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Werner Syndrome Protein—Unwinding Function to Explain Disease

Raymond J. Monnat Jr. and Yannick Saintigny

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Werner syndrome (WS) is one of three heritable human genetic instability/cancer predisposition syndromes that result from mutations in a member of the gene family encoding human RecQ helicases. Cellular defects are a prominent part of the WS phenotype. Here we review recent work to identify in vivo functions of the WS protein and discuss how loss of function leads to cellular defects. These new results provide clues to the origin of cell lineage-specific defects in WS patients and suggest a broader role for Werner protein function in determining disease risk in the general population.

Introduction

Werner syndrome (http://sageke.sciencemag.org/cgi/content/full/sageke;2001/1/ns2) (WS) is growing up. After an uncertain childhood that included almost a half century of neglect (*1*), research on WS has entered a vigorous adolescence. Much of the continuing interest in this autosomal recessive disease (caused by loss-of-function mutations in the *WRN* http://sageke.sciencemag.org/cgi/genedata/sagekeGdbGene;189 gene) has focused on the appearance of premature aging in association with an elevated risk of age-associated diseases such as cancer, atherosclerotic cardiovascular disease, diabetes mellitus, and osteoporosis in affected individuals (*2-4*). The presumption is that a deeper understanding of WS will provide useful new information about the pathogenesis of these clinically important, age-associated disease processes, as well as useful new insights into more general aspects of the biology of human aging (*5, 6*).

Our focus in this Review is on functions of the WRN protein (http://sageke.sciencemag.org/cgi/content/full/2002/13/re2), a DNA helicase, at the cellular level. Our aim is to indicate how loss of function promotes cell- and lineage-specific defects in vivo.

Genetic instability is an important consequence of the loss of WRN function and one of the first abnormal phenotypes to be identified in cells from WS patients (*7*). Genetic instability after the loss of WRN function is a plausible "intermediate" phenotype for experimental analyses, because it identifies an immediate consequence of the loss of WRN function at the cellular level. This phenotype also provides a conceptually useful way to think about the origins of the lineage-specific defects observed in affected individuals. Thus, we consider how genetic instability might arise in the absence of WRN function and the consequences of genetic instability in specific cell lineages during and after development. Also discussed are emerging data that indicate a larger role for WRN function in human health and disease than is suggested by the rarity of the WS clinical phenotype.

We emphasize in this discussion the role of WRN in homologous recombination (HR). Homologous recombination, as suggested by its name, involves the exchange of genetic information between homologous (identical) DNA sequences in the genome. In germ cells, these exchanges promote the reshuffling of genetic information between generations, whereas in somatic cells, HR promotes DNA repair and the successful completion of DNA replication. The recent identification of a role for the WRN protein in HR in human somatic cells was unexpected. A reconsideration of molecular, biochemical, and cytological data on WS in the context of HR begins to explain mechanistic links among recombination, cell viability, and mutagenesis in WS cells. The most surprising aspect of this story is the conclusion that WS disease pathogenesis might be driven by a recombination defect. This conclusion is the opposite of what has been widely assumed—that WS is a hyperrecombination syndrome—and thus of particular heuristic value. The assumption that WS is a hyperrecombination syndrome came initially from the identification of chromosomal rearrangements and extensive deletions in WS cells and cell lines (see below), and the assumption that these genomic rearrangements were the result of excessive recombination. As we discuss below, the deletion mutator phenotype appears to be a consequence of defective, not excessive, HR. A few of the most important aspects of the *WRN* gene, WRN protein, and WS phenotype are summarized below to begin this discussion. Readers are also referred to recent reviews in SAGE KE [see Fry Review (http://sageke.sciencemag.org/cgi/content/full/sageke;2002/13/re2) and Cheng Perspective (http://sageke.sciencemag.org/cgi/content/full/sageke;2003/31/pe22)] and in print that provide additional details and viewpoints on many of the topics discussed here (*4, 8-13*).

The *WRN* Gene and Protein

Positional cloning of the *WRN* (http://sageke.sciencemag.org/cgi/genedata/sagekeGdbGene;189) gene in 1996 provided a strong stimulus for research on in vivo function and disease pathogenesis. The *WRN* locus, situated at chromosomal position 8p12, encodes a 162-kD member of the human RecQ helicase family (*14*). The five members of this family all possess 3′-to-5′ helicase activity as well as adenosine triphosphatase activity (*13, 15*). WRN is unique among the human RecQ helicases in possessing an additional 3′-to-5′ exonuclease activity (*16-18*) (Fig. 1).

The biochemical similarity of human RecQ helicases to homologs identified in prokaryotes (http://sageke.sciencemag.org/cgi/content/full/2003/40/nw137) and single-celled eukaryotes suggested the potential for functional parallelism and helped to identify several model organisms in which to investigate RecQ helicase function [reviewed in (*12, 13*)]. Potential roles for the human RecQ helicases in genome stability assurance were emphasized by the identification of three human RecQ helicase deficiency syndromes: WS, Bloom syndrome (caused by muta-

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tions in the *BLM* http://sageke.sciencemag.org/cgi/genedata/sagekeGdbGene;8 gene), and a subset of Rothmund-Thomson syndrome. In each syndrome, genetic instability and a predisposition to neoplasia result from the loss of function of a different human RecQ helicase protein (*14, 19-21*) (see "A Jump-Start for Replication" http://sageke.sciencemag.org/cgi/content/abstract/2002/13/nw45).

Analyses of WRN activities on defined nucleic acid substrates indicate that WRN can unwind and/or degrade several types of DNA metabolic intermediates. These include three- and four-way DNA junctions and gapped, branched, or unpaired DNA regions [reviewed in (*10*, *22*)]. DNA molecules containing three- or four-stranded junctions arise as part of DNA replication, as a consequence of DNA repair, and during HR. The increasingly rich body of in vitro biochemical data on WRN has been complemented by protein interaction studies that indicate physical and/or functional cross-talk between WRN and general nucleic acid metabolic proteins such as replication protein A (http://sageke.sciencemag.org/cgi/content/full/sageke;2002/13/re2) and more specialized proteins involved in DNA synthesis, recombination, or repair (*10*, *22*). The *WRN* mutations identified in WS patients all truncate and promote loss of the WRN protein from patient cells (*23-26*). The absence of WS-associated missense mutations that selectively inactivate either the WRN exonuclease or helicase activity is noteworthy: This finding suggests that it is the loss of the helicase and exonuclease functions of WRN that leads to WS. We discuss this point further below in light of new experimental results.

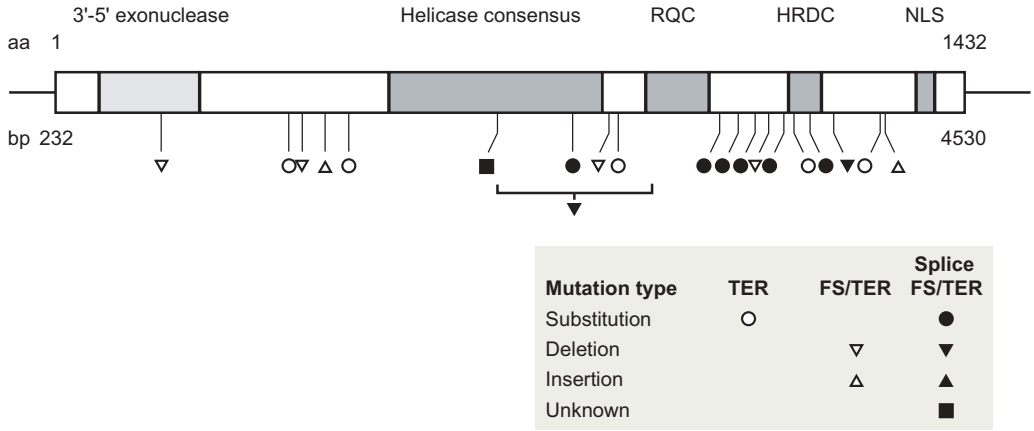

Fig. 1. Domain structure of WRN and spectrum of WS-associated *WRN* mutations. The central box indicates the WRN open reading frame with amino acid residue numbering indicated on top and cDNA base pair coordinates below. The positions of five protein motifs are indicated by shaded boxes and labels: (i) exonuclease domain, (ii) RecQ helicase domain, (iii) RecQ consensus (RQC) domain, and (iv) the helicase and RNaseD-C-terminal (HRDC) domain and the C-terminal nuclear localization signal (NLS). The positions and molecular types of mutations identified in WS patients are indicated by symbols below. All mutations thus far identified truncate the WRN open reading frame and cause loss of the nuclear localization signal. These data have been compiled and are available in the *WRN* Mutation and Polymorphism Database (www.pathology.washington.edu/research/werner/ws_wrn.html), developed and maintained at the University of Washington. TER, termination; FS, frameshift; splice FS/TER, mutations that interfere with splicing and lead to frameshifts with downstream stop codon(s) in the new reading frame.

The Importance of Cellular Phenotype

Three consistent cellular defects have been identified after the loss of WRN function. This triad includes (i) cell proliferation defects that have been best defined in the fibroblast cell lineage (*27*, *28*); (ii) selective sensitivity to a small number of DNA-damaging agents (*29-33*); and (iii) genetic instability (observed at both the cytogenetic and molecular genetic level) in different cell lineages in vivo and in vitro (*34-36*). These aspects of the WS cell phenotype are experimentally tractable, can be easily quantified, and appear to directly reflect WRN function. Thus, cellular phenotype has been an important focus in attempts to understand WRN function.

Analysis of WRN function at the cellular level has also been conceptually important as a way to integrate and interrelate the molecular, biochemical, cytogenetic, and cytological data on WRN function. Further, understanding WRN function at the cellular level is likely to be directly relevant to disease pathogenesis. Thus, an important goal and milestone for current WS research is to develop a sophisticated molecular-level model of WRN function at the level of single cells that is both quantitative and predictive.

WRN Function in HR

The past 5 years have seen a resurgence of interest in HR in human somatic cells (*37-40*). This renewed interest has been driven by several factors, including (i) data indicating the quantitative importance of HR in mammalian DNA break repair [see, for example, (*41*)]; (ii) the critical role played by HR in mammalian development (*42*, *43*) and cell viability (*44*); and (iii) data from bacteria and single-celled eukaryotes that indicate a critical role for HR proteins and HR function in DNA repair and the rescue of stalled replication forks (*45-47*). A final stimulus for renewed interest in mammalian HR comes from the fields of human genetics and genomics: An increasing number of human diseases are being identified that might represent defects in recombination [reviewed in (*48*, *49*)], and a better understanding of HR might provide more efficient ways to modify human genes for therapeutic gain. Moreover, many of the proteins mediating human HR have polymorphic variants with the potential to affect expression and/or function, and several variants have already been associated with disease risk, most notably for neoplasia (*48*, *50*).

Although there have been hints for some time that WRN might play a role in HR [see, for example, (*51*)], the nature of this involvement only became clear when the behavior of "direct repeat" recombination reporter (DR reporter) substrates was examined in WS and control cells (Fig. 2) (*52*). The use of

this type of chromosomally integrated substrate allows the frequency and rate of spontaneous and damage-induced recombination to be quantified and the molecular nature of recombinants to be analyzed to provide mechanistic insight into mammalian recombination pathways (*40*, *53*, *54*).

Two classes of genetically active recombinant molecules can be recovered after DR reporter plasmids undergo recombination: (i) conversion-type recombinant molecules, in which the DR reporter is intact and one of the two reporter alleles has been converted to an active form; and (ii) crossover or "popout" molecules, in which a single active allele remains with the loss of intervening DNA. Either or both recombinant classes can be recovered depending on the structure of the initial or substrate DR reporter plasmid. These two types of recombinant molecules can be generated by several different pathways (Fig. 2). Human and other mammalian cells show a clear preference for the generation of conversion-type events, the most conservative of the potential outcomes that generate active reporter alleles (*53*, *55-57*).

Analyses of spontaneous HR in WS cells using this type of DR reporter have revealed a 25-fold reduction in the rate of generation of viable recombinant daughter cells that retain growth potential as compared with normal cells. This reduction is observed despite an apparently normal rate of generation of recombinant molecules in WS cells. Related to this finding is a reduction in the proportion of viable recombinant cells that harbor conversion-type recombinant molecules (*52*). This WS-associated HR defect can be rescued by expression of catalytically active WRN protein or by the expression of the RusA bacterial resolvase protein, suggesting that WRN, like RusA, functions in the resolution of HR products. Resolution or postsynapsis is the stage of HR when successful recombinant molecules are disentangled and segregated to daughter cells. The reduced cell survival that accompanies the WS HR defect can also be suppressed by dominant negative RAD51 (http://sageke.sciencemag.org/cgi/genedata/sagekeGdbGene;121), the key protein involved in nucleoprotein filament formation, strand invasion, and the search for homology in the early stages of HR (*58*) (Fig. 3). Expression of this dominant negative form inhibits HR. The simplest explanation for these results is that HR initiation is normal in WS cells, but a portion of the products of RAD51-dependent HR cannot be successfully resolved in the absence of WRN function.

Recent data have allowed us to take this story one step further and to address the roles of the WRN exonuclease and helicase functions in HR and in cell survival after DNA damage. By expressing WRN proteins in which single amino acid substitutions were used to inactivate the WRN exonuclease and/or helicase functions, we showed that both catalytic activities were essential for WRN to function in HR resolution. To our surprise, single missense mutant forms of WRN that lacked exonuclease or helicase activity supported normal cell survival levels after DNA damage in the absence of HR. The WS cell phenotypes of an HR defect and reduced cell survival after DNA damage did not differ between WS cells that lacked detectable WRN or that expressed catalytically inactive WRN at physiological levels (*58*, *59*).

These results indicate that catalytic functions [as opposed to postulated scaffolding functions (http://sageke.sciencemag.org/cgi/content/full/2003/31/pe22)] of WRN are critical for HR, and that HR and cell survival can in some cases be separated. Our results thus differ from recently published work by Chen *et al.* proposing an important structural role for WRN in determining the outcome of break repair events (*60*). We think the most likely explanation for this discrepancy is Chen *et al.*'s use of fibroblasts immortalized by expression of the catalytic subunit of telomerase, hTERT (http://sageke.sciencemag.org/cgi/genedata/sagekeGdbGene;205). Although hTERT expression suppresses the growth defect of primary WS fibroblasts, as does the SV40 T antigen expressed in the cells used in our experiments (*61-64*), hTERT also appears to suppress other important aspects of the WS cellular phenotype, such as selective drug sensitivity and the WS HR defect. The expression of hTERT also appears to alter the outcome of some classes of repair event in WS cells, such as plasmid rejoining [see (*65*)], that is likely to depend on nonhomologous DNA end joining (http://sageke.sciencemag.org/cgi/content/full/2003/8/re3) (NHEJ), a repair pathway in which DNA ends are joined without regard for the presence of homologous DNA sequences.

An important conclusion from our experimental results is that both of the WRN catalytic activities need to be lost in order to generate the WS cellular phenotype. This conclusion pro-

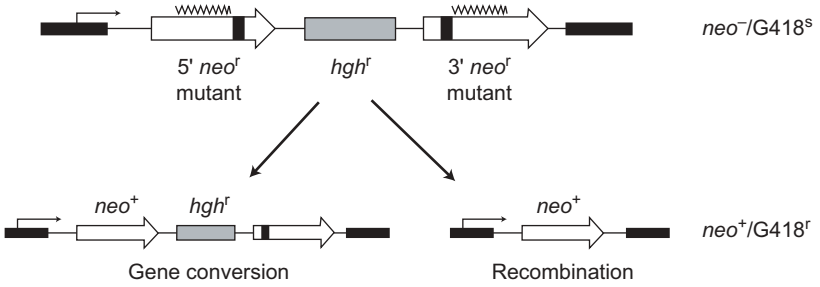

Fig. 2. DR reporter plasmid structure and recombinant classes. Structure of a DR reporter plasmid, pNeoA (*57*). Open arrows indicate direct repeat neomycin phosphotransferase (*neo*) genes inactivated by linker insertions (solid boxes), with overlying wavy lines indicating the region of homology between linker insertion sites. Two major classes of recombinant molecules represent the outcome of recombination pathways that by virtue of gene conversion (left) or crossing over (right) generate an active reporter allele (neo^+) that allows growth in the presence of the drug G418, a neomycin analog. The two outcomes are distinguished by the structure of the resulting recombinant molecule and by whether cells also retain or lose resistance to hygromycin (hyg^r, hygromycin resistance cassette; $G418^s$, G418-sensitive; $G418^r$, G418-resistant). Gene conversion (left), the nonreciprocal transfer of genetic information between two homologous DNA molecules, is thought to be initiated in most cases by DNA breakage. Breaks can be repaired by the classical two-ended double-strand break repair pathway or via single-ended invasion and synthesis-dependent strand annealing. Noncrossover conversion-type events predominate among mitotic recombinant products in mammalian cells. Crossover or popout-type recombinants (right) can arise as a result of intrachromosomal recombination, unequal sister chromatid exchange, or single-strand annealing. These recombination pathways and the genetic requirements for each are discussed in detail in (*40, 54, 116*).

vides a satisfying explanation for the spectrum of *WRN* mutations in WS patients that, as noted above, lack missense mutations that selectively inactivate the WRN exonuclease or helicase activities. The identification of a requirement for both WRN catalytic activities in HR also raises the interesting prospect that inherited or somatically acquired *WRN* missense mutations could selectively cripple the WRN HR function without appreciably affecting cell viability. Such mutations, if they are segregating in the human population, would likely confer a phenotype different from WS [see (*59*) for further discussion].

Resolution and Its Consequences

A role for WRN in the resolution of HR products was unexpected. Successful resolution is important if HR products are to be topologically disentangled and accurately segregated to generate viable recombinant daughter cells (*66*, *67*). In addition to topologically disentangling recombinant DNA duplexes, resolution determines the proportion of crossover and noncrossover recombinants. Crossover recombinants have the potential to promote gene or chromosomal rearrangement and the loss of genetic information. Thus, not surprisingly, the generation of different types of resolution products is under tight control, with mammalian HR strongly favoring the generation of noncrossover products (*40*).

How the resolution of recombinant molecules is managed at the molecular level is best understood in prokaryotes. Successful resolution appears to involve at least three discrete steps: (i) the recognition and binding of DNA molecules containing recombination junctions such as Holliday junctions or D loops; (ii) branch migration of the DNA junctions; and (iii) junction cleavage at or near crossover points, followed by ligation of the now-separated DNA molecules to form intact recombinant DNA duplexes (*46*, *66*-*68*). In *Escherichia coli*, a dedicated molecular machine known as the RuvABC complex binds, branch-migrates, and cleaves Holliday junction-containing products at crossover points to give rise to different classes of recombinant DNA molecules (*68*).

The comparable proteins that mediate resolution in eukaryotes have been intensively sought and are now just beginning to come into focus (*67*). Two human RecQ helicases, WRN and BLM, can bind and branch-migrate model recombination substrates such as Holliday junctions, one of the desired activities for a resolution complex (*69*, *70*). The exonuclease activity of WRN might also be useful for resolving certain types of recombinant products such as D loops (Fig. 4A). However, neither WRN nor BLM can cleave model recombination junctions in vitro, and neither appears to be present in mammalian cell fractions that possess the ability to branch-migrate and cleave model HR junction substrates (*71*, *72*).

Some types of eukaryotic recombination junctions might be cleaved and resolved by the heteromeric protein Mus81-Eme1/Mms4. This endonuclease, first identified in parallel analyses in budding and fission yeast, consists of two proteins that interact to form a structure-specific endonuclease [reviewed in (*67*, *73*)]. Human homologs exist for both proteins, and both proteins appear to be involved in recombination and the response to DNA damage. For example, Mus81 contributes to one of the recombination resolution activities identified by biochemical fractionation of mammalian cell extracts (*72*, *74*). Human Mus81 localizes to sites of DNA damage in the nuclei of HeLa cells and colocalizes in the nucleolus with WRN and BLM (*75*). Moreover, depletion of Mus81 mRNA by RNA interference leads to a reduction in the generation of recombinants (in the same recombination reporter cell lines that were originally used to identify the HR defect in WS cells) and a loss of cell viability (*52*, *76*).

A recent extensive analysis of murine Eme1 indicates that it is part of a structure-specific endonuclease with a preference for 3ρ flap substrates. This type of DNA structure, in which a nicked DNA duplex contains a single-stranded DNA tail that is displaced at the nick, can arise in the context of DNA replication or DNA repair. Murine embryonic stem (ES) cells that lack Eme1 are sensitive to DNA cross-linking agents such as cisplatin (cis-Pt) and mitomycin-C, and exhibit an increased incidence of damage-dependent sister chromatid exchange and elevated levels of both spontaneous and damage-induced chromosomal aberrations as compared with wild-type ES cells (*77*).

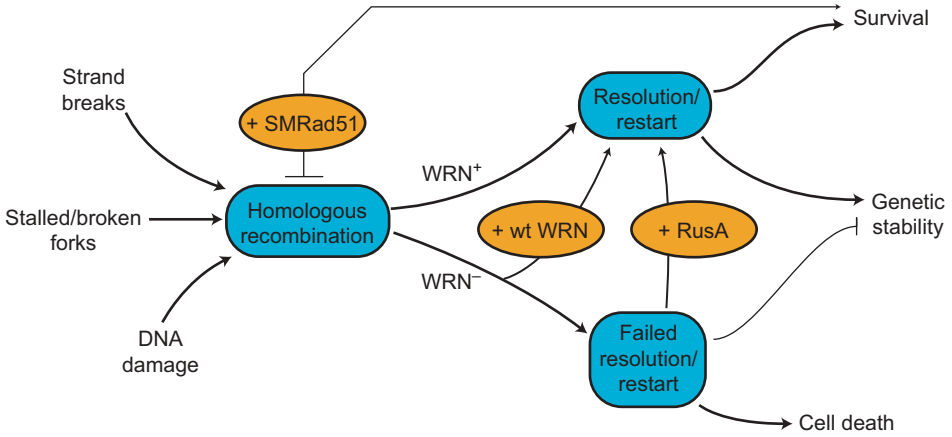

Fig. 3. Model of WRN function in HR. DNA damage, replication, or repair can initiate HR (left). WRN promotes HR resolution or replication restart to ensure cell viability and genetic stability (WRN^+ arrow). In the absence of WRN (WRN^-), HR resolution and/or replication restart fails, leading to mitotic arrest, cell death, and genetic instability. Experimental tests of this model are shown in ovals: Reexpressing WRN protein (+wt WRN) improves both cell survival and the recovery of viable mitotic recombinants, as does expression of the bacterial resolvase protein RusA (+RusA). The dependence of WRN phenotypes on RAD51 pathway function and products can be revealed by expressing a dominant negative form of mammalian RAD51 protein (+SMRAD51) that suppresses mitotic recombination in WRN and controls cells while improving WRN cell survival after cis-Pt-induced damage. Anticipated consequences of survival in the absence of HR function are mutagenesis and genetic instability (*58*).

These and other data indicate that the Mus81-Eme1/Mms4 endonuclease is a plausible candidate for one of the eukaryotic resolution endonuclease activities, despite uncertainty as to the likely in vivo substrates and the quantitative importance of this resolution pathway (*67*).

The recent identification of RAD51C, one of the mammalian RAD51 paralogs, as a candidate resolution protein is intriguing (*78*). The RAD51 paralogs were originally identified on the basis of sequence conservation and the ability to complement the x-ray sensitivity displayed by certain mutant hamster cell lines. These proteins clearly play a role in the maintenance of genome stability and might have functional roles in one or more repair pathways, although the mechanistic details remain obscure (*79*). RAD51C appears to be part of at least two different heteromeric paralog complexes. One of these complexes, RAD51C-XRCC3, is important for both HR resolution and the replication of damaged DNA (*78-81*). Precisely how RAD51C acts to promote resolution, despite apparently lacking branch migration or endonuclease activity (*78*), is puzzling and points to the participation of RAD51C-XRCC3 in one or more resolution complexes. The number, composition, and substrate preference of these resolution complexes may be rapidly discovered, now that several participating proteins, as well as mammalian cell fractions that exhibit resolution activity, have been identified.

Controlling Resolution to Suppress Gene Rearrangements

Recent evidence indicates that RecQ helicases might also play a role in determining the proportion of crossover products in mammalian HR and in mitotic and meiotic HR in yeast (*82*, *83*). Biochemical insight into one mechanism by which crossover suppression might occur in human cells was revealed in recent analyses of the resolution of recombination substrates by recombinant human BLM and DNA topoisomerase IIIα (*84*). This pair of proteins appears to work in concert to resolve substrates containing double Holliday junctions without the exchange of flanking markers. Junction "dissolution," as this type of resolution has been termed, thus differs from the more familiar RuvC- or Mus81-like cleavage reaction discussed above (*67*, *84*). These recent results parallel and extend earlier analyses of *E. coli* RecQ, in which similar catenation-decatenation activities were first described (*85*). One satisfying aspect of this result is that it explains one of the cytogenetic hallmarks of Bloom syndrome: the abnormally high levels of sister chromatid exchanges in cells and cell lines from BLM patients. Sister chromatid exchange crossover products arise at high frequency once crossover product formation is no longer effectively suppressed after the loss of BLM function (*40*, *84*, *86*).

The results summarized above indicate that the WRN and BLM human RecQ helicases might have complementary roles in suppressing gene rearrangement or loss in somatic cells. WRN appears to promote the successful resolution of HR products to favor the generation of viable conversion-type recombinants, whereas BLM acts to suppress the generation of crossover products. This model also explains why BLM and WRN, which are both envisioned to be acting in HR, have divergent loss-of-function HR phenotypes.

The Role of HR in DNA Replication

Many of the proteins that mediate HR in mammalian cells might have an additional role in insuring the completion of DNA replication (http://sageke.sciencemag.org/cgi/content/full/sageke;2003/8/re3). The stalling or disruption of DNA replication forks appears to be common in virtually all organisms and can be accentuated by many forms of DNA damage (*45*, *47*, *87*). The most desirable outcome of this apparently unavoidable event is to promote the successful restart of replication, while suppressing DNA breakage and genome rearrangement. DNA damage that involves both DNA strands (for example, DNA interstrand cross-links) are potent blocks to replication fork progression that may be efficiently and preferentially repaired by HR (*88*). Many other forms of DNA damage trigger HR, and thus might use HR for purposes of repair or to promote damage tolerance (by, for example, lesion bypass) (*40*, *47*).

One example of the latter role for HR proteins and HR func-

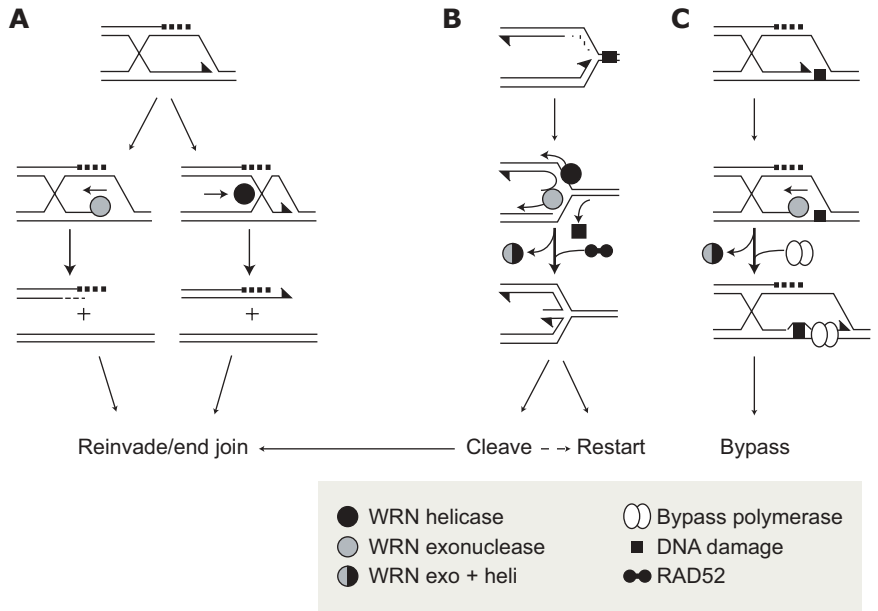

Fig. 4. Potential recombination resolution and replication restart pathways using WRN exonuclease or helicase activity. (**A**) Degradation or unwinding of recombination intermediates (a D loop is shown) by the WRN exonuclease or helicase activities could topologically disentangle molecules to promote resolution. (**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. WRN acting with RAD52 could promote a stable regressed fork that could be cleaved by Holliday junction resolvases to promote end invasion or used directly to reinitiate replication (*90*). (**C**) Disassembly or unwinding of a replication complex stalled at template DNA by WRN exonuclease (shown) or helicase activities 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 (*45, 47, 87*).

tion is in the processing or stabilization of stalled replication forks to either promote or inhibit the initiation of HR. Many of the postulated DNA intermediates in stalled replication forks resemble recombination intermediates, and thus might serve as substrates for recombination protein recognition and processing (*40*, *45*, *47*, *67*, *87*). An explicit role for the *E. coli* RecQ protein in stalled replication fork processing has been proposed (*89*), and similar roles can be envisioned for WRN acting either alone or in conjunction with other proteins such as mammalian RAD52 (http://sageke.sciencemag.org/cgi/genedata/sagekeGdbGene;122) to facilitate fork repair or stabilization (Fig. 4, B and C) (*90*). The WRN exonuclease activity alone, or WRN helicase in conjunction with other nuclease activities, could also act to promote the bypass of DNA damage (Fig. 4, B and C).

Roles for RecQs in the Cross-Talk Between HR and NHEJ

The identification of an HR defect in WS cells provides a ready explanation for the loss of conversion-type recombinants, reduced cell viability, and selective sensitivity to DNA damaging agents that might require HR for repair (*58*). How, given this picture, do we make sense of reports that indicate biochemical and functional interactions between WRN and proteins that function in the NHEJ pathway, such as Ku and DNA-PK_{cs}? [See "Twisted Logic: Discoveries tangle Werner syndrome helicase story" (http://sageke.sciencemag.org/cgi/content/abstract/2002/12/nw40) and "Break Dancing: Werner syndrome protein might keep rowdy enzymes from doing a number on tattered DNA ends" (http://sageke.sciencemag.org/cgi/content/abstract/2002/3/nw8.)] The interrelation of WRN function, HR, and NHEJ is perhaps easiest to understand in the context of a model of substrate trafficking or flux during DNA replication (S phase) (Fig. 5).

In WRN^+ cells, DNA damage or S-phase intermediates that initiate HR or that require HR function are successfully converted to appropriate products. The successful resolution of these products insures high cell viability and minimizes genetic instability or gene rearrangements. In WS cells, in contrast, a portion of HR resolution events fail, giving rise to cells that undergo mitotic arrest and/or harbor DNA breaks or gaps. Such DNA breaks and gaps are substrates for recombination (*40*, *91*). However, in the absence of WRN function, many of these "second tries" are again likely to fail, setting up a futile cycle that leads to mitotic death or apoptosis. DNA breaks that arise directly or indirectly from resolution failure can, in contrast, be captured and joined by NHEJ to restore chromosome integrity and insure high cell viability, albeit at the expense of mutation (*92*).

In this model, NHEJ function is downstream of WRN function in HR. This provides an explanation for the WRN cell phenotype, which most closely resembles an HR (as opposed to NHEJ) defect (*52*, *58*, *93*, *94*). Close coordination of NHEJ and HR function in normal cells makes good teleologic sense by providing redundant ways to recognize, process, and resolve potentially dangerous DNA ends to insure chromosomal integrity and cell viability. The suggestion that WRN might be playing somewhat different roles in HR and in NHEJ, and that the balance might be altered in WRN-deficient or NHEJ-deficient cells, is also consistent with data indicating substantial cross-talk between HR and NHEJ (*95-101*).

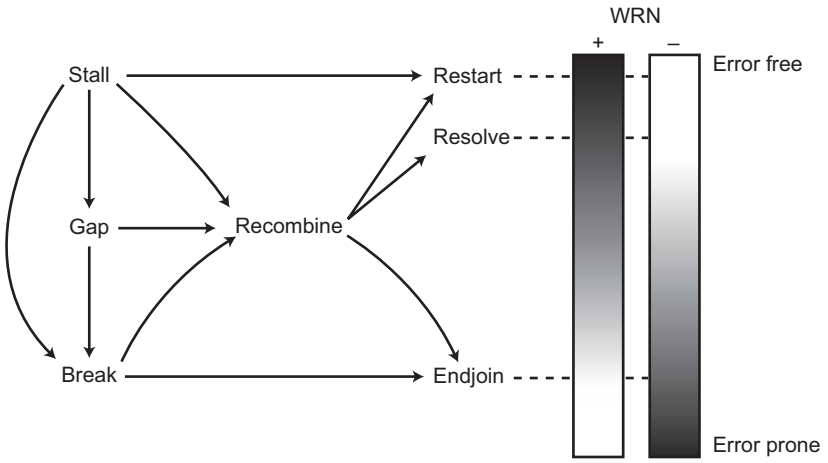

Fig. 5. S-phase substrate trafficking and outcomes. As depicted in Fig. 3, several types of DNA damage, including replication fork stalling, DNA gaps that result from fork stall, and DNA breaks, can initiate HR. HR proteins can funnel these substrates into classical HR and correctly resolve the products to insure high cell viability and chromosomal integrity (center; Fig. 4, A and C). HR proteins together with additional proteins such as DNA polymerases and resolvases may be able to act in concert to promote the restart of replication forks stalled by DNA damage either directly in the absence of HR (top) or via HR (center; see also Fig. 4, B and C). Replication stall and gapped and broken DNA molecules, if not successfully resolved by replication restart or HR, can be captured by NHEJ to insure chromosomal integrity, albeit at the expense of gene rearrangement, loss, or mutagenesis (*40, 47*). The shaded boxes to the right indicate the disposition of substrate in the presence or absence of WRN function.

Some quantitative rigor can be brought to this model by examining HR, cell viability, and mutagenesis data as a function of WRN status (Fig. 6). These data were all derived from the same set of well-characterized human WS and control SV40-transformed fibroblast cell lines (*52*, *58*, *102*). Consequences of the loss of WRN function in fibroblast-lineage cells include (i) a substantial reduction in the probability of continued cell division, as measured by colony forming efficiency (CFE) or more sensitive colony size distribution (CSD) assays; (ii) a decrease in the rate of generation of recombinant daughter cells, together with the apparent loss of conversion-type recombinants; and (iii) a 10- to 100-fold increase in mutagenesis at the X-linked *HPRT* locus (a convenient marker for examining mutagenesis, because loss-of-function mutations at this locus confer resistance to an otherwise toxic compound) (*52*, *58*, *102*). Molecular characterization of the resulting *HPRT* mutations arising in WS cells indicates that they are predominantly large deletions involving the loss of from 1 to >100 kb of chromosomal DNA (*102*, *103*). Most likely, these deletions originate from disrupted DNA replication intermediates or HR repair products that have been captured by NHEJ with the associated loss of intervening DNA (*103*).

One of the appealing aspects of the picture of WRN function

summarized in Fig. 3, Fig. 4, and Fig. 5 is the ability to reconcile, and in part explain, many of the molecular, biochemical, cytological, and cytogenetic features of WS and of WRN function. This functional "snapshot" also begins to reveal the complexity of WRN functional roles in vivo and of functional cross-talk among the nucleic acid metabolic processes that can be influenced by WRN. The consequences of the loss of WRN function in fibroblast-lineage cells are likely to be reflected in other cell types and lineages in vivo, although this assumption needs to be documented. It will be important to replicate the quantitative and molecular data summarized in Fig. 6, using primary cells to assess how the consequences of WRN loss of function are further modified by DNA damage checkpoints and apoptosis, which can be compromised or lost in immortalized cell lines.

WRN Function in Cell Lineages

WRN and the other human RecQ helicases appear to be ubiquitously expressed, whereas HR function appears to be largely limited to dividing cells. These observations lead to an inevitable question: If the functional picture represented in Fig. 3, Fig. 4, and Fig. 5 is accurate, why are dividing cell lineages not selectively affected by the loss of WRN function? We think the most likely explanation is the following (*104*). All cell lineages (or at least the precursors to all cell lineages) undergo mitotic division during development. An absence of WRN function during development thus has the potential to affect all cell lineages by reducing the number of cells available for lineage construction and by "seeding" cell lineages with mutant progeny. How cell loss, genetic instability, and mutation accumulation affect a given cell lineage is likely to depend on (i) how mitotically "deep" the mature cell lineage is; (ii) how much cell editing (or loss of lineage precursor cells) occurs during and after development; (iii) whether there is redundancy of function that is lineage-specific; and (iv) how large the functional reserve is for a given lineage, tissue, or organ.

For example, the central nervous system (CNS) might be protected from the effects of loss of WRN function by virtue of stringent cell editing during development and by a comparative absence of mitotic activity during adult life. Cell editing of neuronal precursors can occur as part of normal CNS development, in which a large number of precursors are generated during the initial stages of neurogenesis, and cells in excess of the number needed to complete development undergo programmed cell death. Cell editing also appears to reflect the selective loss of precursors that lack required functional properties or are damaged. However, subtle defects might emerge over time if WRN is important for neural stem cell function during adult life (*105*). Conversely, cell lineages that divide continuously throughout adult life, such as those that make up the skin, gut, and bone marrow, might be tolerant of the loss of WRN function by virtue of mutation expansion-limiting lineage architecture (*106*), a combination of stringent cell editing and large reserves of stem or lineage repopulating cells. The cell lineages or tissues most susceptible to a loss of WRN function might be those that retain cell division potential, lack stringent cell editing during and after development, and are tolerant of and thus able to accumulate at least some types of genetic instability. The fibroblast lineage and other mesenchymal or mesodermally derived cell lineages might be selectively affected by the loss of WRN function, as suggested by the clinical and cell phenotype of WS, for precisely these reasons.

This line of reasoning leads to two important conclusions. First, we need to know more about the "normal biology" of human cell lineages before we will be able to understand and predict the in vivo consequences of loss of function of WRN or of related proteins. Second, we clearly need new and experimentally tractable cell culture and animal models in which to

	WRN +	***WRN –***
Homologous recombination		
Initiation rate	10^{-4}	10^{-4}
Viable recombinant generation rate	10^{-4}	25-fold lower
Recombinant products		
Conversion type	70%	25%
Crossover type	30%	75%
Cell viability		
Colony-forming efficiency (CFE)		
≥6 cell colonies	39%	24%
≥50 cell colonies	21%	2.4%
Colony size distribution (CSD)	16 cells	4.6 cells
50th percentile		
Mutagenesis		
Forward mutation rate	~10^{-8}	100-fold higher
Mutation molecular type		
Deletion	39%	75%
Non-deletion	61%	25%

Fig. 6. Quantitative aspects of the WS fibroblast phenotype. This integrated data set was developed from the same SV40-transformed fibroblast cell lines. With a loss of WRN function, the recombination initiation rate (recombinant molecules/cell doubling) is unaffected. However, the rate of successful HR resolution as measured by the rate of generation of viable recombinant daughter cells/cell doubling falls 25-fold, together with an apparent loss of conversion-type recombinants (*52*). The HR defect is mirrored by a decline in cell viability in the absence of DNA damage as measured by CFE and CSD assays that measure, respectively, the generation of colonies of 6 or more or 50 or more cells (CFE), or the 50th percentile for number of cells in colonies after a defined growth interval (CSD) (*58*). In conjunction with the loss of viable recombinant daughter cells and reduced cell division potential, the rate of forward spontaneous mutation at the X-linked *HPRT* gene climbs 10- to 100-fold per cell per generation. The predominant type of mutation identified in HPRT-deficient WS cell lines was the large deletion [(*102, 103*); see text for additional discussion]. The mitotic recombination rate is at least two orders of magnitude higher that the rate of forward *HPRT* mutations in *WRN*$^+$ and *WRN*$^-$ cells, and the recombination defect in *WRN*$^-$ cells is accompanied by a large increase in *HPRT* mutagenesis. These results indicate that the recombination initiation rate is comparable in WS and control cells and that in *WRN*$^+$ cells, recombination events rarely give rise to mutations. The recombination and viability deficits obersved in WS cells are further accentuated by DNA damage (*58*, *59*).

study normal lineage biology and to identify lineage-specific aspects of WRN function.

WRN As a Modulator of Disease Risk in Populations

The *WRN* locus resembles many other human genes in that a large number of genetic variants are present in the population [see compilation and links in (*24*) and at the Environmental Genome Project (EGP) Web site (http://egp.gs.washington.edu)]. A subset of these are clearly disease-associated mutant alleles segregating in the human population, whereas the majority are single-nucleotide polymorphisms (SNPs) or sequence variants of uncertain functional importance. The number of these variants is surprisingly large: Recently completed resequencing of the *WRN* exons, promoter region, and downstream untranslated region at the University of Washington as part of the EGP revealed 375 *WRN* sequence variants in 90 different DNA samples contained in the EGP's Polymorphism Discovery Resource (http://locus.umdnj.edu/nigms/products/pdr.html). These variants included a large number of SNPs in addition to nonsynonymous coding region substitutions and a previously reported nonsense mutant allele.

The potential of *WRN* sequence variants to modify human disease risk outside the context of WS is most clearly understood for *WRN* heterozygotes. Heterozygous carriers of single mutant *WRN* alleles appear to be present worldwide at frequencies ranging up to 1:100 [reviewed in (*4*)]. Frequency estimates of heterozygotes in the United States are in the range of ~1:250, leading to estimates of $>10^6$ carriers of mutant alleles in the United States alone. *WRN* heterozygotes exhibit genetic instability in vivo (*107*), and lymphoblastoid cell lines derived from otherwise healthy *WRN* heterozygotes display an intermediate sensitivity to killing by DNA-damaging agents that selectively kill WRN-deficient cells (*31*, *32*).

Heterozygote effects have the potential to influence cancer risk or the outcome of cancer therapy. Several strategies might be helpful in establishing these links (*108*). For example, analysis of DNA derived from cells within clonal tumors might reveal inherited or somatically acquired *WRN* mutations that played a role in either the origin or progression of cancer. It will be interesting to see if sporadic tumors of the types observed in WS patients (*109*, *110*) are enriched for mutations or epigenetic events such as hypermethylation (http://sageke.sciencemag.org/cgi/content/full/2001/1/oa3), with associated silencing of *WRN* expression. The identification of an aberrant or exaggerated response to chemotherapy in tumors that are heterozygous or deficient in WRN function would provide a second route to identification of a role for the WRN pathway in tumor biology. Of note, several clinically important chemotherapeutic agents such as camptothecin, mitomycin-C, and cis-Pt selectively kill cells that are WRN-deficient or haploinsufficient (*31-33*, *111*). The functional consequences of the much larger number of polymorphic *WRN* variants and potential disease associations outside neoplasia are also just beginning to be explored (*24*, *112-115*).

Concluding Remarks

WS is an example of the growing number of heritable human diseases in which a defect in genome stability assurance or genomic "housekeeping" leads to an elevated risk of one or more secondary disease phenotypes such as cancer. WS is further distinguished among the heritable housekeeping defects as one of the increasing number of human cancer predispositions that appear to result from a defect in HR (*49*). In returning to the metaphor with which we began, all parents know that change and surprise are key features of adolescence. Our "adolescent" WS research promises an immediate future that will be challenging, surprising, and rewarding!

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