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The Werner syndrome protein has separable recombination and survival functions[☆]

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Abstract

The Werner syndrome (WS) protein WRN is unique in possessing a 3' to 5' exonuclease activity in addition to the 3' to 5' helicase activity characteristic of other RecQ proteins. In order to determine *in vivo* functions of the WRN catalytic activities and their roles in Werner syndrome pathogenesis, we quantified cell survival and homologous recombination after DNA damage in cells expressing WRN missense-mutant proteins that lacked exonuclease and/or helicase activity. Both WRN biochemical activities were required to generate viable recombinant daughter cells. In contrast, either activity was sufficient to promote cell survival after DNA damage in the absence of recombination. These results indicate that WRN has recombination and survival functions that can be separated by missense mutations. Two implications are that Werner syndrome most likely results from the loss of both activities and their associated functions from patient cells, and that WRN missense mutations or polymorphisms could promote genetic instability and cancer in the general population by selectively interfering with recombination in somatic cells.

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Keywords: Werner syndrome; RecQ; Genetic instability; Homologous recombination

1. Introduction

Werner syndrome (WS) is a human autosomal recessive genetic instability syndrome. The intense interest in this uncommon disease has focused on the appearance of premature aging in affected individuals, together with an elevated risk of important age-associated diseases such as cancer, atherosclerotic cardiovascular disease, diabetes mellitus and osteoporosis [1,2]. Cancer and cardiovascular disease are the leading causes of premature morbidity and death in WS patients, in whom the mean age of death is ~47 years [3,4].

Werner syndrome patients have mutations in the chromosome 8p12 WRN locus that encodes a 1432 residue, 162 kDa member of the human RecQ helicase family [5]. RecQ helicase proteins are widely distributed in nature, and there is good evidence that RecQ helicases play important roles in genome stability maintenance in prokaryotes, in single cell eukaryotes and in mammals [6,7]. The role of human RecQ helicases in genome stability assurance and tumor suppression was highlighted by the identification of Werner syndrome, Bloom syndrome and Rothmund–Thomson syndrome: each of these genetic instability/tumor predisposition syndromes results from loss of function of a different human RecQ helicase protein [7,8].

Biochemical characterization of the human WRN RecQ helicase protein has revealed a unique 3' to 5' exonuclease activity in addition to the 3' to 5' helicase and ATPase activities characteristic of other RecQ helicase proteins [9,10]. Among the WRN mutations that have been identified in WS patients, none selectively inactivates the WRN exonuclease or helicase activities [11]. Thus, the functional importance of these biochemical activities is uncertain, and it is not

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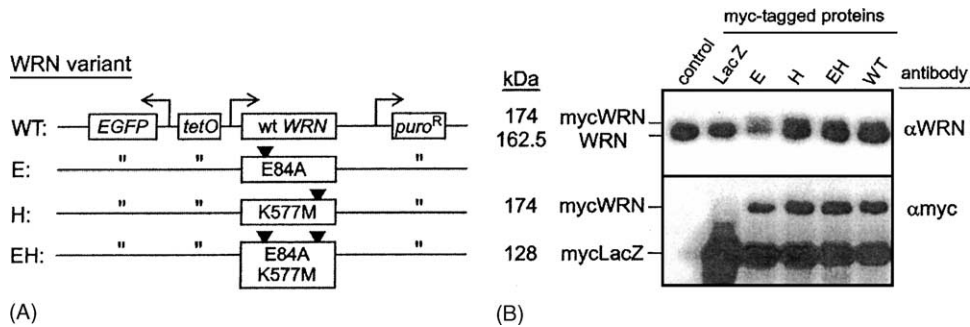


Fig. 1. Transgene expression and detection of genetically characterized WRN proteins. (A) Vectors encoding N-terminal myc epitope-tagged wildtype (WT), exonuclease-deficient (E), helicase-deficient (H) or double missense (EH) WRN. Altered WRN residues are numbered and changes are indicated in single letter amino acid code. Key: *EGFP*, enhanced green fluorescent protein gene; *tetO*, tetracycline operator sequence; *puro^R*, puromycin resistance gene. (B) Control GM639 fibroblast cells were transiently transfected with WRN plasmids in A together with a tTA transactivator plasmid [14]. Transgene-encoded WRN proteins (174 kDa) were detected with WRN (upper panel) or myc epitope (lower panel) antibodies. A myc epitope-tagged 128 kDa bacterial β -galactosidase encoded by pCS2 + c β lacZ plasmid [40] was used as a positive control for Westerns and for transfection efficiency. Protein extracts were prepared 24 h after transfection.

known whether both must be lost to promote WS disease pathogenesis.

We recently identified a role for the human WRN protein in cell survival and homologous recombination after DNA damage [12,13]. In order to determine whether one or both of the WRN catalytic activities were required for *in vivo* function, we quantified cell survival and recombination after DNA damage using cells that expressed wildtype WRN or missense-mutant forms of WRN that lacked exonuclease, helicase or both enzymatic activities (E84A, K577M, and E84A/K577M WRN, respectively; Fig. 1). We found that recombination required both of the WRN biochemical activities. Either activity alone, in contrast, was able to promote cell survival after DNA damage in the absence of recombination. Our results provide new information on WRN function *in vivo* and on the pathogenesis of Werner syndrome, and suggest new ways in which *WRN* mutation or variation may be promoting disease risk in the general population.

2. Materials and methods

2.1. Cell lines and cell culture

Werner syndrome and control SV40 fibroblast cell lines used for these analyses were from unrelated individuals and have been previously described [12,13]. Both WS cell lines contain *WRN* mutations and do not make detectable WRN protein. Cells were grown in Dulbecco-modified Eagle's Medium (DMEM, 4.5 gm/l glucose; Cellgro) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin G sulfate, and 100 μ g/ml streptomycin sulfate in a humidified 37 °C, 7% CO₂ incubator.

2.2. WRN expression vectors

Plasmids expressing divergently transcribed cDNAs encoding myc epitope-tagged WRN proteins or enhanced

green fluorescent protein were constructed from pBI (Clontech; Fig. 1). Transient transfections were performed using SuperFect (Qiagen) according to the manufacturer's protocol: cells ($1-5 \times 10^5$ /10 cm dish) were plated 48 h prior to co-transfection with 10 μ g of WRN expression vector \pm 4 μ g of a *tet*-transactivator (tTA) coding plasmid pUHD15-1 [14]. Stable transfections also were performed using SuperFect: $2-8 \times 10^5$ cells/6 cm dish were transfected with 5 μ g of expression vector, then dilute-plated 24 h after transfection in 10 cm dishes in order to recover puromycin-resistant (0.1–0.4 μ g/ml) colonies.

2.3. Western blot detection of WRN

Cell pellets (3×10^7 cells/ml) were resuspended in lysis buffer (25 mM Tris pH 8, 5 mM EDTA, 600 mM NaCl, 10% glycerol, 0.01 mM DTT, 0.1% NP-40), incubated on ice for 40–60 min, then centrifuged to remove cell debris. Proteins were separated by SDS-PAGE electrophoresis, and then electroblotted onto PVDF membrane (100 V, 90 min) in 25 mM Tris, 192 mM glycine buffer containing 20% (v/v) methanol. Non-specific antibody binding was blocked by incubation in TBS-T buffer (150 mM NaCl, 10 mM Tris pH 8, 0.05% Tween-20) containing 10% (w/v) non-fat dry milk (NFD). WRN was detected with a mouse monoclonal IgG₁ anti-WRN antibody (BD, Transduction Laboratories; 1:1000 dilution). Myc epitope-tagged proteins were detected with 9E10 anti-myc epitope tag-specific IgG₁ hybridoma supernatant [15] at a 1:50 dilution. Bound antibodies were detected with a goat anti-mouse IgG₁-HRP antibody conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc.; 1:2000 dilution) and chemiluminescence detection (ECL + plus, Amersham Biosciences).

2.4. Cell survival and recombination assays

Survival and recombination assays were performed as previously described [13]. Cells were transfected 48 h

prior to DNA damage in order to allow WRN protein expression; treated with *cis*-Pt; and then replated 24 h later in order to determine colony forming efficiency (CFE; 100–1000 cells/6 cm well) or recombinant colony formation (10^5 – 10^6 cells/10 cm dish). CFE plates were grown for 10 days, and recombinant colonies for 10–16 days in the presence of 400–600 μ g/ml G418 (Gibco/BRL), prior to staining and counting. Transient transfections were normalized for the percent transfected cells as revealed by use of a co-transfected bacterial β -galactosidase reporter plasmid. Recombination frequencies were corrected for the intrinsic difference in CFE between WRN-deficient and control cells, and for the selective sensitivity of WRN cells to *cis*-Pt cytotoxicity ([13,21] and additional unpublished results). The statistical significance of differences in survival or recombinant colony generation after DNA damage as a function of genotype and WRN protein expression was determined as previously described [13].

3. Results

In order to determine the requirement for WRN exonuclease and helicase activity *in vivo*, we expressed wildtype or missense-mutant WRN protein in WS or control cells, and then quantified cell survival and recombination after DNA damage. Four different WRN proteins were expressed: wildtype WRN possessing exonuclease and helicase activity, and WRN missense-mutants that lacked exonuclease or helicase activity (E84A or K577M WRN, respectively) or both activities (E84A/K577M WRN; Fig. 1A). The missense mutations used had been previously shown to inactivate the WRN helicase or exonuclease activity [16,17]. A myc epitope tag was added in-frame to the N-terminus of all of the WRN proteins to allow unambiguous detection of transgene-encoded proteins in the presence of native WRN (Fig. 1B). This epitope tag does not interfere with either biochemical activity of WRN or with *in vivo* functioning of WRN [13,18].

The cells used for experiments were SV40-transformed fibroblasts from WS patients or controls. We had previously demonstrated a mutator phenotype and recombination deficits in these cells, as well as selective drug sensitivities that parallel the phenotype of primary WS cells [13,18]. One potentially attractive alternate cell type for the assays described here are telomerase-immortalized WS fibroblasts [19]. Although these cells proliferate indefinitely, we chose not to use them as they no longer faithfully recapitulate the mutator phenotype or selective drug sensitivity profiles observed in primary WS fibroblasts, peripheral blood lymphocytes or B-lymphoblastoid cell lines ([20]; additional results not shown).

The DNA damaging agent chosen for our analyses was *cis*-Pt, one of several DNA cross-linking agents to which WS patient cells and cell lines are selectively hypersensitive [21]. In contrast to several other cross-linking agents, *cis*-Pt does not require metabolic activation, and has a well-defined

DNA damage and mutational spectrum profile (see, for example, [22]). The dose and exposure time used in the experiments reported here (2 μ M *cis*-Pt for 24 h) was chosen on the basis of an extensive series of dose–response and exposure time experiments that revealed consistent, highly significant reductions in cell survival as measured by colony forming efficiency and by colony size distribution (CSD) assays as well as markedly reduced recombination in the absence of WRN function. The survival difference after *cis*-Pt damage was marked: LD₁₀ values for WRN and control cells were 1.0 μ M versus 2.1 μ M, respectively, in experiments that examined dose-dependent killing over a three-log range ([13] additional data not shown).

The survival of control cells after *cis*-Pt damage, as measured by colony-forming efficiency, was unaffected by the transient expression of wildtype, single- or double-missense-mutant forms of WRN ($P = 0.43$ – 0.87 ; Fig. 2A, open bars). In contrast, WS cells had colony forming efficiencies that were significantly lower than control cells prior to and after DNA damage (Fig. 2A, compare C columns; $P = 1.4 \times 10^{-6}$). The expression of wildtype WRN or of either single missense-mutant WRN protein

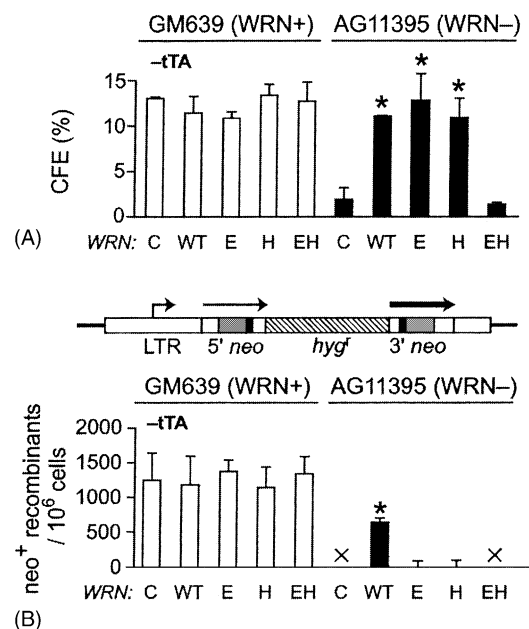


Fig. 2. WRN exonuclease or helicase activities can promote cell survival in the absence of recombination. (A) Survival of control and WS cells measured by colony forming efficiency after WRN expression and *cis*-Pt damage. The improved survival of WS cells expressing WT, E or H WRN was statistically significant (*, see text for P values). (B) Upper panel: structure of the pNeoA recombination reporter plasmid. Arrows indicate direct repeat neomycin phosphotransferase (*neo*) genes inactivated by linker insertions (filled boxes), with crosshatch indicating the region of homology between linker insertion sites. Key: *LTR*, retroviral long terminal repeat promoter; *hyg'*, hygromycin resistance cassette. Bottom panel: frequency of neo⁺ recombinant colonies/10⁶ viable cells after *cis*-Pt damage. Only WT WRN protein led to a significant (*) recovery of neo⁺ recombinants when expressed in WS cells. Error bars are standard deviations for a minimum of two replicate experiments.

in WS cells led to a significant increase in cell survival after DNA damage (Fig. 2A, right panel, filled bars; compare WT, E or H with C; $P = 3.2\text{--}7.2 \times 10^{-6}$). These survival levels were indistinguishable from comparably transfected control cells (Fig. 2A, open bars; $P = 0.30\text{--}0.98$). In contrast, the expression of double missense-mutant WRN did not detectably improve the survival of WS cells (Fig. 2A, right panel, compare C and EH; $P = 0.96$).

Recombination in transiently transfected WS and control cells was measured by determining the frequency of recombination-dependent *neo*⁺ allele generation from a chromosomally integrated recombination reporter plasmid (pNeoA; Fig. 2B, top panel; [13,23]). Control cells display a ~100-fold increase in the frequency of *neo*⁺/G418-resistant recombinant colonies per surviving cell after DNA damage as compared with WS cells (Fig. 2B, bottom panel left; [13] and additional results not shown). No difference in recombinant frequency was observed in control cells transfected with a related control plasmid, or expressing any of the four WRN proteins shown in Fig. 1 ($P = 0.73\text{--}0.88$). In WS cells a statistically significant increase in recombinant colony formation after *cis*-Pt damage was observed only in cells that expressed wildtype WRN protein (Fig. 2B, bottom panel right, compare C and WT; $P = 0.03$; see also Fig. 1 in Supplementary Material).

Cell survival and recombination after DNA damage were also analyzed in clonal derivatives of control or WS cells that stably expressed wildtype or missense-mutant WRN protein (Fig. 3). Control cells that stably expressed single- or double-missense-mutant forms of WRN had survival frequencies that were indistinguishable from cells transfected with a control plasmid or that expressed wildtype WRN (Fig. 3A, left panel open bars; additional results not shown). In contrast, WS cells that stably expressed either single missense-mutant form of WRN had significantly improved cell survival frequencies after *cis*-Pt damage (Fig. 3A, right panel filled bars E or H versus EH; $P = 9 \times 10^{-13}$) to levels that were indistinguishable from control cells (compare E or H CFEs of control cells with WRN-complemented WS cells; $P = 0.19$ and 0.53 , respectively). WS cells that stably expressed double missense-mutant WRN had the same survival as untransfected WS cells lacking WRN protein ($P = 0.78$). None of the stably expressed missense-mutant WRN proteins promoted the generation of *neo*⁺/G418-resistant recombinant colony formation after DNA damage (Fig. 3B; additional results not shown).

Western blot analyses indicated a range of WRN transgene expression levels in cells used for functional analyses (Figs. 4 and 5). In order to determine whether level of expression of WRN proteins also influenced cell phenotype after DNA damage, we quantified cell survival and recombination in control and WS cells after inducing WRN expression with *tet*-transactivator protein [14]. These experiments took advantage of the expression vector construction strategy that placed the WRN open reading frame un-

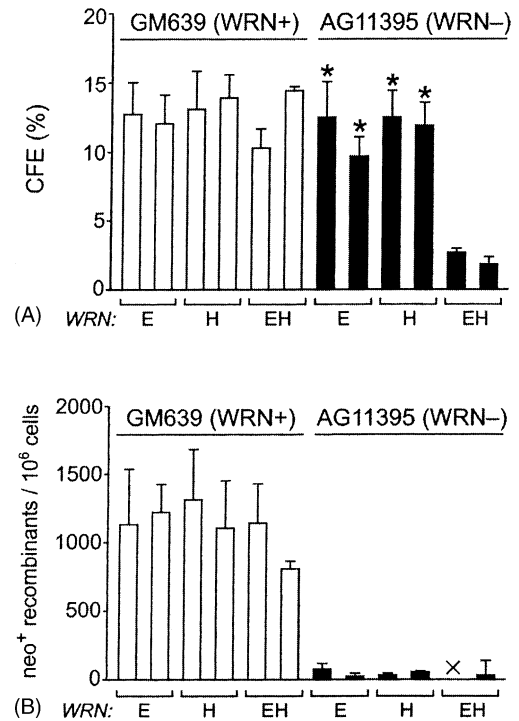


Fig. 3. Stable expression of WRN lacking exonuclease or helicase activity promotes cell survival but not recombination. (A) The colony forming efficiency of independent, WS cell clones expressing E and H WRN was significantly higher (*, see text for P values) than the survival of WS-deficient cells expressing EH (catalytically inactive) WRN. (B) Frequency of *neo*⁺ recombinant colonies/ 10^6 viable cells after *cis*-Pt damage. None of the WRN mutant proteins led to detectable recovery of *neo*⁺ recombinant colonies (see also Fig. 1 in Supplementary Material).

der the control of a CMV minimal promoter and adjacent to a *tet* repressor binding site (Fig. 1A). The rationale for these experiments was provided by previous work showing intermediate sensitivity of WRN heterozygous lymphoblastoid cell lines to several DNA damaging agents including *cis*-Pt [24,25] and the identification of genetic instability *in vivo* in the red blood cell lineage of WRN heterozygotes [26].

Although WRN transgene expression could be induced by tTA expression in both transient and stable transfection experiments to 50% of wildtype levels (Fig. 5A; additional results not shown), we observed no difference in cell survival or in recombination as a function of higher expression of any WRN protein (Fig. 5B; compare with Fig. 2A and B). These results indicate that WRN can promote cell survival even when expressed at low levels, whereas even high level expression of WRN lacking helicase or exonuclease activity cannot promote recombination in the absence of missing WRN catalytic activities. The apparent copy number independence of WRN function in our experiments may reflect the comparatively high level of WRN protein in fibroblast cell lines [27] and/or attenuated DNA damage checkpoint functioning in SV40-transformed fibroblast cell lines (see, e.g. [28]).

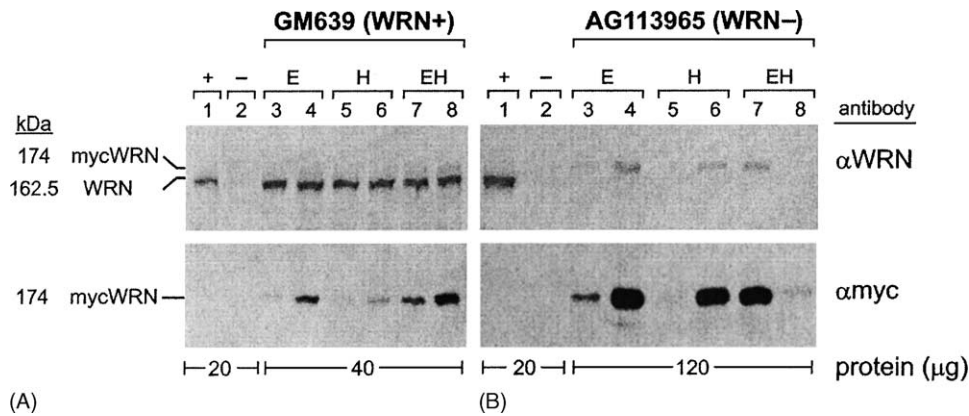


Fig. 4. Western blot analysis of WRN transgene expression in stable transfectants. WRN transgene expression in the pairs of independently derived sublines in the same order shown in Fig. 3 with WRN (upper panels) or myc (lower panels) antibody. Uninduced transgene expression levels ranged from ~2.5 to 25% of wildtype levels after correcting for signal intensity and the different amounts of protein loaded (40 μ g left panels or 120 μ g right panels). Extract controls consisted of 20 μ g of total protein from control (WRN+) and WS cell lines (left two lanes in all panels).

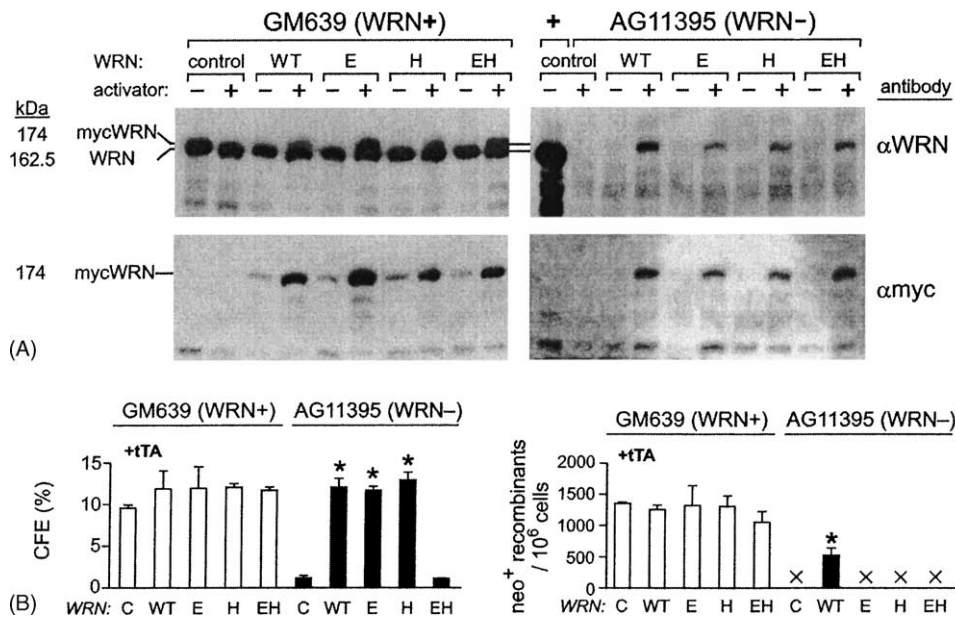


Fig. 5. Cell survival and recombination are not affected by WRN expression level. (A) Western blot analysis of WRN transgene expression in control and WS cells transiently transfected with WRN coding plasmids (Fig. 1A) and a tTA activator plasmid (activator; [14]). WRN expression, detected with WRN (upper panels) or myc epitope tag (lower panels) antibodies, increased in all induced clones. (B) Increased expression of WRN did not improve survival or recombination of WS cells after DNA damage (compare Figs. 2 and 5B). Error bars are standard deviations for a minimum of two replicate experiments. key: (*) statistically significant recovery (see text for *P* values).

4. Discussion

Our results indicate that human WRN protein has recombination and survival functions that differ in their requirement for WRN exonuclease and helicase activities. Recombination is the more stringent of the functions, and requires both activities; either activity, in contrast, supported cell survival after DNA damage. WRN is not unique in having recombination and survival functions that can be separated by mutations. Similar examples are known, among which the most potentially instructive involve members of the RecA/RAD51 strand transferase family (see, e.g. [29]; reviewed in [30,31]).

One important function for WRN in somatic cells appears to be in the resolution of intra-chromosomal recombination products. Single missense-mutant forms of WRN display the same reduced rate of generation of recombinant daughter cells as previously observed in cells lacking WRN protein (Figs. 2, 3 and 5; [12,13]). The likely substrate for WRN in these resolution events are D-loops or Holliday junction-containing intermediates that are generated during gene conversion or synthesis-dependent strand annealing [13,32,33].

Biochemical analyses of WRN action on defined oligonucleotide substrates indicate that the biochemical activities of WRN can compete to unwind or degrade different templates,

and that the outcome is determined by a combination of substrate conformation, the balance of WRN catalytic activities and the presence of additional proteins that have been shown to interact with WRN (reviewed in [9,10]). For example, the requirement for both WRN catalytic activities in recombination may reflect the ability of WRN and RPA to efficiently unwind strand invasion intermediates such as D-loops [34]. It should be possible to determine whether the WRN catalytic activities are required in the same protein complex to promote the resolution of recombination intermediates in vivo, and whether the over-expression of other RecQ helicase proteins or of alternative 3' to 5' exonuclease activities can promote recombination resolution in the absence of, respectively, the WRN helicase or exonuclease activities.

The ability of WRN lacking exonuclease or helicase activity to support cell survival after DNA damage in the absence of recombination was unexpected. One mechanistic inference from these results is that the missense-mutant proteins are likely to be acting on common substrates, as the expression of each single missense-mutant WRN protein gave comparable cell survival frequencies (Figs. 2, 3 and 5). These common substrates are likely to include recombination intermediates or products [12,13].

The biochemical properties of the WRN helicase and exonuclease suggest several models to explain how each activity in isolation could promote cell survival in the absence of recombination (Fig. 6). Isolated exonuclease or helicase activity could, respectively, degrade or unwind recombination intermediates or products that could not otherwise be correctly resolved. This would topologically disentangle the molecules and generate DNA ends that could reinvade or be

captured for DNA end joining (Fig. 6A). Alternatively, the WRN exonuclease or helicase activities could process or stabilize recombination or replication intermediates to promote replication restart, lesion bypass or DNA cleavage (Fig. 6B and C) [33,35]. Each pathway is plausible in light of known biochemical properties and actions of the WRN catalytic activities in vitro (reviewed in [9,10]). Additional biochemical requirements in each of the above survival pathways could be fulfilled by RecQ helicases, topoisomerases, or replication and recombination proteins that have been shown to interact with WRN [7,10]. It is important to emphasize that recombination-independent survival pathways are likely to be error-prone: thus cell survival in the absence of recombination would come at the cost of genome instability.

Our results indicate that both WRN catalytic activities must be lost to give rise to the WS cellular phenotype. The loss of both activities is thus likely required to promote WS pathogenesis. A requirement for the coordinate loss of both of the WRN catalytic activities to fully reveal the WS cellular phenotype provides a facile explanation for the spectrum of WRN mutations found in WS patients. This spectrum consists solely of mutations that lead to WRN loss [11]. A second, less obvious, conclusion is that WRN is unlikely to harbor additional activities that strongly influence cell survival or recombination. This is indicated by the virtually identical recombination and survival phenotypes of WS cells that lack detectable WRN protein, and of the same cells expressing catalytically inactive (EH-mutant) WRN protein (Figs. 2, 3, 5 and Fig. 1 in Supplementary Material). Two alternative explanations that could explain this apparent lack of 'scaffolding' activity are low or no expression of the

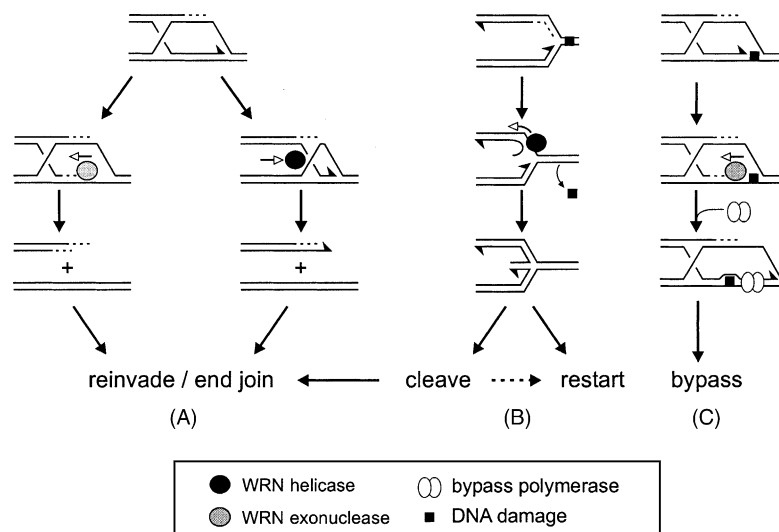


Fig. 6. Potential survival pathways utilizing WRN exonuclease or helicase activity in the absence of recombination. (A) Degradation or unwinding of recombination intermediates (a D-loop is shown) by WRN possessing only exonuclease (○) or helicase (●) activity could topologically disentangle molecules for reinvade or end-joining. (B) Degradation or unwinding of replication forks stalled by DNA damage (■) could remove bound proteins, stabilize the fork for restart after repair, or promote regression and cleavage to generate free DNA ends. Lagging strand unwinding is shown as an example. (C) Disassembly or unwinding of a replication complex stalled at template DNA damage during recombination. WRN exonuclease or helicase activity could promote assembly of a bypass complex (open ovals) containing one or more specialized DNA polymerases. All three pathways depicted here are likely to be error-prone (see text).

EH-mutant protein or mislocalization of the mutant protein. Both are unlikely in light of our Western data and the ability of each of the single missense-mutant proteins to efficiently promote cell survival after DNA damage.

The most intriguing implication of our work is that *WRN* missense mutations or polymorphisms that affect catalytic activity have the potential to promote human disease risk by interfering with recombination. This mechanism may be important in the general population as WS-associated *WRN* mutations are common (to <1/100 in, e.g. Japan; [2], and over 375 *WRN* polymorphisms and potential missense mutations of uncertain functional importance have already been identified ([11]; additional unpublished results).

Clinical expression of selective loss of the *WRN* recombination function would most likely be a genetic instability or cancer predisposition syndrome. One place to look for such selective *WRN* functional deficits in association with *WRN* missense mutations or polymorphisms is in otherwise normal individuals who have multiple primary neoplasms of the types observed in WS patients, e.g. osteosarcoma [36,37]. If identified, additional *WRN*-linked recombination deficiency syndromes would join WS and a growing number of human disease predispositions that result from defects in recombination pathways that act to insure genomic stability and suppress the risk of cancer [38,39].

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