-strand break and blocked replication fork by modulating related proteins, including BRCA1 and WRN. In response to γ-irradiation, BRCA1 and MRE11-RAD50-NBS1

cooperate with each other to form irradiation-induced foci (IRIF) (Wu et al., 2000; Zhong et al., 1999). WRN also interacts with the complex, and hydrourea-induced foci of WRN was shown to be dependent on NBS1 (Cheng et al., 2004; Franchitto and Pichierri, 2004; Pichierri and Franchitto, 2004). Autonomous accumulation of WRN at laser-induced damage sites through its HRDC domain suggests that the accumulation of WRN at double-strand breaks is an initial response of WRN to double-strand breaks in living cells. Because the accumulation of WRN at sites of laser-induced double-strand breaks is independent of replication and other interacting proteins (Figs 5, 7), the HRDC domain-dependent accumulation of WRN at double-strand breaks is followed by interaction with other proteins at the site of replication. Further analysis is necessary to identify the relationship between double-strand breaks accumulation and replication-dependent foci formation of WRN in cells and the steps after accumulation of WRN at double-strand breaks for forming IRIF.

Werner syndrome cells show replication defects and altered telomere dynamics leading to the shortening of telomeres. A mouse model with both *Wrn* and *Terc* (encoding the telomerase RNA component) deficiencies was shown to exhibit accelerated replicative senescence of cells with Werner-like premature aging phenotypes (Chang et al., 2004). Because the telomere exhibits a double-strand breaks-like structure, it is tempting to suppose that WRN accumulates via the HRDC domain at shortened telomeres after replication in late S phase and may contribute to the recovery of telomere length together with other proteins. Further studies of the functions of WRN at double-stand breaks in cells will help us to understand the molecular basis of the phenotype of WS patients.

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