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ORIGINAL ARTICLE

# The Werner syndrome protein is required for recruitment of chromatin assembly factor 1 following DNA damage

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**The Werner syndrome protein (WRN) and chromatin assembly factor 1 (CAF-1) are both involved in the maintenance of genome stability. In response to DNA-damaging signals, both of these proteins relocate to sites where DNA synthesis occurs. However, the interaction between WRN and CAF-1 has not yet been investigated. In this report, we show that WRN interacts physically with the largest subunit of CAF-1, hp150, *in vitro* and *in vivo*. Although hp150 does not alter WRN catalytic activities *in vitro*, and the chromatin assembly activity of CAF-1 is not affected in the absence of WRN *in vivo*, this interaction may have an important role during the cellular response to DNA replication fork blockage and/or DNA damage signals. In hp150 RNA-mediated interference (RNAi) knockdown cells, WRN partially formed foci following hydroxyurea (HU) treatment. However, in the absence of WRN, hp150 did not relocate to form foci following exposure to HU and ultraviolet light. Thus, our results demonstrate that WRN responds to DNA damage before CAF-1 and suggest that WRN may recruit CAF-1, via interaction with hp150, to DNA damage sites during DNA synthesis.**
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## Introduction

The eukaryotic nuclear genome is assembled into the nucleoprotein structure termed chromatin. The basic repeating unit of chromatin is the nucleosome, which comprises 147 bp of DNA wrapped around an octamer of histone proteins (two molecules each of histones H3, H4, H2A and H2B) (Luger *et al*., 1997). The majority of chromatin is assembled immediately following DNA replication. This is mediated in part by the histone chaperone chromatin assembly factor 1 (CAF-1) that deposits histones H3 and H4 onto newly replicated DNA *in vitro* (Smith and Stillman, 1989). CAF-1 is a heterotrimeric protein that is highly conserved from yeast to humans (Kaufman *et al*., 1995, 1997; Tyler *et al*., 1996, 2001; Verreault *et al*., 1996). CAF-1 has been shown to localize to sites of DNA replication (Krude, 1995). Yeast lacking CAF-1 have global under-assembly of their genome into chromatin (Adkins *et al*., 2004), and reduction of CAF-1 activity in tissue culture cells leads to reduced packaging of the genome into chromatin, replication defects and S-phase arrest (Krude, 1999; Hoek and Stillman, 2003; Ye *et al*., 2003; Nabatiyan and Krude, 2004). CAF-1 has also been implicated in DNA repair via the nucleotide excision repair (NER) pathway. NER repairs single-stranded DNA lesions such as those incurred by exposure to ultraviolet light (UV) (Prakash and Prakash, 2000), and CAF-1 is capable of assembling chromatin coupled with NER *in vitro* (Gaillard *et al*., 1996). CAF-1 has also been localized to DNA templates undergoing NER, in a manner dependent upon proliferating-cell nuclear antigen (PCNA) (Moggs *et al*., 2000; Green and Almouzni, 2003). Accordingly, yeast deleted for CAF-1 components are hypersensitive to UV irradiation, indicating the importance of CAF-1 in surviving UV-induced DNA damage (Kaufman *et al*., 1997). It has been reported recently that CAF-1 has a role in the assembly of chromatin following or during the repair of double-strand DNA damage (Linger and Tyler, 2005).

Werner syndrome (WS) is an autosomal recessive disorder with distinct premature aging features and with a high incidence of age-associated diseases including cancer (soft tissue) and diabetes mellitus type II (Hickson, 2003; Bohr, 2005; Ozgenc and Loeb, 2005). The gene defective in WS, *WRN*, encodes a protein of the RecQ family of DNA helicases (Yu *et al*., 1996; Hickson, 2003; Opresko *et al*., 2003), which possesses DNA-dependent ATPase, 3′-5′ helicase and 3′-5′ exonuclease activities. The substrate specificity of WRN helicase and exonuclease activities has been determined *in vitro*, and includes a variety of intermediates produced during DNA replication, recombination and repair (Shen *et al*., 1998; Xue *et al*., 2002). WRN has been shown to predominantly localize to the nucleolus, but after certain types of DNA damage, leaves the nucleolus and forms distinct foci in the nucleoplasm (Sakamoto *et al*., 2001; Karmakar and Bohr, 2005; Li *et al*., 2005). The formation of these foci likely reflects an important stage in the DNA damage response. Although the number of WRN-containing nuclear foci increases after replication fork arrest and upon DNA damage (Szekely *et al*., 2000; Sakamoto *et al*., 2001), the significance of the formation of these foci and how they are regulated remain to be elucidated.

WRN physically and functionally interacts with proteins that 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 more than one role in DNA repair (Hickson, 2003; Opresko *et al*., 2004b). WRN has been shown to interact with Ku and DNA-PKcs (Li and Comai, 2000; Orren *et al*., 2001; Karmakar *et al*., 2002a, b; Karmakar and Bohr, 2005; Li *et al*., 2005). Consistently, WS 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 compared to controls (Yannone *et al*., 2001; Cheng *et al*., 2004). WS cells also display extensive deletions at non-homologous joined ends as well as non-homologous chromosome exchanges (Oshima *et al*., 2002). These results suggest that WRN may be involved in processing ionizing radiation-induced double-strand breaks. In agreement, WRN also interacts physically with the Mre11-Rad50-NBS1 complex, which functions in homologous recombination for double-strand break processing (Cheng *et al*., 2004). WRN may also play a role in base excision repair as WRN stimulates DNA polymerase $\beta$-strand displacement synthesis via its helicase activity (Harrigan *et al*., 2003, 2006). The sensitivity of WS cells to cross-linking drugs (Poot *et al*., 2001) and a very recent report on the WRN response to UV treatment (Guay *et al*., 2006) suggest that WRN might also be involved in the repair process that removes cross-linking induced lesions. Increasing evidence also supports the hypothesis that WRN plays a direct role in telomere maintenance (Opresko *et al*., 2004a; Laud *et al*., 2005).

Here we present evidence that suggests a cross-talk between WRN and CAF-1 via its largest subunit hp150. We show that WRN interacts physically with hp150, *in vitro* and *in vivo*. In the absence of WRN, hp150 did not re-localize to form nuclear foci following treatment of cells with hydroxyurea (HU), whereas in hp150 RNAi knockdown cells, WRN did respond to the same treatment. Thus, our results indicate that before CAF-1, WRN responds to DNA damage. WRN may recruit CAF-1, via hp150, to DNA damage sites to assemble the newly synthesized DNA into chromatin following DNA synthesis, so that the epigenetic state of the chromatin is re-established, and to prevent the DNA from further damage.

## Results

*WRN and hp150 interact* in vitro *and* in vivo

We previously reported that BLM, another member of the human RecQ helicase family, interacts physically and functionally with hp150, the largest subunit of CAF-1 (Jiao *et al*., 2004). As WRN is also involved in the maintenance of genome stability and re-localizes to sites of DNA synthesis following DNA damage similar to CAF-1 (Green and Almouzni, 2003; Jiao *et al*., 2004; Karmakar and Bohr, 2005; Li *et al*., 2005), we examined for possible interactions between WRN and CAF-1 during DNA replication and/or repair.

To test for a physical interaction between WRN and hp150 *in vitro*, we employed the Far-Western assay. As shown in Figure 1b, purified recombinant hp150 protein interacted directly with full length WRN (aa 1–1432) and two fragments WRN P1 (aa 523–1227) and WRN P2 (aa 523–1148) (lanes 1–3). These fragments comprise the helicase, RQC and HRDC (Helicase RNaseD C-terminus domain) (WRN P1), and helicase and RQC (WRN P2) domains of WRN, respectively (Figure 1e; Lee *et al*., 2005). In contrast, hp150 did not interact with the N-terminal WRN fragment, LGS-WRN (aa 1–368), which contains the exonuclease domain (Figure 1b, lane 4), nor with the unrelated bacterial helicase UvrD (Figure 1b, lane 5). In the reverse Far-Western, full-length WRN bound to hp150 and its known partner DNA polymerase $\beta$ (pol $\beta$) (Figure 1c, lanes 2 and 3; Harrigan *et al*., 2003), but not to DNA polymerase lambda (pol $\lambda$) (Figure 1c, lane 1). All the purified proteins used in the Far-Western assay are shown in Figure 1a.

We next performed co-immunoprecipitation experiments to investigate whether WRN and CAF-1 formed a complex *in vivo*. In untreated HeLa cells, we were not able to detect a WRN-hp150 complex by co-immunoprecipitation using anti-hp150 antibodies (Figure 1d, lower panel, lane 3). Similarly, we did not detect a WRN and hp150 interaction in a yeast two hybrid assay (data not shown). However, in extracts from HU-treated HeLa cells, anti-hp150 antibodies co-immunoprecipitated WRN (Figure 1d, lower panel, lane 4). To eliminate the possibility that WRN and hp150 may associate through DNA, the immunoprecipitation was performed in the presence of ethidium bromide (EtBr),

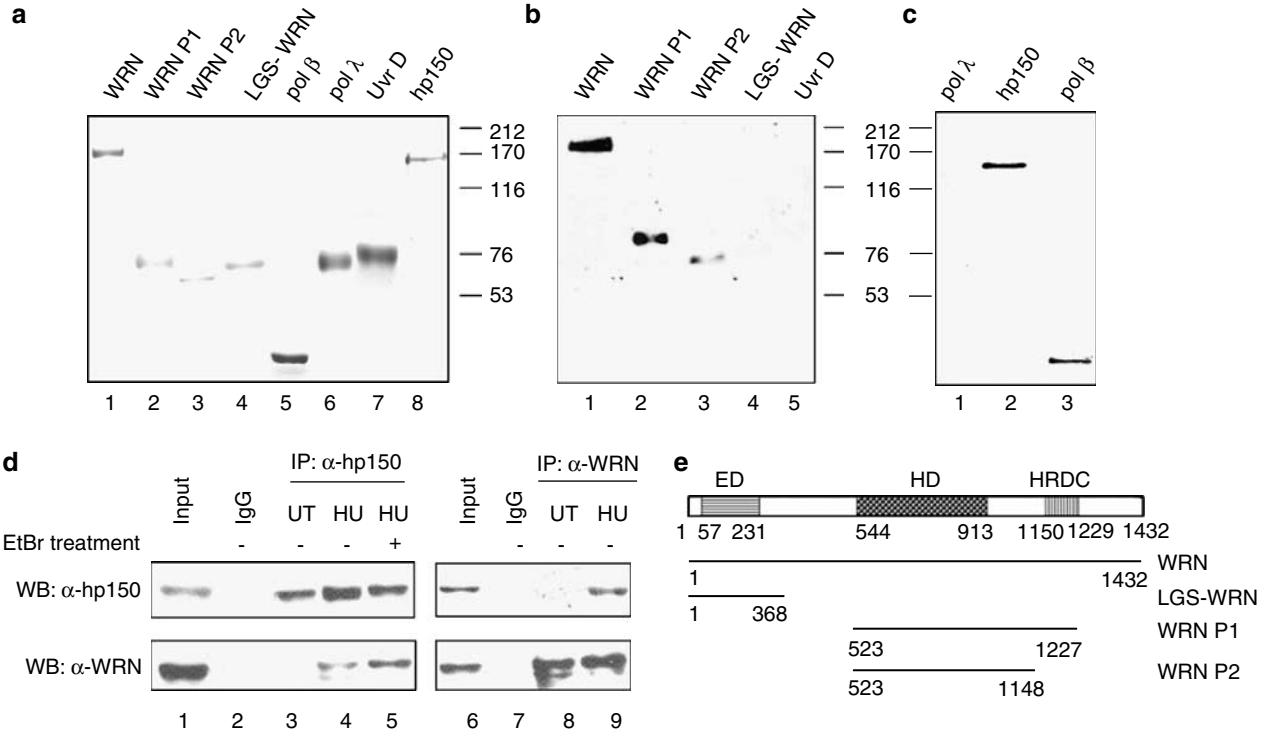



**Figure 1** WRN physically interacts with the largest subunit of CAF-1, hp150, *in vitro* and *in vivo*. (**a**) SDS–PAGE and Coomassie staining showing the proteins used in the Far-Western assay. Lanes 1–8 represent, respectively, the following proteins: full-length WRN, WRN P1 (aa 523–1227), WRN P2 (aa 523–1148), LGS-WRN (aa 1–368), pol $\beta$, pol $\lambda$, UvrD and hp150. Molecular weight markers are indicated on the right. The gel was stained with Coomassie blue. (**b**) Far-Western analysis. WRN proteins (full-length and truncated) and UvrD as indicated were subjected to SDS–PAGE, transferred to a nitrocellulose membrane, and incubated with hp150. Anti-hp150 antibodies were used for detection of the hp150 interacting proteins. Molecular weight markers are indicated on the right. (**c**) pol $\lambda$, hp150, and pol $\beta$ as indicated were subjected to SDS–PAGE, transferred to a nitrocellulose filter, and incubated with WRN. Anti-WRN antibodies were used for detection of the WRN interacting proteins. Molecular weight markers are indicated on the left. (**d**) Left panels: anti-hp150 antibodies (lanes 3–5) or control (IgG) antibodies (lane 2) were used to immunoprecipitate proteins from either untreated (UT) or HU-treated (HU) HeLa nuclear extracts in the absence (lanes 2–4) or presence (lane 5) of EtBr and subjected to Western blotting using anti-hp150 or anti-WRN antibodies as indicated. Right panels: anti-WRN antibodies (lanes 8–9) or control IgG antibodies (lane 7) were used to immunoprecipitate proteins from either untreated (UT) or HU-treated (HU) HeLa nuclear extracts. In both the left and right panels, 1/10 of the nuclear extracts were used as input control (lanes 1 and 6). (**e**) Representation of different WRN fragments used in the Far-Western assay. ED: exonuclease domain, HD: helicase domain, HRDC: helicase RNaseD C-terminus domain.

a DNA-intercalating agent that can dissociate proteins from DNA and has often been used to identify DNA-independent protein associations (Lai and Herr, 1992). The anti-hp150 antibody co-immunoprecipitated WRN in the presence of EtBr (Figure 1d, lower panel, lane 5), suggesting that WRN forms a DNA-independent complex with hp150. Likewise, in a reverse immunoprecipitation experiment, anti-WRN antibodies also co-immunoprecipitated hp150 from the HU-treated (Figure 1d, top panel, lane 9), but not from the untreated HeLa cells (Figure 1d, top panel, lane 8). Thus, we conclude from our Far-Western assay and co-immunoprecipitation experiments that WRN and hp150 interact directly *in vitro* and after exposure to HU *in vivo*, and that the helicase-RQC domain of WRN (aa 523–1148) is necessary and sufficient to bind hp150.

### *WRN and CAF-1 co-localize in human cells in response to DNA damaging agents*

Based on the physical interaction between WRN and hp150 shown above, we used indirect immunofluorescence microscopy to analyze analyse whether WRN and CAF-1 co-localize in human cells. Consistent with the reports of others (Krude, 1995; Marciniak *et al*., 1998; von Kobbe *et al*., 2002), in untreated HeLa cells approximately 10% of cells contained hp150 nuclear foci while whereas only about 3% of the cells exhibited distinct nuclear foci containing WRN. The majority of WRN occupied the nucleoli (Figure 2, upper panels and Table 1). In untreated HeLa cells, we did not observe any obvious co-localization of WRN and hp150 (Figure 2, upper panels and Table 1), consistent with the immunoprecipitation experiments. However, following exposure to 10 mM HU, an agent that inhibits ribonucleotide reductase and therefore halts DNA replication fork progression, the majority of WRN re-localized from the nucleoli to the nucleoplasm to form discrete nuclear foci at sites of stalled DNA replication forks (Figure 2, middle panels; Franchitto and Pichierri, 2004). Approximately 74% of the cell population contained WRN foci with a mean number of 36 foci per cell (Table 1). Similarly, HU treatment also resulted in hp150 relocation to sites of DNA replication fork

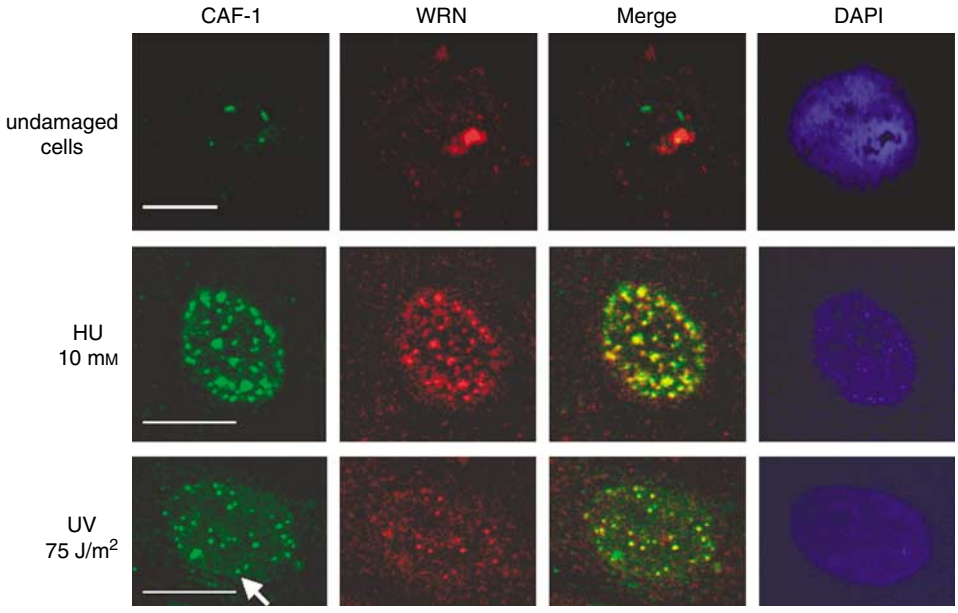



**Figure 2** WRN and CAF-1 colocalize in HU- and UV-treated HeLa cells. HeLa cells cultured on slides were either untreated (upper panels) or treated with 10 mM HU (middle panel, 20 h) or 75 J/m$^2$ UV (lower panels, 4–6 h recovery). Following treatment, cells were processed using anti-WRN (red) and anti-hp150 (green) antibodies. In the merged pictures, yellow represents the colocalization of WRN and CAF-1. DAPI staining of the nucleus is indicated in blue. The white arrow indicates the minimum concentrated signal to be a focus. Scale bars represent 10 $\mu$m.

**Table 1** WRN and CAF-1 colocalization in HeLa cells after HU and UV treatment

| *Treatment* | *None* | *HU* | *UV* |
|---|---|---|---|
| Percentage of cells containing WRN foci | 3 | 74 | 46 |
| Percentage of cells containing CAF-1 foci | 10 | 65 | 36 |
| Mean no. of WEN foci/cell (range) | 2 (0–12) | 36 (0–83) | 11 (0–36) |
| Mean no. of CAF-1 foci/cell (range) | 3 (0–25) | 27 (0–61) | 8 (0–33) |
| Percentage of cells containing WEN and CAF-1 colocalizing foci | | | |
| 0 (%) | 99 | 60 | 79 |
| 1–5 (%) | 1 | 9 | 8 |
| More than 5 (%) | 0 | 31 | 13 |
| Mean no. of WRN and CAF-1 co-localizing foci/cell (range) | 0 (0–3) | 16 (0–55) | 7 (0–28) |

In each case, 200 cells were scored.

stalling (Figure 2, middle panels; Jiao *et al*., 2004), and 65% of cells contained CAF-1 foci with a mean number of 27 foci per cell (Table 1). Among all the cells we analyzed, 40% showed co-localization of WRN and hp150 with a mean number of 16 co-localizing foci per cell (Figure 2, middle panels and Table 1). These results suggest that WRN and CAF-1 function together in the recovery of DNA replication fork stalling. To investigate the kinetics of formation of WRN and CAF-1 foci and the co-localization of these two proteins in HeLa cells, we performed immunofluorescence studies 1, 2, 4 and 20 h after HU treatment. As shown in Figure 3a and Table 2, WRN and CAF-1 co-localized 1 h after HU treatment, however, in a lower percentage of cells and with a fewer number of co-localizing foci per cell compared to 20 h, the co-localization increased with the longer time of HU treatment, suggesting that the WRN and CAF-1 interaction is involved more in restoring stalled replication forks and/or repairing lesions induced by HU, than in establishing DNA replication forks. Table 2 also shows that the increase in WRN foci is faster than that of CAF-1 foci, especially in the first 4 h, indicating that WRN is recruited to the damaged sites earlier than CAF-1. In contrast to the observed increase of CAF-1 associated foci, the total level of CAF-1-hp150 protein did not increase in cells 20 h after HU treatment (Figure 3b). This result indicates that the increase of CAF-1 foci may reflect re-location of the protein to detergent-insoluble nuclear matrix associated regions in response to HU, rather than increased expression of the protein.

We also irradiated cells with UV, which generates the formation of covalent bonds between adjacent thymine bases, producing photoproducts. These photoproducts do not base-pair normally and, if not removed in time, may cause distortion of the DNA helix and stalling of replication forks. Four to 6 h after recovery from UV exposure, we observed WRN and hp150 co-localization

in 21% of cells with a mean number of 7 co-localizing foci per cell, while whereas 36% and 46% of the cells contained hp150 and WRN foci, respectively, each with 8 and 11 foci per cell on avarage (Figure 2, bottom panels and Table 1). Thus, our results indicate that WRN and CAF-1 may also function in a common pathway after UV treatment.

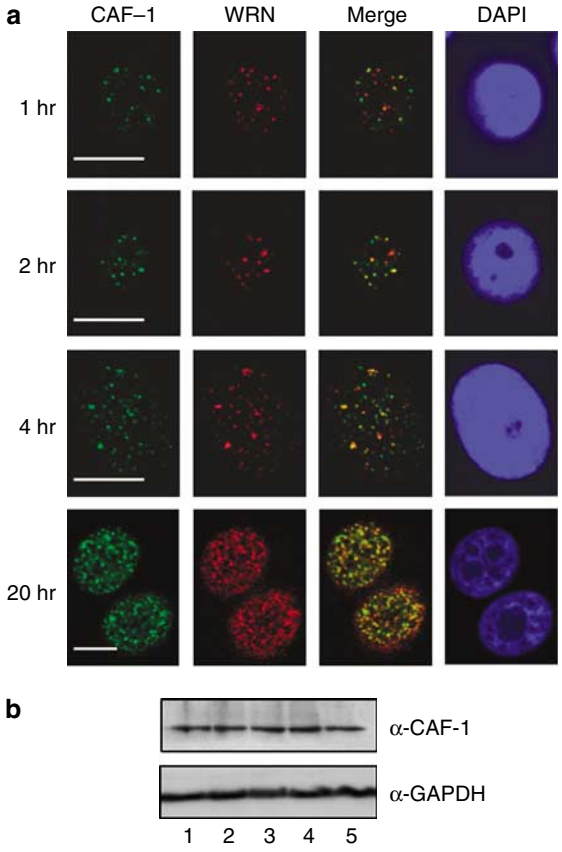



**Figure 3** Kinetics of WRN and CAF-1 colocalization in HeLa cells after HU treatment. (**a**) HeLa cells cultured on slides were treated with 10 mM HU for 1, 2, 4 or 20 h, before processing with anti-WRN (red) and anti-hp150 (green) antibodies. In the merged pictures, yellow represents the colocalization of WRN and CAF-1. DAPI staining of the nucleus is indicated in blue. Scale bars represent 10 $\mu$m. (**b**) Western blot showing the amount of CAF-1-hp150 in HeLa cells at various time points after addition of HU. Lane 1, untreated. Lanes 2–5 represent the amount of CAF-1-hp150 1, 2, 4, and 20 h after HU treatment. Glyceraldehyde-3-phosphate dehydrogenase is the loading control.

*The hp150 protein does not affect the enzymatic activities of WRN*

Given that WRN and hp150 interacted *in vitro* and *in vivo*, and also co-localized following exposure to DNA damaging agents, we investigated whether one influenced the enzymatic activity of the other. As shown in Figure 4a, the presence of hp150 did not affect WRN helicase activity on forked DNA duplexes *in vitro* (lanes 4–8 and 10–14) compared to WRN protein alone (lanes 3 and 9). As WRN helicase activity is ATP dependent, it was not surprising that the presence of hp150 had no obvious influence on the ability of WRN to hydrolyze hydrolyse ATP (Figure 4b, lanes 2–7). Distinct from other RecQ helicases, WRN also possesses exonuclease activity. Therefore, we next examined whether this activity was altered by the presence of hp150. As shown in Figure 4c, increasing the amounts of hp150 had no effect on the exonuclease activity of WRN, either in the presence (lanes 3–8) or in the absence (lanes 10–15) of ATP. We conclude from these experiments that the catalytic activities of WRN are not affected by the presence of hp150.

*CAF-1 activity is not altered in WRN deficient cells*

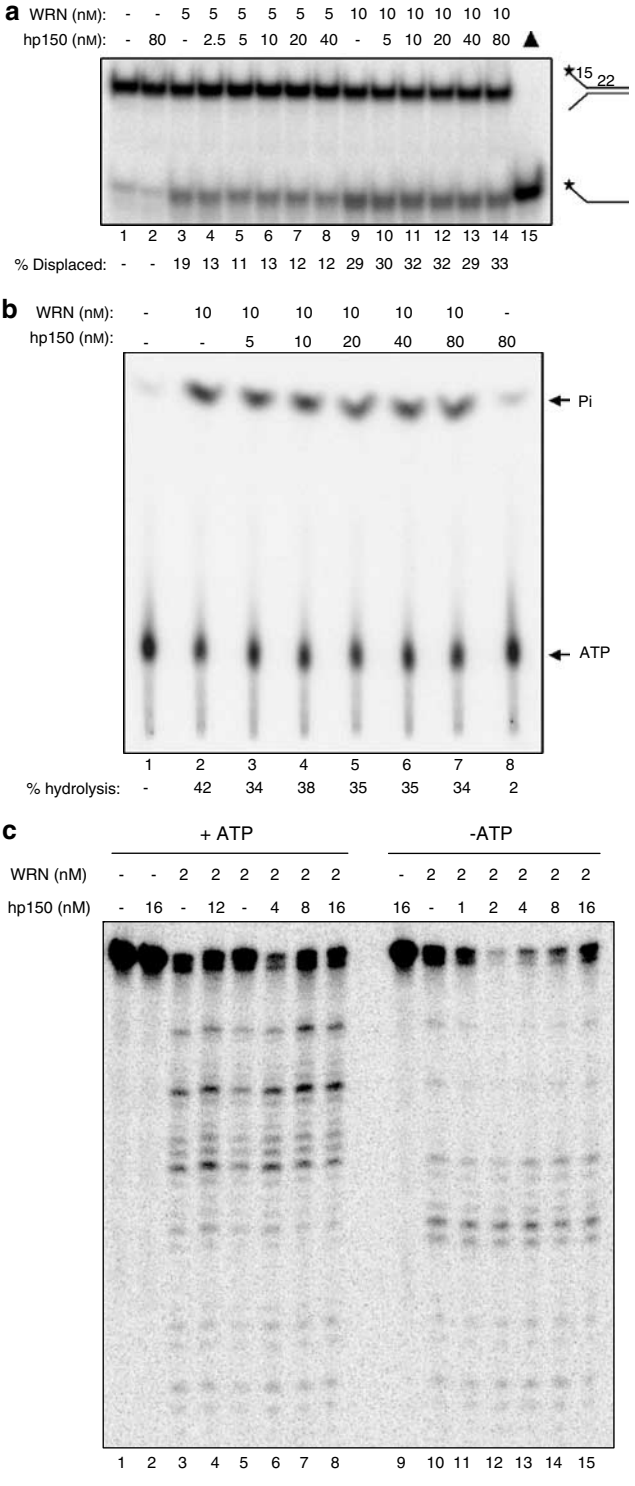
We used an *in vitro* chromatin assembly assay coupled to DNA repair of UV-damaged DNA (Gaillard *et al*., 1996; Jiao *et al*., 2004) to detect CAF-1 activity *in vivo*. This assay takes advantage of the simultaneous analysis of DNA repair and chromatin assembly processes on the same damaged circular DNA template. Cytosolic (lacking CAF-1 proteins, Figure 5b, lane 1 and data not shown) and S100 (lacking hp150, but not hp 60, Figure 5b, lane 2 and data not shown) extracts from HeLa cells were not sufficient to mediate chromatin assembly/supercoiling (Figure 5a, lanes 1 and 2). When the S100 extract was supplemented with recombinant hp150, the chromatin assembly activity was restored (Figure 5a, lane 3), demonstrating that the recombinant hp150 used in our study was functional. Likewise, when the cytosolic extract was supplemented with HeLa

**Table 2** Kinetics of WRN and CAF-1 colocalization in HeLa cells after HU treatment

| *Hours after HU treatment* | *0 h* | *1 h* | *2 h* | *4 h* | *20 h* |
|---|---|---|---|---|---|
| Percentage of cells containing WRN foci | 3 | 8 | 16 | 27 | 75 |
| Percentage of cells containing CAF-1 foci | 10 | 13 | 15 | 18 | 66 |
| Mean no. of WRN foci/cell (range) | 2 (0–12) | 5 (0–21) | 7 (0–24) | 10 (0–38) | 35 (0–77) |
| Mean no. of CAF-1 foci/cell (range) | 3 (0–25) | 4 (0–24) | 5 (0–27) | 7 (0–31) | 27 (0–58) |
| Percentage of cells containing WRN and CAF-1 co-localizing foci | | | | | |
| 0 (%) | 99 | 95 | 88 | 84 | 58 |
| 1–5 (%) | 1 | 4 | 10 | 11 | 10 |
| more than 5 (%) | 0 | 1 | 2 | 5 | 32 |
| Mean no. of WRN and CAF-1 colocalizing foci/cell (range) | 0 (0–3) | 2 (0–10) | 3 (0–13) | 5 (0–18) | 17 (0–56) |

In each case, 200 cells were scored.

nuclear extract, providing a CAF-1 source, chromatin assembly also occurred (Figure 5a, lane 4). However, one could ague that the chromatin assembly activity from HeLa nuclear extracts is not necessarily attributed to CAF-1 and may come from another chromatin assembly factor(s). To exclude this possibility, we performed RNAi experiments to knockdown endogenous hp150 before making nuclear extracts. As shown in Figure 5 (b, lane 5) transfection of hp150-specific small interfering RNA oligos efficiently eliminated hp150 protein in HeLa cells (lane 5), while whereas the control oligos did not (lane 6). Supplementing the cytosolic extracts with nuclear extracts from hp150-RNAi cells did not result in supercoiling (Figure 5a, lane 5). In contrast, nuclear extracts from control RNAi experiments clearly displayed chromatin assembly activity



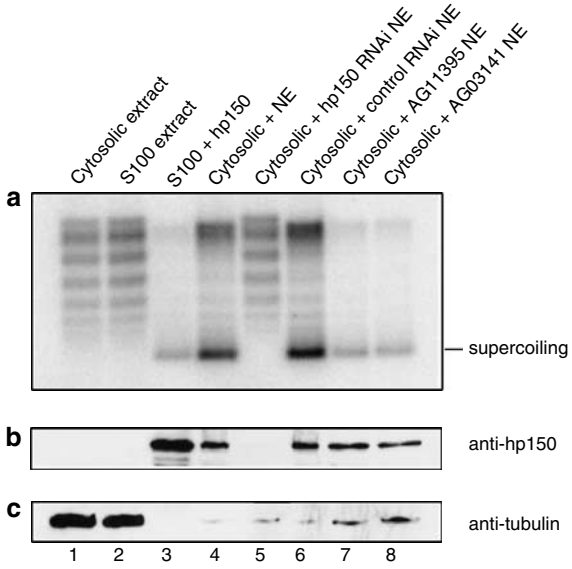




**Figure 5** DNA repair-coupled chromatin assembly activity of CAF-1 does not require the presence of WRN. (**a**) Chromatin assembly assay using cytosolic, S100, or nuclear extracts (NE) as indicated and 75 ng of 500 J/m$^2$ UV-irradiated plasmid DNA. Lanes 1 and 2 are cytosolic and S100 extract controls. Lane 3, S100 extracts plus purified hp150. Lane 4, cytosolic plus NE. Lanes 5–8 contain cytosolic extracts plus NE from hp150 RNAi, control RNAi, WS (AG11395) or WS (AG03141) cells, respectively. (**b**) Western blot to detect the presence of hp150. Loading order is: 1, cytosolic extract; 2, S100 extract; 3, recombinant hp150;4, Normal HeLa nuclear extract; 5, HeLa nuclear extract after RNAi of hp150;6, HeLa nuclear extract after control RNAi;7, nuclear extract from WRN-deficient AG11395 cells and 8, nuclear extract from WRN deficient AG01341 cells. (**c**) Western blot to detect the presence of tubulin (loading control). The loading order is the same as in **b**.

**Figure 4** WRN helicase, ATPase and exonuclease activities are not affected by hp150 *in vitro*. (**a**) Helicase assay. WRN (5 or 10 fmol) or hp150 (2.5–80 fmol) as indicated were incubated with a radiolabeled forked duplex substrate for 15 min at 37°C. Reaction products were separated on 12% native gels and visualized by a PhosphorImager. Lane 1, substrate alone.; lane 15, heat-denatured substrate. The quantification of displaced single-stranded DNA is indicated below each lane. (**b**) ATPase assay. WRN (10 nM) or hp150 (5–80 nM) as indicated were incubated with M13mp18 DNA cofactor and radiolabeled ATP for 15 min at 37°C. The percentage of ATP hydrolysis was quantified and indicated below each lane. (**c**) Exonuclease assay. WRN (2 nM) or hp150 (1–16 nM) as indicated were incubated with a radiolabeled forked duplex substrate in the absence (lanes 1–8) or presence (lanes 9–15) of ATP for 15 min at 37°C. Reaction products were separated on 14% denaturing gels and visualized by a PhosphImager. Lane 1, substrate alone.

(lane 6). Therefore, we conclude that the chromatin assembly activity from the nuclear extracts in this assay was CAF-1 specific. To this end, we examined the CAF-1 dependent chromatin assembly activity in cells lacking WRN. Using nuclear extracts from two WRN deficient cell lines, AG11395 and AG03141, we found that CAF-1 chromatin assembly activity was not affected in the absence of WRN (Figure 5a, lanes 7 and 8). Thus, these experiments indicate that CAF-1 activity is not dependent on WRN *in vivo*.

### *WRN is required for CAF-1 re-localization in response to HU treatment*

Since the enzymatic activities of WRN and CAF-1 were not dependent on each other, yet they interacted following exposure to HU, we hypothesized that the interaction would be important in response to DNA replication fork blockage and DNA damaging signals. Similar to the situation in HeLa cells, HU treatment resulted in re-localization of WRN and hp150 to sites of stalled DNA replication forks in a normal human fibroblast cell line (Figure 6, upper panels, AG10076). In these cells, 68% contained WRN nuclear foci, 63% contained hp150 foci, whereas 36% of cells showed WRN and hp150 co-localization (Table 3 and Figure 6, upper panels). These numbers were comparable to what we observed in HeLa cells suggesting that WRN and CAF-1 behave similarly in different human cell lines.

More importantly, in the WRN deficient AG11395 cells, the profile of hp150 staining was different from normal human fibroblasts following HU and UV treatment (Figure 6). The percentage of WRN deficient cells that contained discrete nuclear hp150 foci decreased from 63 to 2–3% after HU treatment (Table 3). As expected, we did not observe nuclear staining of WRN in cells from a WS patient (Figure 6, second and last rows). Thus, our results demonstrate that the presence of WRN is required for CAF-1 to relocate in the cells after HU treatment. They also suggest that WRN may recruit CAF-1 to sites where DNA replication is stalled and that both WRN and CAF-1 are important for the recovery of stalled replication forks. This idea is confirmed by the fact that CAF-1 foci formation and CAF-1/WRN co-localization was restored following HU treatment when the WRN deficient cells were corrected with WRN expressing plasmids (Figure 6, third row and Table 3). To show that it is WRN that recruits CAF-1, and not CAF-1 that recruits WRN, we examined WRN localization in cells after HU treatment in the absence of hp150. Following transfection of RNA interfering oligos, endogenous hp150 was not detected by immunofluorescence (Figure 6 and Table 3). However, the staining of WRN, especially the relocation of WRN to partially form nuclear foci in response to HU treatment, still occurred, suggesting that CAF-1 is not required for re-localization of WRN to sites of DNA replication blocks.

## Discussion

Most lines of evidence presented thus far are compatible with a multifaceted role for WRN in the resolution of alternative DNA structures in a variety of DNA synthetic processes such as replication, repair and recombination (Bohr, 2005; Ozgenc and Loeb, 2005). For example, WRN is proposed to function during DNA replication to clear the path for the replicative apparatus by resolving alternative DNA structures that would otherwise impede the progression of replication (Franchitto and Pichierri, 2004; Sharma *et al*., 2004a; Ozgenc and Loeb, 2005). On a cellular level, WS cells are

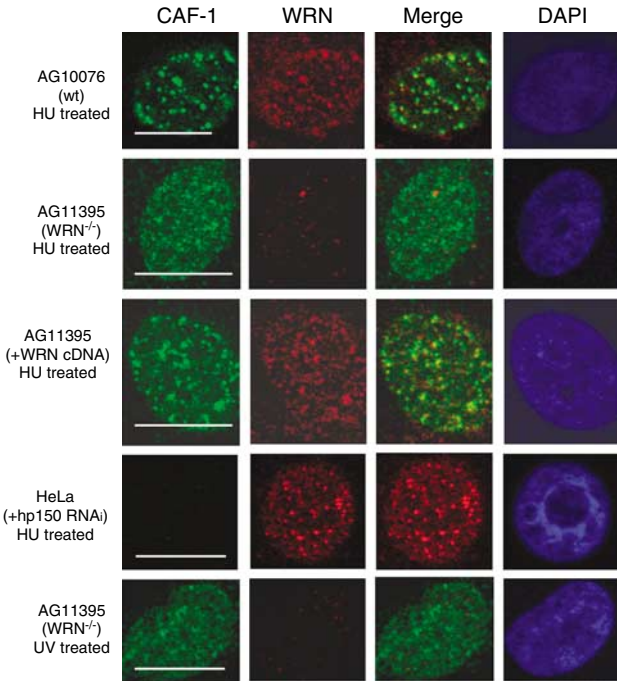



**Figure 6** WRN is required for CAF-1 relocalization in response to DNA replication fork blockage by HU and UV. Wild-type (AG10076), WS (AG11395), WS cells corrected with WRN cDNA, and HeLa cells transfected with hp150 RNAi oligos as indicated were treated with HU or UV and processed for localization of hp150 (green) or WRN (red). The yellow in the merged pictures demonstrates the co-localization of WRN and CAF-1. DAPI staining indicates the nucleus (blue). Scale bars represent 10 $\mu$m.

**Table 3** Colocalization of WRN and CAF-1 in response to HU treatment in WS and CAF-1 RNAi cells

| *Cell type* | *AG10076(wt)* | *AG11395(WS)* | *AG11395 (WRN cDNA corrected)* | *AG03141 (WS)* | *Hpl5Q RNAi* |
|---|---|---|---|---|---|
| Cells containing WRN foci (%) | 68 | No staining | 32 | No staining | 71 |
| Cells containing CAF-1 foci (%) | 63 | 3 | 29 | 2 | 1 |
| Cells containing co-localizing foci (%) | 36 | 0 | 18 | 0 | 0 |

In each case, 200 cells were scored.

hypersensitive to some, but not all, types of DNA-damaging agents. WRN is clearly involved in DNA repair processes, but more work is needed to establish its *in vivo* role in DNA transactions.

*In vivo*, DNA is wrapped with histones and other proteins into chromatin, a structural organization that on the one hand protects DNA from being damaged, and on the other hand inhibits DNA-dependent reactions such as replication, repair and recombination (Wolffe, 1998). More importantly, it is crucial that after every DNA transaction, the epigenetic state of the chromatin be reset so that the genome remains fully functional. Therefore, it is likely that proteins involved in DNA metabolic processes, such as WRN, coordinate with chromatin remodeling factors or chromatin assembly factors to ensure full restoration of the chromatin structure after each *in vivo* DNA transaction. The first possible connection between DNA synthesis and a protein likely to be implicated in chromatin structure restoration has been identified through studies of CAF-1. The involvement of CAF-1 in both DNA replication and nucleotide excision repair (NER) are dependent on the presence of proliferating-cell nuclear antigen (PCNA) (Shibahara and Stillman, 1999; Green and Almouzni, 2003). Here, we report the interaction of CAF-1 and the RecQ DNA helicase, WRN. We show that WRN interacts physically with the largest subunit of CAF-1, hp150, *in vitro* and *in vivo*. The results of our interaction region mapping indicate that WRN interacts with hp150 via the region (aa 523–1148) comprising the helicase and RQC domains of WRN (Figure 1e). Interestingly, this interacting domain is different from the one associating with BLM, which interacts with hp150 via two separate sites, the first located near the *N*-terminus of BLM (aa 131–333) and the second located in the middle of the helicase domain (aa 782–952) (Jiao *et al*., 2004).

Our results also show that hp150 did not affect the helicase, exonuclease or ATPase activities of WRN *in vitro*. The chromatin assembly activity of CAF-1 was also not affected in the absence of WRN *in vivo*. However, in the absence of WRN in human cells, hp150 did not relocate to form foci following treatment with HU. In hp150 RNAi knockdown cells, WRN responded normally after HU treatment, demonstrating an epistatic relationship between WRN and CAF-1 in the process of DNA replication and/or repair. Thus, before CAF-1, WRN responds to DNA damage and may recruit CAF-1 to the DNA-damaged sites during DNA synthesis in order to restore the altered chromatin structure. We note that the localization pattern of WRN in hp150 RNAi cells (HeLa) was slightly different from that of WRN in wild-type cells (AG10076) after HU treatment (Figure 6). However, by comparison with untreated HeLa cells (Figure 2), the punctuate nuclear staining (rather than nucleolar) observed after HU indicates WRN re-localization in response to HU treatment.

PCNA is essential in recruiting and modulating the activity of CAF-1, at least *in vitro*, and it is likely that WRN and PCNA act together to recruit CAF-1 in a cooperative way. It is also known that when hp150 is down-regulated, PCNA can still be recruited to sites where DNA is damaged (Green and Almouzni, 2003). It would therefore be valuable to test how CAF-1 responds to HU treatment in the absence of PCNA, unfortunately, no such PCNA-deficient cell lines are currently available. Nevertheless, we examined the PCNA localization in HU-treated HeLa cells. Figure 7 shows that PCNA colocalizes partially with both WRN and CAF-1 following HU treatment. This is in agreement with previous reports that PCNA associates with CAF-1 in DNA replication (Moggs *et al*., 2000), after UV exposure (Green and Almouzni, 2003) and in the

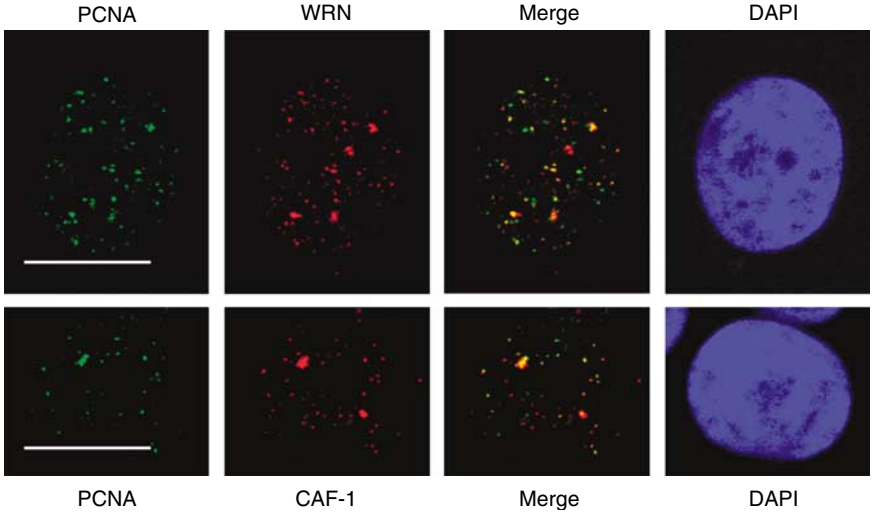



**Figure 7** PCNA partially colocalizes with WRN and CAF-1 in HU -treated HeLa cells. HeLa cells cultured on slides were treated with 10 mM HU. Following treatment, cells were processed using PCNA (green) and anti-WRN (red, upper panels) or anti-CAF-1 (red, lower panels) antibodies. In the merged pictures, yellow represents the colocalization of PCNA and WRN (upper panels) or PCNA and CAF-1 (lower panels). DAPI staining of the nucleus is indicated in blue. Scale bars represent 10 $\mu$m.

process of double-strand breaks repair (Nabatiyan *et al*., 2006), and that PCNA interacts with WRN during replication (Rodriquez-Lopez *et al*., 2003). However, how WRN and PCNA coordinately interact with CAF-1 remains to be elucidated. One possibility could be that like in the case of FEN-1 (Sharma *et al*., 2005), WRN and PCNA have combined effect on the function/localization of CAF-1.

We have previously reported that CAF-1, via its largest subunit hp150, interacts physically and functionally with BLM (Jiao *et al*., 2004). Interestingly, hp150 is not the first common interaction partner of WRN and BLM. BLM and WRN both interact with replication protein A (RPA) (Garcia *et al*., 2004; Doherty *et al*., 2005), p53 (Spillare *et al*., 1999; Wang *et al*., 2001; Yang *et al*., 2002), flap endonuclease-1 (FEN-1) (Sharma *et al*., 2004a, b) and TRF2 (Opresko *et al*., 2002; Bradshaw *et al*., 2005). Moreover, BLM and WRN also interact with each other (von Kobbe *et al*., 2002). Our data suggest that it is possible that BLM, WRN and CAF-1 form a complex, at least transiently, to accomplish certain cellular functions such as the resolution of stalled replication fork intermediates. However, neither WRN nor BLM colocalized 100% with CAF-1, suggesting that the association of CAF-1 with either BLM or WRN is very dynamic. At any given time, only a proportion of WRN colocalized with CAF-1 (Table 1). Therefore, the WRN and CAF-1 interaction may also function independently from the BLM and CAF-1 interaction. This is also supported by other observations that BS cells and WS cells are sensitive to a different spectrum of DNA-damaging agents and display different phenotypes (Hickson, 2003; Jiao *et al*., 2004; Ozgenc and Loeb, 2005).

In BLM-deficient cells, the CAF-1 localization to nuclear foci following HU treatment is not as robust as in wild-type cells (Jiao *et al*., 2004). However, in WRN-deficient cells, CAF-1 did not respond normally to HU treatment (Figure 6 and Table 3). This raises the possibility that WRN plays a more important role in recruiting CAF-1 to stalled replication forks. Given the observation that in unstressed cells, BLM, but not WRN, colocalizes with CAF-1 (Jiao *et al*., 2004), it is very possible that WRN and CAF-1 interact only in DNA repair-related pathway(s), which is dependent on an activity of WRN which acts before CAF-1. Our observations are consistent with those reported by the Almouzni group that CAF-1 is not recruited to UV-damaged sites in cells from several XP complementation groups, including those deficient in the incision steps of NER (Green and Almouzni, 2003). Thus, CAF-1 functioning as a chromatin assembly factor plays an important role in restoring the structure of chromatin following DNA repair.

## Materials and methods

*Cell culture and transfection*

HeLa, AG10076 (SV40-transformed normal human fibroblasts), AG11395 (SV40-transformed WS fibroblasts) and AG03141 (hTERT-immortalized WS fibroblasts) cells (von Kobbe *et al*., 2003) were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum. The WRN expression plasmid that contains the full-length human WRN cDNA cloned into the pCDNA3.1 vector (Invitrogen, Basel, Switzerland) was transfected into AG11395 cells using the conventional calcium phosphate transfection method. For HU treatment, cells were incubated with 10 mM HU for 1, 2, 4 or 20 h. UV treatment was performed with 50–100 J/m$^2$ at 254 nm and the recovery time was 4 h. After treatment, cells were either fixed for immunostaining or subjected to fractionation.

*Far-western analysis*

This assay was performed essentially as described previously (Wu *et al*., 2000; Pedrazzi *et al*., 2003). Briefly, 6 pmol of full-length WRN, truncated WRN proteins (LGS-WRN, WRN P1 and WRN P2), and control protein UvrD were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose filters. After renaturation and blocking steps, the filters were incubated for 60 min in a solution containing hp150 (1 $\mu$g/ml) in Tris-buffered saline supplemented with 0.25% milk, 0.3% Tween 20, 1 mM dithiotherlotol (DTT) and 1 mM phenylmethylsulfonyl fluoride . After extensive washing, conventional Western blotting was performed to detect the presence of hp150 (with anti-hp150 mAb, Ab-3, Oncogene research products). For the reverse direction of Far-Western analysis, filters containing hp150, pol $\beta$ (positive control) and pol $\lambda$ (negative control) proteins were incubated with WRN before blotting with anti-WRN antibody (ab-200, Abcam).

*Preparation of nuclear extracts and co-immunoprecipitation*

Nuclear extracts were prepared as follows: cells (100% confluence) were harvested, washed in phosphate-buffered saline (PBS), re-suspended in buffer A (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, protease inhibitors) and then centrifuged for 1 min at 2000 r.p.m. The cell pellet was then re-suspended in buffer A+ (buffer A supplemented with 0.1% Noniodet P 40) for 5 min on ice and centrifuged at 10 000 r.p.m., for 5 min. The supernatant contained the cytosolic extract. The pellet was washed in buffer A and then re-suspended in buffer C (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, protease inhibitors) for 15 min, with rolling at 4°C. The samples were then centrifuged at 14 000 r.p.m. for 5 min. The supernatant contained the nuclear extract and the NaCl concentration was adjusted to 120 mM with buffer D (20 mM HEPES, pH 7.9, protease inhibitors). Before immunoprecipitation, nuclear extracts from HeLa cells were incubated with or without ethidium bromide (100 $\mu$g/ml) for 1 h. Two hundred microgram of nuclear extract from HeLa cells was incubated with either 2 $\mu$g anti-hp150 antibody (Ab-3, Oncogene research products) or 2 $\mu$g control IgG in IP buffer (20 mM HEPES, pH 7.5, 120 mM KCl, 5 mM $MgCl_2$, 0.1% (v/v) NP-40, protease inhibitors) at 4°C for 3 h in a total reaction volume of 200 $\mu$l. Forty microliters IP buffer-equilibrated protein G-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) were then added, and the mixture was incubated for 3 h at 4°C. The beads were washed five times with wash buffer (IP buffer supplemented with an additional 20 mM KCl) before the protein complexes bound to the beads were eluted and split into two portions for SDS–PAGE. 10 $\mu$g of nuclear extract were used as the input. Western blot analysis with an anti-WRN antibody (ab-200,

Abcam) and an anti-hp150 polyclonal antibody (gifts of Geneviève Almouzni) were used to detect proteins immunoprecipitated by Ab-3 (anti-hp150, Oncogene). ECL detection was performed according to the manufacturer's instructions (Amersham Pharmacia Biotech). Ab-200 (anti-WRN, Abcam) was used for the reverse IP in the above IP buffer containing 120 mM KCl.

*Immunofluorescence microscopy*
The indirect immunofluorescence assay was performed as described previously (Jiao *et al*., 2004; Petkovic *et al*., 2005). Briefly, cells grown on glass slides were incubated with 0.5% Triton X-100 in PBS for 5 min, fixed with 2% paraformaldehyde in PBS (20 min at room temperature (RT)), and permeabilized with 0.5% Triton X-100 in PBS (20 min at RT). After the blocking step (0.1% Tween-20, 5% bovine serum albumin (BSA) in PBS, 20 min at RT), slides were incubated with a mixture of different rabbit and mouse antibodies. Rabbit antibodies were detected with Cy3-conjugated goat anti-rabbit IgG (Jackson Laboratories, Newmarket, Suffold, England; 1:200 in blocking buffer), and mouse antibodies were detected with FITC-conjugated goat anti-mouse IgG (Jackson Laboratories 1:100 in blocking buffer). To visualize nuclear DNA, slides were incubated with 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI, 0.4 $\mu$g/ml). After washing, slides were mounted with Vectashield (Vector Laboratories, Peterborough, England) and viewed under a confocal microscope Leica TCS 4D. Images were processed by Imaris software. For statistical analysis, a minimum of 200 cells was counted in two independent experiments. Anti-WRN was purchased from Abcam (rabbit Ab-200) and anti-hp150 (mAb, Ab-3) from Oncogene research product, whereas polyclonal anti-hp150 was a generous gift from Dr G Almouzni. Anti-PCNA was kindly provided by Dr U Hubscher (mAb, PC10).

*Helicase assay*
Helicase reactions were performed as described previously (von Kobbe *et al*., 2004). Briefly, oligonucleotide 22Fork3 was 5′ end-labeled with [$^{32}$P]ATP and T4 polynucleotide kinase (New England Biolabs, Ipswich, MA, USA) and annealed to 22Fork4. Reactions (20 $\mu$l) were performed in helicase reaction buffer (40 mM Tris-HCl, pH 8, 4 mM $MgCl_2$, 5 mM DTT and 0.1 mg/ml BSA) and contained ATP (2 mM), DNA substrate (1 nM) WRN and hp150 as indicated. Samples were incubated for 15 min at 37°C and terminated by the addition of stop dye (50 mM ethylenediaminetetraacetic acid (EDTA), 40% glycerol, 0.9% SDS, 0.05% bromophenol blue and 0.05% xylene cyanol). Products were run on a 12% native polyacrylamide gel and visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

*Exonuclease assay*
Exonuclease reactions were performed as described previously (von Kobbe *et al*., 2004). Briefly, oligonucleotide 34ForkA was 5′ end-labeled and annealed to 34ForkB. Reaction (10 $\mu$l) were performed in helicase reaction buffer (see above) and contained ATP (2 mM), DNA substrate (1 nM), WRN and hp150 as indicated. Samples were incubated at 37°C for 15 min and terminated by the addition of formamide stop dye (80% formamide, 0.5X tris-borate-EDTA (TBE), 0.1% bromophenol blue and 0.1% xylene cyanol). Products were heat-denatured at 90°C for 5 min and run on a 14% denaturing polyacrylamide gel. Radioactive products were visualized using a PhosphorImager.

*ATPase assay*
ATPase reactions (10 $\mu$l) were performed in helicase reaction buffer (see above) and contained M13mp18 DNA cofactor (150 ng), [$^{32}$P]ATP (1 $\mu$Ci), and ATP (50 $\mu$M). Reactions were incubated for 30 min at 37°C and terminated by the addition of 5 $\mu$l of 0.5 M EDTA. Samples were separated on a polyethylenimine/cellulose thin layer chromatography plate (Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA) developed in 1 M formic acid/0.8 M LiCl. Radioactive products were visualized using a PhosphorImager.

*Chromatin assembly assay*
An *in vitro* chromatin assembly assay using a human cell-free system was utilized to analyse the activity of recombinant hp150 and the CAF-1 activity from various cells on DNA repair-coupled chromatin assembly (Gaillard *et al*., 1996). Briefly, UV (500 J/m$^2$) irradiated circular pBluescript plasmid DNA was incubated with cytosolic or S100 extract derived from HeLa cells (Martini *et al*., 1998) supplemented with nuclear extracts from either HeLa or WS cells or purified recombinant hp150 for 3 h at 37°C. The extent of supercoiling was examined by agarose gel electrophoresis followed by drying of the gel and exposure to X-ray film. Incorporation of ($\alpha$-$^{32}$P) dCTP indicated newly synthesized DNA after NER.

*RNA interference*
For short interfering RNA (siRNA) experiments, siRNA oligos were from Qiagen (Basel, Switzerland) and the targeting sequences of each were control GL3 (directed against firefly luciferase): CUUACGCUGAGUACUUCGAdTdT; 150-1, AGGGGAAAGCCGAUGACAUdTdT (Hoek and Stillman, 2003). The siRNAs were transfected in HeLa cells using a 200 nM siRNA concentration with Oligofectamine (Invitrogen) according to the manufacturer's instructions and harvested after 24 h.

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