Werner syndrome helicase activity is essential in maintaining fragile site stability

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WRN is a member of the RecQ family of DNA helicases implicated in the resolution of DNA structures leading to the stall of replication forks. Fragile sites have been proposed to be DNA regions particularly sensitive to replicative stress. Here, we establish that WRN is a key regulator of fragile site stability. We demonstrate that in response to mild doses of aphidicolin, WRN is efficiently relocalized in nuclear foci in replicating cells and that WRN deficiency is associated with accumulation of gaps and breaks at common fragile sites even under unperturbed conditions. By expressing WRN isoforms impaired in either helicase or exonuclease activity in defective cells, we identified WRN helicase activity as the function required for maintaining the stability of fragile sites. Finally, we find that WRN stabilizes fragile sites acting in a common pathway with the ataxia telangiectasia and Rad3 related replication checkpoint. These findings provide the first evidence of a crucial role for a helicase in protecting cells against chromosome breakage at normally occurring replication fork stalling sites.

Introduction

Werner syndrome (WS) is a human autosomal recessive disorder. Affected individuals prematurely exhibit many age-related pathologies as well as a high predisposition for cancer development (Martin and Oshima, 2000; Oshima, 2000). The gene mutated in WS, *WRN*, encodes a nuclear protein that is a member of the RecQ family of DNA helicases and possesses two enzymatic activities: an ATP-dependent 3′–5′ DNA unwinding activity (Gray et al., 1997; Suzuki et al., 1997) and a 3′–5′ exonuclease activity residing in the amino-terminal region (Huang et al., 1998). Cultured cells derived from WS patients show a wide genomic instability manifested as spontaneous chromosomal abnormalities and large deletions in many genes (Salk, 1985; Gebhart et al., 1988; Fukuchi et al., 1989), which may represent an important determinant of the increased risk of cancer (Goto et al., 1996; Moser et al., 2000; van Brabant et al., 2000). RecQ helicase family members are implicated in several biochemical processes such as DNA replication, recombination, and repair but the precise molecular function of WRN is not well elucidated. Also, the functional significance of each WRN biochemical activity and whether loss of one or both leads to WS pathogenesis is not fully understood. In vitro studies have shown that forked duplexes resembling DNA structures arising during replication, recombination, and repair are resolved by the coordinated action of WRN activities (Shen and Loeb, 2000; Opresko et al., 2004). Interestingly, recombination requires both WRN activities, whereas single helicase or exonuclease activity is sufficient to protect cells against toxic insults (Swanson et al., 2004). Other studies indicated that WRN helicase activity has a role in the prevention of telomere dysfunction (Bai and Murnane, 2003; Cheng et al., 2004).

Mounting evidence strongly supports the idea that WRN may play a critical role in the rescue of stalled replication forks. First, S phase prolongation is observed in WS cells together with extreme sensitivity to drugs that block replication fork progression (Poot et al., 1999, 2001; Pichierri et al., 2000a,b). Second, WRN shows a great substrate preference toward complex DNA secondary structures, which represent a roadblock for DNA replication (Shen and Loeb, 2000; Brosh et al., 2002). Third, WRN is required for fruitful recovery from replication fork arrest (Pichierri et al., 2001; Sakamoto et al., 2001; Baynton et al., 2003). Furthermore, WRN has been recently found to interact or colocalize with proteins involved either in the intra-S or replication checkpoint and is targeted by the replication checkpoint kinase ataxia telangiectasia and Rad3 related (ATR; Baynton et al., 2003; Pichierri et al., 2003; Cheng et al., 2004; Franchitto and Pichierri, 2004).

Fragile sites are replication-delayed genomic regions particularly sensitive to partial inhibition of DNA synthesis by aphidicolin (Glover et al., 1984). Previous studies proposed that the stalling of replication forks may correlate with DNA

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Abbreviations used in this paper: ATR, ataxia telangiectasia and Rad3 related; LCL, lymphoblastoid cell line; WS, Werner syndrome.

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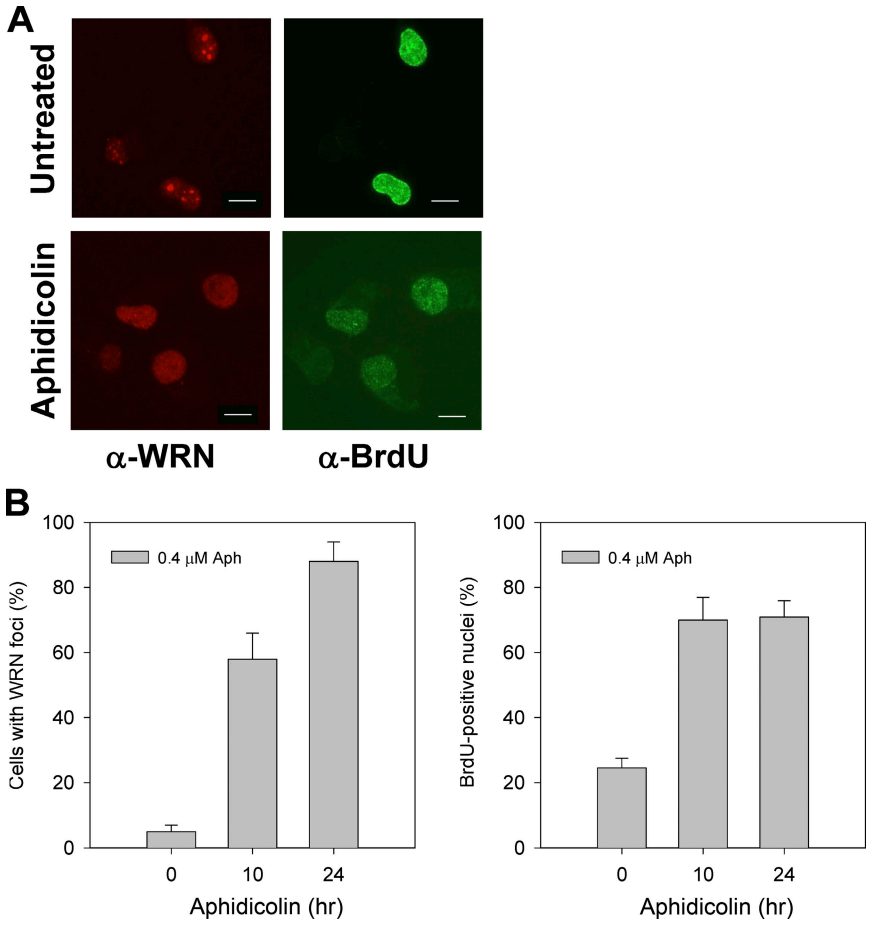

Figure 1. **WRN forms foci in response to aphidicolin-induced replication slowdown.** (A) Colocalization of WRN-positive nuclei with S phase cells. Wild-type fibroblasts were treated with 0.4 μM aphidicolin for 24 h, pulse-labeled with 3 μg/ml BrdU for 30 min before fixation, and double immunostained with α-WRN and α-BrdU as described in Immunofluorescence. Bars, 5 μm. (B) Percentage of cells showing WRN foci in response to 0.4 μM aphidicolin (Aph) treatment (left) and percentage of BrdU-positive nuclei (right). Incorporation of BrdU was evaluated by immunofluorescence using specific antibodies. Data are presented as means from three independent experiments. Error bars represent standard error.

breaks and chromosomal rearrangements occurring at common fragile sites (Arlt et al., 2003; Schwartz et al., 2006). Although extensive knowledge of the molecular determinants underlying common fragile site instability is still missing, computational analysis performed on a subset of these genomic sequences has suggested that common fragile sites could be regions enriched in clusters of highly flexible ataxia telangiectasia sequences (Mishmar et al., 1998; Zlotorynski et al., 2003). These sequences show in silico the propensity to form stable secondary DNA structures that perturb replication, contributing to genome fragility. Very little is known about the molecular mechanisms involved in their stability but it is thought that ATR and other proteins working in the response to replication stress are implicated (Casper et al., 2002; Arlt et al., 2004; Howlett et al., 2005; Musio et al., 2005). More recently, it has been proposed that homologous recombination and, to a lesser extent, nonhomologous end joining are required for fragile site stability after aphidicolin-induced replication slowdown (Schwartz et al., 2005).

These findings led to the conclusion that chromosomal breakage occurring at fragile sites is the end result of incorrect recovery from replication fork stalling at these loci. Taking into account the fact that fork stalling is a very frequent and dangerous event that occurs naturally during normal DNA replication, common fragile sites may represent a useful means to study mechanisms underlying replication fork recovery in vivo.

In this study, we found that WRN was implicated in the response to the partial inhibition of DNA synthesis induced by low doses of aphidicolin. Using cells from WS patients or fibroblasts in which endogenous WRN was down-regulated by RNA interference, we have shown that the loss of functional WRN leads to common fragile site instability with or without aphidicolin treatment. WRN helicase rather than exonuclease activity seems to play the main role in stabilizing fragile sites. Furthermore, we suggest that WRN and ATR act in a common pathway preventing accumulation of DNA gaps and breaks at common fragile sites.

Results

WRN accumulates into nuclear foci after aphidicolin-induced replication delay

It has been demonstrated that WRN is mainly located in the nucleoli and relocalizes to nuclear foci after DNA damage or replication fork arrest (Sakamoto et al., 2001; Baynton et al., 2003; Pichierri et al., 2003; Otterlei et al., 2006). This subnuclear redistribution seems to be a general behavior of WRN in response to DNA damage or replication arrest. Thus, we wanted to verify whether WRN was relocalized into nuclear foci in response to partial inhibition of DNA replication.

Wild-type fibroblasts were exposed to 0.4 μM aphidicolin and fixed at different time points (Fig. 1). Before fixation, cells were detergent-extracted to visualize only the chromatin-associated

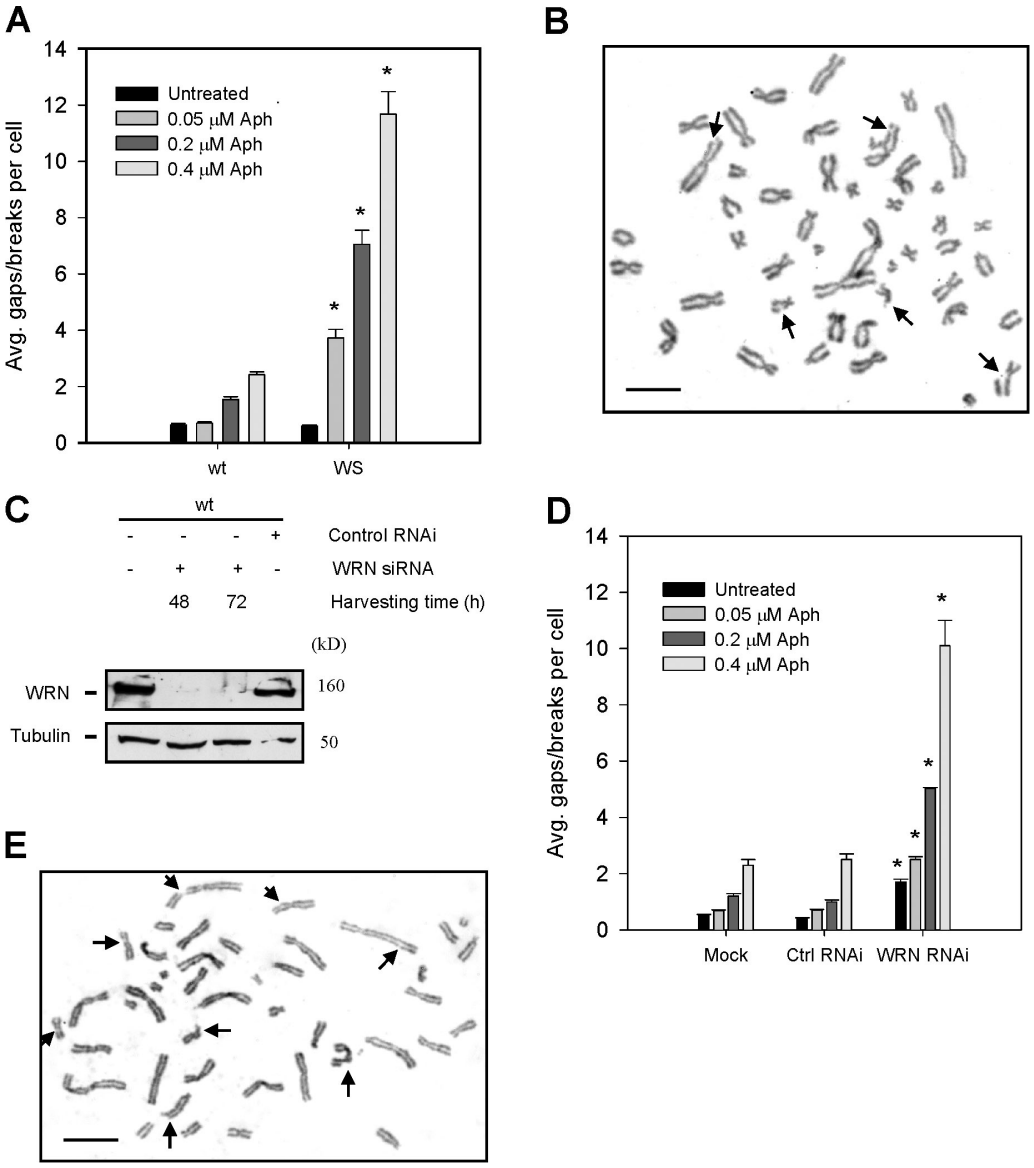

Figure 2. **WRN deficiency leads to increased spontaneous and aphidicolin-induced DNA chromosomal aberrations.** (A) Mean overall chromosome gaps and breaks per cell in wild-type (wt) and WS cells. Cells were treated with different doses of aphidicolin (Aph) 24 h before harvest. Data are presented as means of three independent experiments. Asterisks indicate that the result is statistically significant compared with the wild type; $P < 0.05$ by *t* test. (B) Representative Giemsa-stained metaphase of WS fibroblasts treated with 0.2 μM aphidicolin. Arrows indicate chromosomal aberrations. (C) Western blotting probed with α-WRN showing the reduction in the WRN protein level in wild-type fibroblasts transfected with control siRNA or siRNAs directed against WRN and harvested 48 or 72 h after interference. Tubulin was used as loading control. (D) Mean overall chromosome gaps and breaks per cell in wild-type fibroblasts (mock), fibroblasts transfected with control siRNA, or fibroblasts in which WRN was abrogated by RNAi (WRN RNAi). Cells were treated with different doses of aphidicolin 24 h before being harvested. Data are presented as means of three independent experiments. Asterisks indicate that the result is statistically significant compared with the wild type; $P < 0.05$ by *t* test. Error bars represent standard error. (E) Representative Giemsa-stained metaphase of fibroblasts in which WRN was abrogated by RNAi and treated with 0.4 μM aphidicolin. Arrows indicate chromosomal aberrations. Bars, 2.5 μm.

WRN, the fraction that is thought to be localized at stalled replication forks. Aphidicolin-induced replication slowdown resulted in a marked relocalization of WRN into diffuse subnuclear foci (Fig. 1 A) and the percentage of cells with WRN foci increased in a time-dependent manner, reaching ~80% at 24 h (Fig. 1 B). Interestingly, the percentage of nuclei with diffuse WRN foci matched the percentage of cells in S phase as demonstrated by BrdU incorporation (Fig. 1, A and B), which suggests that relocalization is linked to replication inhibition induced by aphidicolin. Altogether, our results indicate that WRN is implicated in the response to replication slowdown induced by aphidicolin.

Cells lacking functional WRN show increased sensitivity to aphidicolin

To test the hypothesis that WRN plays a role in the maintenance of fragile site stability, we first investigated the sensitivity of WS cells to aphidicolin-induced replication slowdown. We exposed wild-type and WS fibroblasts to different concentrations of the drug and, 24 h later, metaphase chromosomes were collected and scored for total gaps and breaks (Fig. 2, A and B; and Fig. S1, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200705126/DC1). A dose-dependent induction of chromosome gaps and breaks was observed in both cell lines, with WS

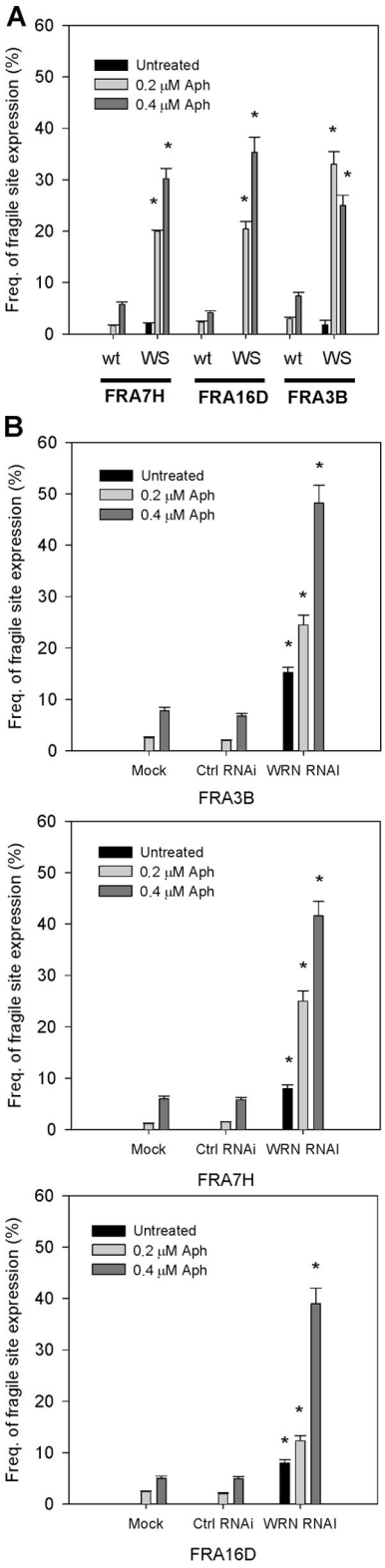

Figure 3. **Enhanced common fragile site expression in WRN-deficient cells.** (A) Frequency of fragile site FRA3B, FRA7H, and FRA16D expression in wild-type (wt) and WS cells. Cells were treated with two doses of aphidicolin (Aph) and harvested 24 h later. Frequency of fragile site induction is presented as the percentage of chromosome 3, 7, or 16 homologues with gaps and breaks at FRA3B, FRA7H, and FRA16D, respectively. Data are presented as means of three independent experiments. (B) Frequency of fragile site FRA3B, FRA7H, and FRA16D expression in wild-type fibroblasts (Mock), fibroblasts transfected with control siRNA, or fibroblasts in which WRN was abrogated by RNAi (WRN RNAi). In treated samples, different doses of aphidicolin were added 48 h after interference and left until harvesting 24 h later. The frequency of fragile site induction is presented as the percentage of chromosome 3, 7, or 16 homologues with gaps and breaks at FRA3B, FRA7H, and FRA16D, respectively. Data are presented as means of three independent experiments. Asterisks indicate that the result is statistically significant compared with the wild type; $P < 0.05$ by *t* test. Error bars represent standard error.

fibroblasts showing an approximately sixfold increase in chromosomal damage in comparison to their wild-type counterparts.

Because there might be a compensation for WRN deficiency in cells derived from WS patients, we explored the effect of aphidicolin treatment in cells in which endogenous WRN was knocked down. Human wild-type fibroblasts were transfected with control siRNA and siRNAs directed against WRN and the reduction of WRN protein level was verified by Western blotting (Fig. 2 C). Depletion of WRN resulted in an enhancement of aphidicolin-induced chromosomal instability similar to that observed in WS cells (Fig. 2, D and E). Interestingly, the abrogation of functional WRN increased spontaneous DNA gaps and breaks (Fig. 2 D). Moreover, a higher chromosomal sensitivity to aphidicolin was observed in a lymphoblastoid cell line (LCL) derived from a WS patient (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200705126/DC1).

Altogether, these results reveal that WS cells are extremely sensitive to aphidicolin treatment and that the loss of WRN is responsible for chromosome instability.

WRN-deficient cells have enhanced instability at common fragile sites

To determine whether the increase in chromosomal gaps and breaks after aphidicolin exposure observed in WS cells takes place at specific DNA regions, we examined by FISH the induction of the most frequently expressed common fragile sites, FRA3B, FRA7H, and FRA16D, in wild-type and WS fibroblasts (Fig. S3, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200705126/DC1). At both doses of aphidicolin, WS cells showed a higher number of gaps and breaks occurring at fragile sites in comparison with control cells (Fig. 3 A). Fragile site induction in WS cells increased in a dose-dependent manner and was about six times higher than in wild-type cells. The FRA3B site seems to be particularly sensitive, possibly because of the elevated percentage of hyperdamaged metaphases that were not included in the count (Fig. S4 A).

To confirm these observations, we repeated the experiment in wild-type fibroblasts in which endogenous WRN was down-regulated. Cells transfected with WRN siRNA displayed enhanced expression of fragile sites after aphidicolin exposure (Fig. 3 B). Interestingly, a higher level of fragile site induction was observed even in the absence of aphidicolin treatment. We established that although aphidicolin-induced total gaps and breaks per cell were more elevated in WS cells than in wild-type cells, the percentage of total breaks attributable to FRA7H and FRA16D was similar with or without the addition of the drug (Table I). Fragile site expression was also analyzed in LCLs and the results were consistent with those obtained in fibroblasts (Fig. S2 B). Altogether, these findings provide

Table I. **Fragile site expression in wild-type and WRN-deficient (WRNi) cells**

Cell line	Treatment	Mean gaps and breaks per cell	Percentage of FRA7H loci with a break	Percentage of total breaks attributable to FRA7H	Percentage of FRA716D loci with a break	Percentage of total breaks attributable to FRA16D
Wild type	−APH	0.5	0	0	0	0
	+APH	0.6	1	3.3	1.1	3.6
WRNi	−APH	1.3	8	12.3	7	10.7
	+APH	2.2	16	14.5	13	11.8

APH, 0.05 μM aphidicolin.

evidence that WRN influences the stability of common fragile sites both during normal DNA synthesis and in response to replication perturbation.

WRN helicase activity is required for fragile site stability

To determine whether one or both WRN enzymatic activities could affect fragile site stability, we produced WS defective cell lines stably expressing wild-type WRN or mutant forms of WRN affecting either helicase or exonuclease activity. Missense mutations have been previously introduced in WRN to inactivate the exonuclease or helicase activity (Gray et al., 1997; Huang et al., 1998; Cheng et al., 2004). Western blotting analyses showed that the levels of WRN protein expressed in WS cells transfected with wild-type *WRN* cDNA (Fig. 4 A, WS*WRN*) and WRN lacking exonuclease (WRN-E84A) or helicase (WRN-K577M) activity were comparable to that of control cells (GM3675). Immunofluorescence staining of WRN protein revealed the proper pattern of subnuclear localization, i.e., mainly in the nucleoli under unperturbed conditions and diffused in the nucleoplasm after camptothecin-induced replication stress (Fig. 4 B). Hypersensitivity to camptothecin, a characteristic cellular phenotype of WS cells, was tested and, as expected (Swanson et al., 2004), expression of wild-type WRN or missense mutant forms of WRN resulted in reduced cell death, reaching values similar to that of control cells (Fig. 4 C).

In comparison with wild-type cells (WS*WRN*), the expression of missense mutant forms of WRN protein in WS cells (WRN-E84A and WRN-K577M) led to a significant increase in chromosomal damage after aphidicolin exposure (Fig. 4 D). However, FISH analyses performed on metaphases after 24 h of treatment indicated that the induction of FRA3B, FRA7H, and FRA16D was enhanced in a statistically significant manner only in WS and WRN-K577M cells (Fig. 4 F). Thus, we conclude that the maintenance of common fragile site stability requires a WRN protein with intact helicase activity.

WRN and ATR regulate fragile site stability in a common pathway

It has been reported that the ATR replication checkpoint is crucial for the maintenance of common fragile site stability after replication inhibition as well as under unperturbed conditions (Casper et al., 2002). Because WRN is targeted by ATR upon replication stress (Pichierri et al., 2003; Otterlei et al., 2006), we investigated the link between WRN and ATR in the stabilization of fragile sites. After down-regulation of WRN, ATR, or both genes by RNAi in wild-type fibroblasts, we verified that the reduction in the corresponding protein levels (Fig. 5 A) was not detrimental to cell growth at least within the period of the assay (not depicted). We then treated cells with 0.05 or 0.4 μM aphidicolin for 24 h and harvested them for chromosome preparations. Metaphases were examined for total gaps and breaks and then for the expression of FRA7H and FRA16D. Aphidicolin increased the levels of gaps and breaks in cells deficient of WRN or ATR compared with the control cells (Fig. 5 B). However, the concomitant depletion of WRN and ATR did not result in more chromosome damage than single deficiencies and fragile site induction was similar in cells in which both WRN and ATR were down-regulated by RNAi either under unstressed conditions or after aphidicolin application (Fig. 5, B and C).

These data support the conclusion that WRN and ATR participate in a common pathway safeguarding fragile site stability.

Discussion

In this paper, we describe how WRN deficiency results in a great enhancement of chromosome aberrations and common fragile site expression after aphidicolin-induced replication slowdown. Most importantly, we demonstrate that loss of WRN function induces accumulation of chromosome gaps and breaks that specifically localize at common fragile sites even under unperturbed cell growth; i.e., in the absence of treatment. Consistently, exposure to aphidicolin at a dose that induces common fragile sites determines an extensive relocalization of WRN to nuclear foci in replicating cells. Finally, we present evidence that indicates that the helicase activity of WRN but not its exonuclease function is essential to prevent common fragile site expression and that ATR and WRN act in a common pathway to stabilize such genomic regions.

Several pieces of evidence indicate that common fragile sites are genomic regions where DNA replication is slowed and eventually stalled at poorly defined DNA structures (Glover et al., 1984, 2005; Casper et al., 2002). The aphidicolin doses used in this study slow down replication but do not completely arrest DNA polymerases and are thought to interfere significantly only with replication of common fragile sites (Glover et al., 1984). Thus, our findings strongly correlate WRN function with these naturally occurring replication fork stalling sites, which further supports the hypothesis that this RecQ helicase is crucial for genome integrity whenever replication forks stall, even during unperturbed cell growth. However, although WRN has been proposed to be involved in the rescue of stalled replication forks

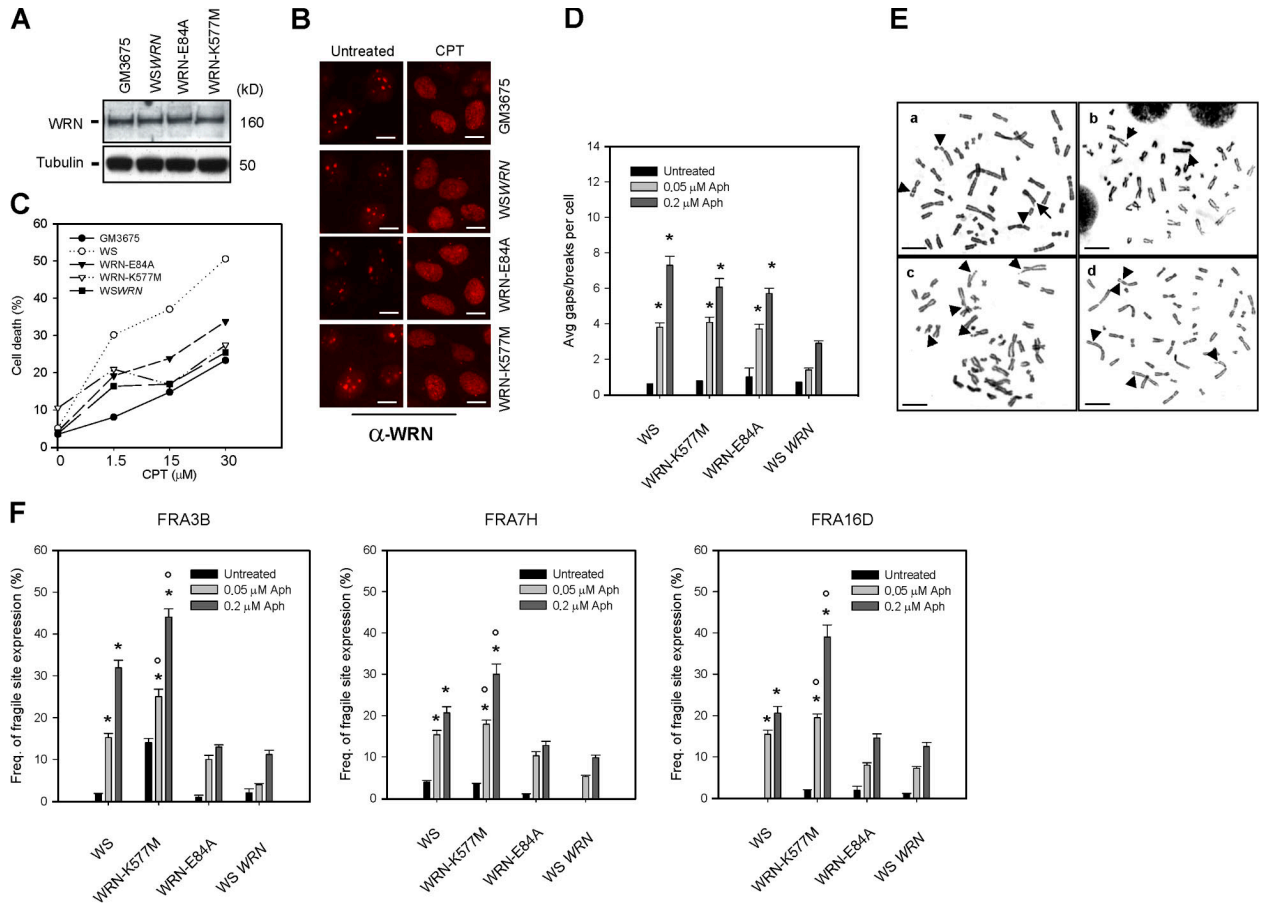

Figure 4. **Impaired WRN helicase activity is responsible for common fragile site instability.** (A) Western blotting analysis showing the expression of WRN protein in cells stably expressing wild-type WRN (WS*WRN*) or missense mutant forms of WRN with impaired exonuclease (WRN-E84A) or helicase (WRN-K577M) activity. GM3675 fibroblasts were used as a positive control. The membrane was probed with α-WRN. Tubulin was used as a loading control. (B) Subnuclear localization of WRN in response to camptothecin-induced replication stress. Indirect immunofluorescence staining was achieved using the same antibody as in Western blotting. (C) Sensitivity of cells to camptothecin-induced replication stress. Cell death was evaluated by the trypan blue method as described in Cell death evaluation. The percentage of cell death was indicated for each dose of camptothecin. (D) Mean overall chromosome gaps and breaks per cell in WS cells, WS cells expressing mutant forms of exonuclease (WRN-E84A) or helicase (WRN-K577M) activity, and WS cells in which wild-type WRN was reintroduced (WS*WRN*). Cells were exposed to different doses of aphidicolin (Aph) 24 h before harvest. Data are presented as means of three independent experiments. (E) Representative Giemsa-stained metaphases of WS fibroblasts (a), WS cells transfected with wild-type *WRN* cDNA (WS*WRN*; b), or WRN lacking helicase (WRN-K577M; c) or exonuclease (WRN-E84A) activity (d), or treated with 0.2 μM aphidicolin. Arrows indicate chromosomal aberrations. (F) Frequency of fragile site FRA3B, FRA7H, and FRA16D expression in WS, WRN-E84A, WRN-K577M, and WS*WRN* cells. Samples were treated with different doses of aphidicolin and left until harvesting 24 h later. Frequency of fragile site induction is presented as the percentage of chromosome 3, 7, or 16 homologues with gaps and breaks at FRA3B, FRA7H, and FRA16D, respectively. Data are presented as means of three independent experiments. Asterisks indicate that the result is statistically significant compared with the wild type; $P < 0.05$ by *t* test. Error bars represent standard error. Bars, 2.5 μm.

by either a recombinogenic or nonrecombinogenic pathway (Ozgenc and Loeb, 2005), WRN exerted its protective role on a specific subset of replicating regions. Our data suggest that WRN is most probably required specifically at slow-replicating sites to prevent their instability. Of particular interest are the results demonstrating that WRN helicase rather than exonuclease activity plays a crucial role in the maintenance of common fragile site stability. Indeed, these naturally occurring slow-replicating zones might be the only physiological targets of the WRN caretaker function; the secondary structures thought to accumulate at these sites (Zlotorynski et al., 2003; Schwartz et al., 2006) could also represent potential in vivo substrates of WRN helicase activity. Previous studies have suggested that WRN helicase activity may efficiently resolve unusual DNA structures at telomeric sequences to facilitate replication fork progression (Mohaghegh et al., 2001; Crabbe et al., 2004; Multani and Chang, 2007). Furthermore, WRN is required in vitro to support DNA polymerase δ in duplicating substrates forming G4 DNA from expanded triplet repeats (Kamath-Loeb et al., 2001). Thus, WRN may function as an accessory helicase specifically involved in the resolution of those unusual DNA structures that can arise at common fragile sites as well as other genomic sites such as telomeres and could otherwise impede normal replication. In this context, WRN would exert a function similar to that of the yeast Rrm3 protein, a DNA helicase implicated in the maintenance of genome stability (Ivessa et al., 2000; Torres et al., 2004b). Even though Rrm3 has an opposite polarity compared with WRN, Rrm3 yeasts show some features resembling WRN deficiency such as replication delay, replication fork pausing or collapse, accumulation of DNA breakage, and premature aging

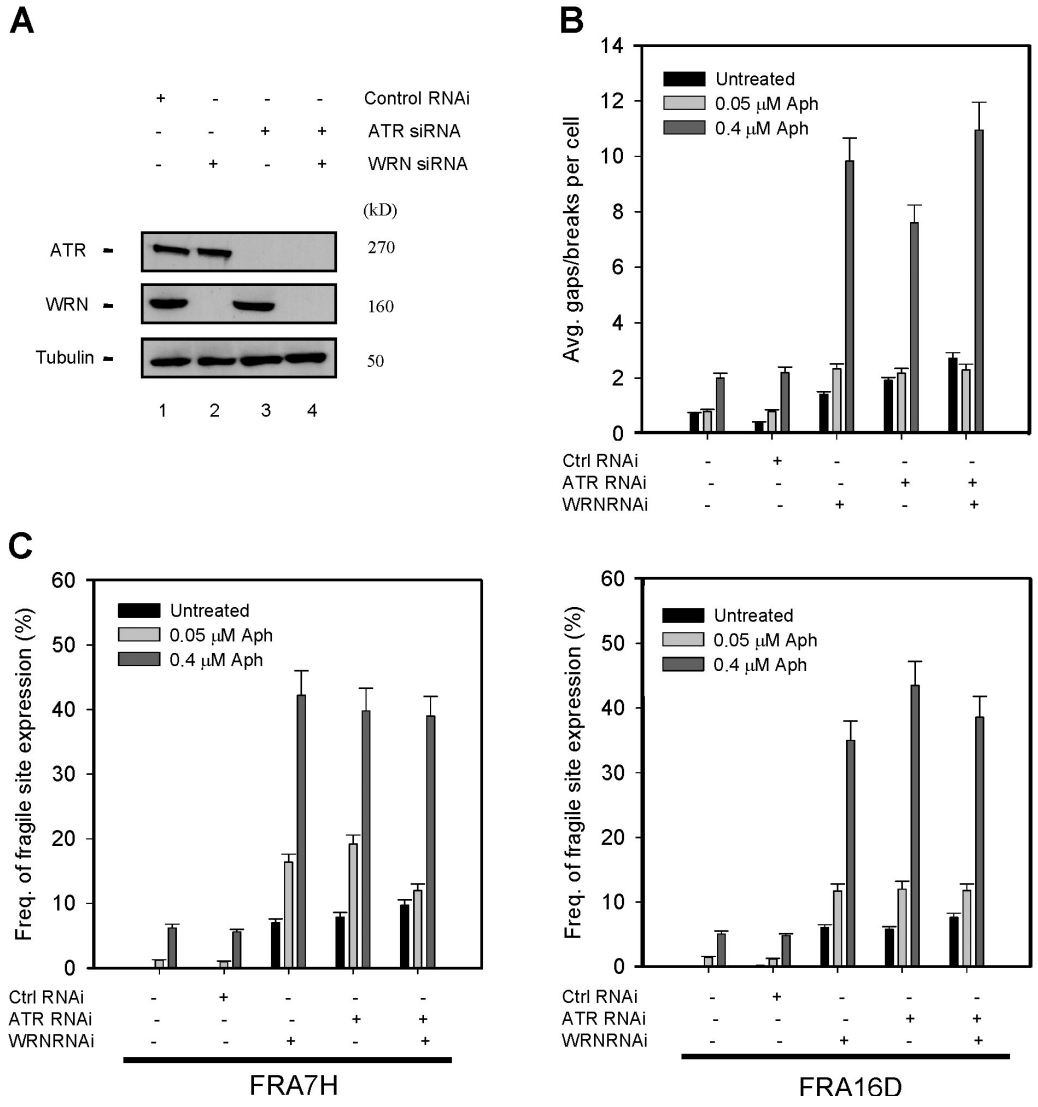

Figure 5. **The effect of ATR and WRN down-regulation on fragile site expression.** (A) Western blotting analysis of protein depletion after transfection of wild-type cells with no siRNA (lane 1) or siRNAs directed against ATR (lane 2), WRN (lane 3), or both (lane 4). The membrane was probed first with α-ATR and then stripped and reprobed with α-WRN, showing the reduction in the corresponding protein levels in wild-type fibroblasts transfected with no siRNA or siRNAs directed against WRN, ATR, or both and harvested 48 h after interference. Tubulin was used as loading control. (B) Mean overall chromosome gaps and breaks per cell in cells interfered with control siRNA or siRNAs against ATR and/or WRN. For site fragile induction, different doses of aphidicolin (Aph) were added 24 h before harvest. Data are presented as means of three independent experiments. For statistical analysis, single mutants are compared with the double knockdown. (C) Frequency of gaps and breaks at specific fragile sites FRA7H and FRA16D in the wild type and fibroblasts depleted of ATR and/or WRN and treated for 24 h with different doses of aphidicolin. Fragile sites were identified by FISH using probes specific to these sites as described in the FISH section. Frequency of fragile site induction is presented as the percentage of chromosome 7 or 16 homologues with gaps and breaks at FRA7H and FRA16D, respectively. Data are presented as means of three independent experiments. For statistical analysis, single mutants are compared with the double knockdown. Error bars represent standard error.

(Ivessa et al., 2000; Torres et al., 2004a,b; Azvolinsky et al., 2006). Similarly, it has been proposed that Rrm3p is needed mainly to help fork progression by removing obstacles such as proteins or particular DNA structures at telomeres or along other difficult-to-replicate regions (Ivessa et al., 2002, 2003; Azvolinsky et al., 2006; Boule and Zakian, 2006). Interestingly, both WRN enzymatic activities are required for recombination-related functions, either after DNA damage (Saintigny et al., 2002) or at telomere sequences in cells that are engaged in the alternative lengthening of telomere pathway (Laud et al., 2005), whereas the helicase activity seems to be sufficient to prevent instability at common fragile sites. Thus, even though recombination has been implicated in the stability of common fragile sites (Schwartz et al., 2005), the function of WRN at these naturally occurring fork stalling sites could be unrelated to its proposed recombinogenic function.

Our results indicate that exonuclease deficiency leads to a significant enhancement of chromosome gaps and breaks at levels comparable to those observed in the absence of helicase activity. However, these aberrations are not localized at the three common fragile sites analyzed. A possible explanation for this is that some fragile sites may be more sensitive to the absence of the exonuclease activity of WRN than others. Alternatively, aphidicolin treatment could sensitize other fragile genomic regions to break in the absence of the exonuclease function of WRN.

Indeed, several genomic regions that are not classified as common fragile sites have the potential to undergo breakage, such as ataxia telangiectasia–rich palindromic regions or closely spaced Alu sequences that can form hairpin structures (Freudenreich, 2007). Interestingly, correct repair of double strand breaks arising at Alu-formed hairpins requires the nuclease activity of the MRE11 complex (Lobachev et al., 2002). Because it has been reported that WRN and the MRE11 complex might cooperate in response to DNA damage (Cheng et al., 2004; Franchitto and Pichierri, 2004), it is tempting to speculate that the nuclease activities of WRN and MRE11 could regulate breakage at noncommon fragile sites under replication stress.

Furthermore, we found that WRN regulates fragile site stability, acting in a pathway associated with ATR-mediated checkpoint response. Our analysis reveals that WRN deficiency recapitulates ATR defects in terms of fragile site instability either upon aphidicolin treatment or under unperturbed conditions. According to the model proposed by Casper et al. (2002), ATR is activated after replication stress to block cell cycle progression to stabilize and then rescue stalled replication forks, promoting the restart of DNA synthesis. Similarly, WRN appears to be essential for fruitful rescue from replication fork arrest (Pichierri et al., 2001; Sakamoto et al., 2001; Baynton et al., 2003) and is targeted for phosphorylation by ATR upon replication arrest (Pichierri et al., 2003; Otterlei et al., 2006). Hence, it is likely that WRN helicase could be required to collaborate with ATR in the recovery of stalled forks at fragile sites, possibly resolving aberrant DNA structures arising as a consequence of the characteristic DNA sequence of these regions. It is noteworthy that ATR deficiency affects not only the stability of stalled forks but also the inhibition of DNA synthesis (Abraham, 2001), whereas loss of WRN function does not influence the checkpoint branch that triggers cell cycle progression after replication stress (Franchitto and Pichierri, 2004). Thus, it is conceivable that the common function of WRN and ATR is unrelated to cell cycle arrest and more strictly correlated to the branch of the replication checkpoint involved in the stabilization of stalled forks.

It has been recently shown that instability at common fragile sites is a hallmark of early precancerous lesions (Gorgoulis et al., 2005) and it is widely accepted that most gross chromosomal rearrangements accumulating in solid tumors originate from fragile sites (Arlt et al., 2006). WS is a cancer-prone and chromosome fragility syndrome characterized by gross chromosomal rearrangements (Martin and Oshima, 2000; Oshima, 2000). Because instability of common fragile sites is readily detected in cells depleted of WRN even under normal division, it is possible that chromosomal instability observed in WS cells could correlate with breaks accumulating at these sites. However, a recent study suggests that most of the chromosomal abnormalities arising in WS cells could be related to erosion of telomeric sequences (Crabbe et al., 2007). These hypotheses are not necessarily incompatible. Indeed, both the common fragile site and telomere stabilities that might require the helicase activity of WRN to clear the way for the replisome and chromosomal rearrangements observed in WS are most likely derived from a common protective mechanism at telomeric and nontelomeric sequences. Consistently, we also observe instability at common fragile sites in Epstein-Barr virus–transformed lymphoblasts derived from WS patients, which are telomerase proficient and thus protected from telomere erosion.

In summary, this study provides additional insights into the mechanisms underlying common fragile site stability and suggests that WRN helicase activity is a key factor in the maintenance of integrity of these specific DNA regions. This supports the hypothesis that WRN may function in the resolution of problems arising in response to alterations in DNA replication and gives insights into the in vivo substrates of this genome caretaker protein. Failure to preserve fragile site stability may have a causative role in the chromosomal abnormalities observed in WS cells.

Materials and methods

Cell lines and culture conditions

Wild-type (9173675) and WS fibroblasts (AG11395) were obtained from Coriell Cell Repositories. The AG11395 cell line carries an Arg368 stop mutation that gives rise to a truncated protein.

Full-length cDNA encoding wild-type or missense mutant forms of WRN with inactive exonuclease (WRN-E84A) or helicase (WRN-K577M) activity (provided by J. Oshima, University of Washington, Seattle, WA) were subcloned into a pLXSP expression vector (provided by S. Soddu, Regina Elena Cancer Institute, Rome, Italy). The recombinant vectors were transfected into Phoenix packaging cells (provided by S. Soddu) by the standard Ca_2PO_4 method and, 24 h later, WS cells (AG11395) were infected with retroviral supernatant. Puromycin-resistant colonies were isolated and Western blotting analyses were performed to assess the expression of WRN protein.

Fibroblasts were maintained in DME (Invitrogen) supplemented with 10% FBS (Boehringer Mannheim). All cell lines were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Chemicals and treatments

Aphidicolin, camptothecin, and BrdU were obtained from Sigma-Aldrich. Aphidicolin was dissolved in DMSO as a stock solution (10 mg/ml) and stored at −20°C. Camptothecin was dissolved in DMSO and a stock solution (2.5 mM) was prepared and stored at –20°C. BrdU was dissolved in sterile PBS as a stock solution (3 mg/ml) and stored at −20°C. After treatments, cells were cultured in complete medium at 37°C until they were processed.

Immunofluorescence

Cells grown on 22 × 22-mm glass coverslips were treated with aphidicolin and harvested at the indicated times. For WRN staining before fixation, cells were subjected to in situ fractionation essentially as described previously (Mirzoeva and Petrini, 2001), with the exception that the NaCl concentration used in the cytoskeleton buffer was 150 mM. Staining with rabbit polyclonals anti-WRN (1:500; Novus BioLabs) was performed for 2 h at RT in 1% BSA/PBS. Species-specific fluorescein- or Texas red–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were applied for 1 h at RT followed by counterstaining with 0.5 μg/ml DAPI in DABCO. Secondary antibodies were used at a 1:500 dilution. Slides were analyzed with a microscope (Leica) equipped with a charge-coupled device camera (Photometrics). Images were acquired as grayscale files using the Metaview software (MDS Analytical Technologies) and then processed using Photoshop (Adobe). For each time point, at least 200 nuclei were examined by two independent investigators and foci were scored at 100×. Only nuclei showing more than five bright foci were counted as positive. Parallel samples incubated with either the appropriate normal serum or only with the secondary antibody confirmed that the observed fluorescence pattern was not attributable to artifacts.

Fragile site induction and slide preparation

Fragile sites were induced by treating cells with different concentrations of aphidicolin (0.05, 0.2, and 0.4 μM). Cell cultures were incubated with 0.2 μg/ml colcemid at 37°C for 3 h until harvesting. Cells for metaphase preparations were collected according to standard procedure. In brief, the cellular pellet was resuspended in prewarmed hypotonic solution (0.075 M KCl in distilled water) and incubated at 37°C for 18 min followed by multiple changes of fixative solution (3:1 methanol/acetic acid). Cell suspension was dropped onto cold, wet slides to make chromosome preparations.

The slides were air dried overnight and stored at −20°C until analysis. For each condition of treatment, the number of breaks and gaps was observed on Giemsa-stained metaphases.

FISH

Bacterial artificial chromosomes (BACs) mapping to fragile or nonfragile site regions (provided by D. Toniolo, Dibit-HSR, Milan, Italy; and M. Rocchi, University of Bari, Bari, Italy) were used as probes for FISH analyses. A mix of the BACs 94D19, 149J4, and 48E21 were used for FRA3B, BAC36B6 (RP-11) was used for FRA7H, and BAC264L1 (RP-11) was used for FRA16D. Probes were labeled with a digoxigenin-11-dUTP nick translation kit (Roche) according to the manufacturer's instructions. FISH experiments were performed according to standard protocols (Wilke et al., 1996). FISH signals were detected by incubation with anti–digoxigenin-rhodamine Fab fragments (Roche). Chromosomes were counterstained with DAPI. Hybridized metaphases were analyzed with an epifluorescence microscope equipped with a charge-coiled device camera. Images were acquired as grayscale files using Metaview software and processed using Photoshop. For each time point, at least 100 chromosomes were examined by two independent investigators and chromosomal damage was scored at 100×.

RNAi

WRN and ATR expression were knocked down by transfection with SMARTpool siRNAs (Thermo Fisher Scientific) at the final concentration of 10 nM. Transfection was performed using a HiPerFect reagent (QUIAGEN) according to the manufacturer's instructions. As a control, an siRNA duplex directed against GFP was used.

Western blotting

Cells were washed with PBS and lysed in standard RIPA buffer (PBS, 1% NP-40, 0.5% sodium dehoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml PMSF, 1 mM sodium orthovanadate, and 1 mM NaF). Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose (PROTRAN; Whatman). Incubation with antibodies was performed for 2 h at RT. Proteins were visualized using ECL+ according to the manufacturer's instructions (GE Healthcare) and normalized to the tubulin level in each extract. Antibodies used for Western blotting were commercially obtained for WRN (1:4,000; Novus BioLabs), ATR (1:15,000; Bethyl Laboratories, Inc.), and β-tubulin (1:15,000; Sigma-Aldrich). Horseradish peroxidase–conjugated goat specie-specific secondary antibodies (Santa Cruz Biotechnology, Inc.) were used at a dilution of 1:1,000.

Evaluation of S phase cells

To quantify S phase cells, normal and WS fibroblast cell lines were pulse labeled for 30 min with 30 μg/ml BrdU and then exposed to a high dose of aphidicolin (0.4 μM) and harvested after different recovery periods (10 or 24 h). Samples were processed for immunodetection of BrdU incorporation essentially as described previously (Pichierri et al., 2001). For each time point, at least 500 interphase cells were scored to evaluate the percentage of labeled nuclei. Only nuclei displaying more or less uniform BrdU labeling in the entire volume were considered to be actively replicating.

The percentage of cells undergoing DNA synthesis at each time point was calculated as a fraction of the treated cells versus untreated controls.

Cell death evaluation

Cells were plated in 6-well dishes at a concentration of 3×10^5 per well and treated with 1.5, 15, or 30 μM of camptothecin for 2 h. Cell death was evaluated by counting cells using the trypan blue exclusion method. The trypan blue solution (0.4%; Invitrogen) was diluted with an equal volume of PBS. Cells were detached and stained with 0.2% trypan blue solution directly. The number of blue cells was scored under a phase-contrast optical microscope.

Statistical analysis

To analyze total gaps, breaks, and frequency of fragile site expression and cell death, the *t* test was used. We always compared data from WRN- and/or ATR-deficient cells to their relevant controls. All the reported data are presented as means of at least three independent experiments.

Cell lines and culture conditions

A normal (SNW646) LCL was obtained from the International Registry of Werner syndrome (G. Martin, University of Washington). WS LCL (AG14426) was obtained from Coriell Cell Repositories. The AG14426 cell line carries an Arg369 stop mutation and gives rise to a truncated protein. The WS1*WRN* was generated by transfection by electroporation of linearized pcDNA3.1*WRN* plasmid expressing wild-type *WRN* cDNA.

All LCLs were routinely maintained in exponential growth in RPMI 1640 medium (Invitrogen) supplemented with 12% heat-inactived fetal calf serum (Boehringer Mannheim) by a daily dilution to 3.5×10^5 cells per milliliter.

Online supplemental material

Fig. S1 contains images of metaphase chromosomes showing chromosomal aberrations induced by aphidicolin in wild-type and WS fibroblasts. Fig. S2 shows additional data confirming enhanced expression of fragile sites after aphidicolin treatment in a WS lymphoblast cell line. Fig. S3 shows images of metaphase chromosomes expressing fragile sites induced by aphidicolin in wild-type and WS fibroblasts. Fig. S4 shows the percentage of hyperdamaged cells in WS fibroblasts after aphidicolin treatment. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200705126/DC1.

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