Published in final edited form as:

DNA Repair (Amst). 2010 March 2; 9(3): 331–344. doi:10.1016/j.dnarep.2009.12.011.

Roles of Werner Syndrome Protein in Protection of Genome Integrity

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Overview

Werner syndrome protein (WRN) is one of a family of five human RecQ helicases implicated in the maintenance of genome stability. The conserved RecQ family also includes RecQ1, Bloom syndrome protein (BLM), RecQ4, and RecQ5 in humans (see additional reviews in this issue), as well as Sgs1 in *Saccharomyces cerevisiae*, Rqh1 in *Schizosaccharomyces pombe*, and homologs in *Caenorhabditis elegans*, *Xenopus laevis*, and *Drosophila melanogaster* [1]. Defects in three of the RecQ helicases, RecQ4, BLM, and WRN, cause human pathologies linked with cancer predisposition and premature aging [1–5]. Mutations in the *WRN* gene are the causative factor of Werner syndrome (WS). WRN is one of the best characterized of the RecQ helicases and is known to have roles in DNA replication and repair, transcription, and telomere maintenance [1–6]. Studies both *in vitro* and *in vivo* indicate that the roles of WRN in a variety of DNA processes are mediated by post-translational modifications, as well as several important protein-protein interactions [1,2,7]. Many of these functions of WRN in genome maintenance, as well as the clinical characteristics of WS, have been recently reviewed [8–12]. In this work, we will summarize some of the early studies on the cellular roles of WRN and highlight the recent findings that shed some light on the link between the protein and its cellular functions with the disease pathology.

Molecular Genetics of Werner Syndrome (WS)

WS is a rare autosomal recessive progeroid disorder characterized by the development of cataracts, changing of skin conditions, bird-like facies, atypical short stature, and premature graying or thinning of the hair [9]. Patients also often develop hypogonadism, osteoporosis, diabetes mellitus, arteriosclerosis [13], and cancers, particularly sarcomas [14]. Onset of symptoms usually occurs in the third decade of life, and health subsequently declines with median age at death between 47–54 years [13]. Because WS presents with early-onset of conditions commonly seen in the aged, it is a good model system for the study of mechanisms of normal aging [15,16].

WRN Biochemistry

The causative factor of the majority of WS is mutation in the *WRN* gene, which codes for a member of the highly conserved RecQ family of helicases. While several different mutations within the gene are seen in WS, most result in production of truncated WRN protein [13].

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Human WRN possesses 3'–5'ATP-dependent helicase, 3'–5' exonuclease, and single-stranded DNA annealing activities [17–23]. Early gel filtration chromatography studies indicated that purified full length WRN exists as a trimer [24], and a fragment containing only the exonuclease domain equilibrates between a trimeric and hexameric form [25]. On the other hand, current electron microscopy data indicates that WRN is likely found as a dimer in solution, yet behaves as a tetramer in complex with DNA [26]. Still, while unwinding DNA, WRN acts as a monomer [27]. Together these results suggest that WRN's oligomeric state may be dependent on whether or not it is catalytically active and how it is interacting with DNA.

Orthologs of human WRN have been identified recently and characterized in other eukaryotes, including *Caenorhabditis elegans* (CeWRN-1) [28,29], *Drosophila melanogaster* (DmWRN) [30–32], and *Xenopus laevis* (focus forming activity 1 (FFA-1)) [33]. *Xenopus* FFA-1 is active as a helicase, while the exonuclease has not yet been investigated [33]. Similarly, the CeWRN-1 is active as a 3'–5'ATP-dependent helicase, though it lacks the 3'–5' exonuclease [28]. In contrast, in *Drosophila* DmWRN is active as a 3'–5' exonuclease [30] but the WRN helicase homolog has yet to be identified [31,32]. Nevertheless, cellular studies indicate that depletion or mutation of WRN increases sensitivity to DNA damage and results in increased genome instability [29–32]. While the enzymatic activities of the WRN orthologs in *Xenopus*, *C. elegans*, and *Drosophila* may not be entirely conserved with the human WRN, each is clearly important for genome maintenance.

WRN is active in resolving a variety of DNA substrates, including forks, flaps, displacement loops (D-loops), bubbles, Holliday junctions, and G-quadruplexes (G4), all of which represent intermediates in DNA replication and repair processes [34,35]. The roles of WRN in these pathways will be described later in detail. Importantly, the enzymatic activity of WRN on DNA substrates can be modulated by post-translational modifications [7]. The helicase and exonuclease activities of WRN are inhibited by oxidation [36]. WRN catalytic activity is also inhibited by both DNA-PK serine/threonine phosphorylation [37,38] and c-Abl tyrosine phosphorylation [39]. Conversely, p300 acetylation stimulates WRN helicase and exonuclease [40]. Additionally, WRN is subject to sumoylation, although the affect of this on its activities has yet to be determined [41]. Presumably, post-translational modification of WRN regulates its roles in multiple DNA transactions.

Epigenetic Regulation and Cancer

Recent studies have also shown an epigenetic component of WRN regulation [42,43]. Epigenetic regulation, specifically hypermethylation of tumor suppressor genes, is tightly linked with cancer [44]. In primary tumors, hypermethylation of the *WRN* promoter is common and correlates with lower levels of WRN protein expression. This hypermethylation inactivates the gene and results in increased chromosomal instability [42]. Inactivation of WRN in cancer cells increases susceptibility to the cytotoxic effects of topoisomerase inhibitors commonly used as chemotherapeutic agents [42,45]. Moreover, knockdown of WRN protein expression induces cell death and growth arrest in several human cancer cell lines, consistent with a tumor-suppressor like role of WRN. Conversely, following WRN depletion, cells that persist in growth have detectable levels of WRN protein indicating that WRN may also promote tumor proliferation [43]. It is likely that the roles of WRN in preserving genome stability and facilitating DNA repair must be balanced to allow for normal cell growth while protecting against cancer formation [43]. Together, these results suggest that WRN is a potential target for anti-tumor therapies [42,43,45].

In addition to the general association of WRN protein expression with cancer, recent analyses of *WRN* single nucleotide polymorphisms (SNPs) show a connection with cancer susceptibility [46,47]. An increased risk of breast cancer is seen among Chinese patients with the *WRN* Leu1074Phe SNP [46] and among German patients with the *WRN* Cys1367Arg SNP [47]. Yet,

Cys1367Arg and Leu1074Phe SNPs do not notably affect the helicase or exonuclease activity of WRN [48,49]. Yet, it is possible that these polymorphisms interrupt the C-terminal WRN protein interaction with mediators of damage signaling and DNA repair, including p53 and BRCA1 [50,51], thus contributing to cancer susceptibility [47]. On the other hand, WRN Cys1367Arg SNP in a Taiwanese population is not associated with prevalence of breast cancer [52]. Also, analysis of Brazilian glioma patients did not detect any links between WRN Cys1367Arg SNP and development of gliomas [53]. Taken together these results suggest that correlation of WRN SNPs with cancer susceptibility is dependent on racial background and also on type of cancer.

Nevertheless, WRN SNPs are correlated with other pathologies characteristic of the WS phenotype. The WRN Cys1367Arg SNP may confer protection for diabetes mellitus [54], sarcomas [55], and non-Hodgkin lymphoma [56]. Moreover, both WRN Cys1367Arg and Leu1074Phe are associated with protection against cardiovascular disease [57,58]. Healthy individuals with WRN 1367Cys and 1074Phe display increased levels of plasminogen activator inhibitor type 1 (PAI-1), an inhibitor of fibrinolysis [59]. Additionally, WRN regulates expression of PAI-1 [59]. Consistent with this, PAI-1 levels are elevated in WS cells [60], which may correlate with occurrence of atherosclerosis in WS. More work is needed in this area to detect associations between WRN SNPs and aspects of the aging phenotype.

Atypical Werner Syndrome

While Werner syndrome is most often associated with mutations of the WRN gene, a select number of cases have been identified in which patients are normal for WRN but instead have mutations in the lamin A/C gene (*LMNA*). This condition is referred to as atypical WS [61]. Patients with atypical WS display some of the common WS clinical features, including bird-like facies, osteoporosis, diabetes mellitus, and atherosclerosis [61–64]. Initial analysis of the genetic causes of atypical WS, revealed a set of heterozygous missense mutations within conserved residues of the *LMNA* gene [61,63]. Recent studies of patients with atypical WS detect two novel mutations in *LMNA*: a previously unidentified missense mutation [64] and a deletion mutation [62]. While it is clear that *LMNA* mutations are linked with atypical WS, the mechanisms for the biological manifestation of the *LMNA* gene mutations has yet to be elucidated. Moreover, the molecular basis for the pathologies associated with WS also requires further study. Consequently, investigation of the functions of WRN is important for understanding both the progression of WS, as well as the progression of the normal aging processes.

Participation of WRN in DNA Replication and Cell Cycle Progression

To understand the link between the pathology of WS and the cellular roles of WRN, studies have been focused on investigating the biological consequences of WRN depletion on cell survival and cell cycle progression. Collectively, these cellular analyses reveal a role of WRN in DNA replication [6,12]. Early examination of cells derived from WS patients showed a delay in S-phase progression compared to normal cells [65–69]. The delay has been attributed to either decreased rates of DNA extension [70] and replication fork propagation [68] or to disruptions in replication initiation or origin firing [66,71–73]. More recent studies also demonstrate that replication fork elongation is slowed in WRN depleted fibroblasts treated with the replication inhibitors methylmethane sulfonate (MMS) and hydroxyurea (HU). Moreover, in the WRN depleted cells there is a more pronounced cell cycle delay in S-phase and G₂-phase following exposure to MMS and HU [74].

Recovery from Replication Fork Stalling

Several groups have shown that WRN cells are sensitive to treatment with replication inhibitors and DNA damaging agents that cause replication fork stalling [65,67,75–77]. The focus of many current investigations has largely been on the response of WRN deficient cells to these replication disruptions, and the role of WRN in recovery from replication-dependent DNA damage (Figure 1). During replication, topoisomerases relieve supercoiling in the DNA that occurs as a result of strand separation [78,79]. Incomplete topoisomerase release from DNA, such as occurs upon treatment with topoisomerase inhibitors, leads to formation of covalent topoisomerase-DNA complexes that can pose a barrier to replication and can result in the formation of strand breaks [79,80]. When exposed to topoisomerase I inhibitor topotecan (TPT), cells with a knockdown of WRN have a greater arrest in S-phase and inhibition of replication compared to control cells. This effect is specific for topoisomerase I inhibitors since the effects are not seen when cells depleted of WRN are treated with the topoisomerase II inhibitor etoposide (ETO). In WRN knockdown versus wild-type cells, there is an increased propensity for conversion of TPT-induced single strand breaks (SSBs) into double strand breaks (DSBs), suggesting that WRN prevents SSBs at replication forks from being converted into DSBs [81]. In addition, DSBs accumulate in WRN deficient cells in response to HU treatment, which induces replication fork stalling. These DSBs form as a result of collapsed replication forks, as indicated by proliferating cell nuclear antigen (PCNA) release from chromatin during S-phase. In the absence of WRN, stalled replication forks are processed via a compensatory pathway, which can be dependent on Mus81 endonuclease, causing DSB formation [82]. Together the results indicate that WRN functions in protecting cells from DSB formation that can occur as a result of replication fork stalling and collapse.

Moreover, WRN is involved in the response to replication fork stalling induced by agents that generate crosslinks within the DNA. Chromium is an environmental genotoxin known to affect DNA replication through formation of interstrand crosslinks that inhibit polymerase elongation of the DNA [83,84] and also through creation of DSBs during S-phase that can lead to replication fork collapse [83,85,86]. Cells depleted of WRN are hypersensitive to chromium exposure, showing increased cell cycle arrest and cell death compared to wild-type cells. In WRN deficient cells exposed to chromium, WRN colocalizes with damage sites, as detected by phosphorylated histone H2AX (γ -H2AX) foci. These cells display a longer recovery time for stalled replication forks and repair of DSBs than control cells. Altogether the results indicate that WRN is involved in the recovery and/or repair of chromium-induced replication stress and DNA damage during replication [87]. One potential avenue of WRN participation in the restart of damage-induced stalled replication forks is through processing of regressed forks [88]. When damage is encountered at the fork, replication halts and the fork regresses into a chicken foot structure. In this structure, the lagging strand serves as a template for leading strand synthesis. Subsequently, WRN can mediate reverse branch migration of the chicken foot to bypass the damage, which can then be repaired by alternate pathways (Figure 1) [88].

S-Phase Checkpoints

Signaling checkpoints are activated in response to DNA damage at stalled forks to arrest replication and facilitate repair [89]. During S-phase, this signaling is mediated by the ataxia telangiectasia mutated (ATM) and ATM and Rad3 related protein (ATR) kinases [90]. While ATM is primarily involved in the response to DSBs [91,92], ATR more frequently facilitates the response to stalled replication forks [93]. During S-phase WRN interacts with both ATM and ATR. ATM and WRN colocalize with replication foci during S-phase [94]. Yet, the association of WRN with the S-phase checkpoint is dependent on formation of DSBs. Activation of ATM and its downstream checkpoint targets is impaired in WRN deficient cells exposed to psoralen plus UVA (PUVA), which generates interstrand crosslinks and subsequent DSBs during S-phase [94]. Therefore, in response to replication-dependent interstrand

crosslink-induced DSBs, WRN facilitates initiation of the intra-S-phase checkpoint through ATM activation [94].

Furthermore, WRN is phosphorylated in an ATM/ATR-dependent manner after damage associated with arrest of S-phase [95]. In addition, WRN is found in complex with members of the Rad52 epistasis group and ATR upon replication arrest caused by mitomycin C generated interstrand crosslinks [96]. Conversely, initiation of the ATR signaling cascade also occurs in the absence of WRN after c-Myc-induced DNA damage activates an intra-S-phase checkpoint [97]. Besides its roles in regulating transcription [98], c-Myc has recently been implicated in replication through its association with replication origins and through its interaction with the pre-replication complex [99]. Cells overexpressing c-Myc accumulate more DNA damage at replication sites in the absence of WRN, indicating that WRN protects against c-Myc-induced DNA damage during S-phase [97]. These results indicate that WRN plays an important role in reducing DNA damage stress sustained during S-phase. Overexpression of c-Myc leads to an accelerated S-phase, which may lead to formation of abnormal replication structures. It is conceivable that WRN may function in repair of these structures [97].

Presumably, WRN has both an indirect role in facilitating the recovery of stalled replication forks through signaling pathways and a direct role through enzymatic processes. In response to exposure to replication fork stalling by aphidicolin treatment, WRN deficient cells display an increase in fragile site instability and breaks compared to wild-type. Still, even in the absence of treatment, WRN deficient cells are more susceptible to DNA breaks suggesting that WRN is involved in maintenance of fragile sites [100]. It has been shown that fragile sites are susceptible to slowing of replication fork progression and to replication fork stalling even in the absence of treatment with replication inhibitors [101–103]. Studies show that it is the helicase activity of WRN which is responsible for the protection of genome stability at fragile sites [100]. WRN helicase may resolve DNA structures and facilitate polymerase replication at fragile sites (Figure 1) [100]. Earlier studies demonstrated that WRN does stimulate DNA polymerase δ (Pol δ) synthesis through structured regions, such as G4 containing DNA [104].

Interactions with Replication Proteins

WRN association with some of the major players in replication, specifically in lagging strand synthesis, also supports a role for it during replication; these interactions include Pol δ [105, 106], PCNA [24,107,108], flap endonuclease 1 (FEN1) [88,109–113], and replication protein A (RPA) [21,114–116]. Notably, the interaction with FEN1 implicates WRN function in replication of the lagging strand, as well as in base excision repair (discussed in a later section). However, WRN is not able to stimulate FEN1 cleavage of structured substrates [110], unlike BLM which resolves foldbacks allowing for FEN1 processing [117]. It is possible, then, that the WRN-FEN1 interaction is important either for processing other types of structured substrates during lagging strand synthesis or for alternative DNA transactions such as repair. Alternatively, WRN may resolve structured DNA which poses as a barrier to replicative polymerases, as mentioned above. In support of this notion, the *Xenopus* homolog of WRN FFA-1 associates both with replication foci [33,118,119] and also with Pol δ and RPA on chromatin upon replication blockage [120].

While it is evident from biochemical interactions and cellular studies that WRN is important for replication and S-phase progression, the effects of WRN depletion are most evident when DNA damage is associated with replication fork collapse. Consequently, although WRN has a prominent role in replication processes, it is also associated with repair of DNA damage.

Multiple Roles of WRN in DNA Repair

Endogenous and exogenous agents, chemicals, and ionizing radiation (IR) constantly damage cellular DNA [121], resulting in numerous DNA lesions throughout the genome. If not repaired properly, these lesions can eventually lead to point mutations, errors in DNA replication, DNA strand breaks, and induction of the stress response. Cells have different pathways to remove and/or repair different types of lesions to maintain the stability of the nuclear genome [122]. The major DNA repair pathways are base excision repair (BER), nucleotide excision repair (NER), double strand break repair (DSBR), and mismatch repair (MMR). BER repairs oxidative DNA base lesions such as 8-oxoguanine (8-oxodG), alkaline base damages, and SSBs. Bulky helix-distorting DNA lesions and crosslinks are repaired by NER. While DSBR repairs strand breaks in the DNA backbone, MMR is responsible for repairing single nucleotide mismatches and small insertion-deletion mispairs [2]. In addition to their functions in DNA replication, RecQ helicases are believed to play important roles in DNA repair processes. Among all the RecQ helicases, WRN's involvement in various repair pathways is most prominent, as it interacts with a number of repair proteins [7]. Notably, WRN interacts with the tumor suppressor p53 [50,123–125], which can activate repair and/or initiate apoptosis following DNA damage, and WS cells have attenuated p53-mediated apoptotic response [126]. Furthermore, WS cells are sensitive to DNA damaging agents such as camptothecin (CPT), 4-nitroquinoline 1-oxide (4-NQO), and MMS, and are slightly sensitive to IR [127–132]. They also accumulate more oxidative lesions after hydrogen peroxide treatment than normal fibroblasts [133]. The specific roles of WRN in different DNA repair pathways are discussed below (Figure 2).

Base Excision Repair (BER)

Oxidative DNA base modifications are caused by endogenous reactive oxygen species (ROS), normal by-products of mitochondrial oxidative phosphorylation and other metabolic processes, and are removed by BER [134,135]. The involvement of WRN in BER is evident from the fact that WS cells are sensitive to hydrogen peroxide [133]. WRN siRNA treated human primary fibroblasts also accumulate increased damage after oxidative stress [136]. These *in vivo* studies are supported by *in vitro* examination of WRN knockdown cells, showing reduced BER activity [132]. WRN has also been implicated in BER of methylation-induced DNA damages and SSBs. A number of methylating agents, such as MMS [129,132], methyl-lexitropsine, and telozolomide [137], sensitize WRN knockdown cells. WS cells are also sensitive to SSB producing agents like CPT and 4-NQO [127,130,131]. In brief, these results imply that WS cells are susceptible to agents that produce DNA damage, and lack of BER in these cells may be responsible for aspects of the aging phenotype.

The BER pathway proceeds either via short-patch (SP-BER), which involves single-nucleotide replacement, or via long-patch (LP-BER), which involves multiple nucleotide strand displacement. WRN physically and functionally interacts with proteins involved in both the BER pathways. DNA Polymerase β (Pol β) is mainly involved in SP-BER, though it can be involved in LP-BER as well [138,139]. The strand displacement activity of Pol β is stimulated by the WRN helicase activity [140]. WRN helicase activity also cooperates with its exonuclease activity to assist Pol β activity at 3'-mismatches [132]. *In vitro* evidence indicates that WRN exonuclease can act as an autonomous proofreading enzyme for Pol β during LP-BER [141]. Furthermore, FEN1, which removes the protruding ssDNA flap generated by DNA strand displacement during LP-BER, is stimulated by WRN [110]. Apurinic/apyrimidinic endonuclease 1 (APE1), the endonuclease that incises abasic sites during BER, inhibits WRN; this interaction could possibly prevent promiscuous unwinding of DNA repair intermediates [142]. Although WRN does not appear to interact with human OGG1, the major glycosylase for 8-oxodG in human cells, it does interact *in vivo* and *in vitro* with NEIL1 [143]. NEIL1 is

a human glycosylase for formamidopyrimidine (fapy) lesions [143,143,144]. Consistent with this interaction, fapy lesions accumulate in cells deficient in WRN [143]. WRN also functionally interacts with polyADP ribose polymerase (PARP-1) which ribosylates a large number of cellular proteins during DNA repair. The ribosylate activity is diminished in WRN-deficient cells, suggesting that PARP-1 is activated or stimulated by WRN [145].

Recent studies by Blander *et al.* suggested acetylation of WRN *in vivo* [146]. This process can either enhance the interaction of WRN with proteins that drives it to the nucleoplasm or diminishes its interaction with proteins holding it to the nucleolus. Furthermore, examination of the effect of acetylation on WRN catalytic activity and BER shows that acetylated WRN enhances the strand displacement activity of Pol β more efficiently than the unacetylated WRN [40]. The enhancement of Pol β strand displacement DNA synthesis after MMS treatment is also higher in WRN positive cells versus WRN knockdown cells. Collectively, these results suggest that acetylation is associated with activity of Pol β and that this process requires the presence of WRN [40]. Additionally, Li *et al.* recently showed that WRN-mediated cellular response to DNA damage is regulated by SIRT1 deacetylation [147]. Translocation of WRN from the nucleolus to the nucleoplasm is also affected by down regulation of SIRT1 [147]. The requirement of SIRT1-mediated deacetylation to stabilize WRN protein is supported by work from the Setou group [148]. They found that SIRT1 inhibitors diminish WRN levels. Likewise, WRN level is decreased in *Sirt1*-deficient mice [148]. Another recent study by Michishita *et al.* shows that at telomeric chromatin, SIRT6 deacetylates histone H3 lysine 9, which is required for the stable association of WRN [149]. This result further suggests that acetylation/deacetylation is a key mediator of WRN's participation in DNA damage repair processes.

Double strand break repair (DSBR)

DSBs are mainly induced by IR. They are recognized by DNA damage sensing proteins, leading to formation of DNA repair foci enriched in γ -H2AX. Rapid accumulation of WRN at laser-induced DSBs has been shown, and it remains at the DSB site for at least for 4 h [150]. WRN depleted cells are very sensitive to a well known DSB generating agent, hydroquinone. A recent study by Galvan *et al.* has demonstrated that WRN silenced HeLa cells accumulate more DSBs upon hydroquinone treatment than WRN positive HeLa cells. Additionally, an elevated DNA damage response was indicated by more than five-fold induction of γ -H2AX compared to the control cells [151]. This result is supported by another study where short hairpin RNA was used to silence endogenous WRN levels in the human HL60 acute promyelocytic cell line. These cells are also highly susceptible to hydroquinone-induced cytotoxicity and show an elevated DNA damage response [152]. Moreover, WS cells show phenotypes such as non-homologous chromosome exchanges and large chromosomal deletions, caused by deficiency of DSBR [5]. Collectively, these results suggest an important role of WRN in DSBR.

DSBs are repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR) processes. WRN is known to physically and functionally interact with two key proteins involved in NHEJ, Ku and DNA-PKcs [37,38,153]. In HeLa cells, Ku was identified as the tightest binding protein to the C-terminus of WRN [153]. However, Ku also interacts with the N-terminus or exonuclease terminus [154]. This protein enhances the exonuclease activity of WRN [153] and enables it to digest some substrates which WRN alone cannot process [155]. For example, 8-oxodG and 8-oxoadenine (8-oxoA) lesions block WRN exonuclease activity, but in the presence of Ku, WRN can digest strands containing these lesions [154,156]. Recently, Bukowy *et al.* reported the same results with strands containing 5-hydroxy uracil (5-OHU) and 5-hydroxy cytidine (5-OHdC) lesions [157]. Physical interaction between DNA-PKcs and WRN has been reported by several groups. However, it is not clear if the interaction is direct or mediated by Ku [37,38]. The DNA-PK complex has

been shown to regulate WRN phosphorylation *in vivo* and *in vitro* [37,38,158]. Notably, the serine/threonine phosphorylation of WRN by the DNA-PK complex inhibits the WRN helicase and exonuclease activities. WRN also interacts with another essential factor of NHEJ, the XRCC4/Ligase IV (X4L4) complex [159]. X4L4 binds with WRN, stimulating its exonuclease activity. The interaction between X4L4 and WRN may enable WRN exonuclease to serve as a DNA end-processing factor during NHEJ [159]. Systems deficient in NHEJ core components show hypersensitivity to IR treatment. Saintigny *et al.* observed that SV-40 transformed WS fibroblasts display a mild, but significant sensitivity to IR, compared with the appropriate control fibroblasts [160]. A similar result was obtained by Yannone *et al.*, comparing IR sensitivity of hTERT WS fibroblasts with WS fibroblasts expressing exogenous WRN [38]. Additionally, Sallmyr *et al.* found an alternative NHEJ pathway for DSB repair in chronic myeloid leukemia cells, involving DNA ligase III α and WRN [161]. Taken together, these results suggest that WRN is probably not an essential component of NHEJ, but is more likely acting as an accessory protein during this process and is involved in alternative NHEJ pathways.

Besides its involvement in NHEJ pathway of DSB repair, WRN also physically and functionally interacts with several important proteins in the HR pathway. After replication arrest induced by HU, WRN co-localizes with Rad51, which is a key player in the strand invasion event during HR [162]. Although no direct interaction between WRN and Rad51 has been reported, WS cells show elevated levels of Rad51 foci. WRN increases the efficiency of Rad52-mediated strand annealing [163], whereas, Rad52 both inhibits and enhances WRN helicase activity in a DNA structure-dependent manner. These results suggest that Rad52 and WRN may cooperatively facilitate rescue of stalled or blocked DNA replication forks. WRN colocalizes with Rad54, another key protein in this pathway, in response to replicative stress [96]. WRN associates with the Mre11-Rad50-NBS1 complex via NBS1, an essential component of HR [164]. Through this interaction, DNA unwinding by WRN is stimulated suggesting that WRN may be involved in resolving recombination intermediates. WRN also interacts physically with BRCA1, the possible HR factor, and BRCA1 stimulates WRN helicase activity [51]. Furthermore, WS cells are deficient in removal of DNA interstrand crosslinks, a process that requires recombination [51,94,165]. Consistent with an involvement in DSB repair, there is growing evidence that WRN interacts with topoisomerases. Interplay between topo I and WRN has been reported and WRN is involved in repair of topo I-inhibitor induced DNA damage [81,166]. Interaction between WRN and topo III is also suggested by Aggarwal *et al.* [167]. Their recent study demonstrated that WRN can function in a genetic pathway that affects topo III related phenotypes. Another RecQ helicase, BLM, functions in HR mediated DSB repair [168]. BLM is known to physically and functionally interact with WRN, inhibiting its exonuclease activity [169]. Thus, these two RecQ helicases could work together in DSB repair. Supporting this hypothesis, synergistically increased hypersensitivities to 4-NQO, CPT, MMS, and UV, were observed in *Wrn*^{-/-}/*Blm*^{-/-} double mutant chicken DT40 cells compared to the single mutants [129]. However, further studies are required to explore the associations and exact roles of these two proteins in HR.

Involvement of WRN in Transcription

The first indication of the involvement of WRN in transcription comes from the fact that purified recombinant WRN protein has been demonstrated to unwind not only a duplex DNA but also an RNA-DNA heteroduplex [23,170]. Association of WRN with transcription was also suggested by the result that WRN overexpression affects p-53 mediated transcription [50]. Furthermore, Shiratori and colleagues have shown that WRN leaves the nucleoli upon inhibition of ribosomal RNA (rRNA) transcription following actinomycin-D treatment [171]. They have also demonstrated that the level of rRNA transcription in WS fibroblasts is significantly lower than that in wild-type fibroblasts, and the reduced level of rRNA can be rescued by expression of wild-type WRN in WS fibroblasts [171]. The functional interaction

between WRN and rRNA was further supported by the observation that methylation of rRNA genes is accelerated in WS fibroblasts [172]. Furthermore, the efficiency of RNA polymerase II (RNA pol II) transcription is reduced by about 50% in permeabilized WS cells and extracts from WS cells compared to normal cells, implicating WRN in RNA pol II-dependent transcription. Additionally, RNA pol II-dependent transcription can be enhanced by the addition of recombinant WRN protein. Deficiencies in RNA polymerase I (RNA pol I) transcript level were not observed in this study [173]. In contrast, WRN was co-immunoprecipitated with an RNA pol I subunit in another study [171]. It was suggested that the ongoing transcription of RNA pol I controls nucleolar accumulation of WRN in serum-stimulated cells. Likewise, RNA pol I inhibition or serum starvation leads to translocation of WRN from the nucleolus to the nucleus [171]. A recent study by Lutomska *et al.* has shown a defect in the RNA pol I transcription in response to distinct growth factors in WS cells [174]. WRN also stimulates promoter clearance of RNA pol I transcription and binds to the active fraction of rDNA in quiescent cells. Furthermore, the role of WRN in the differential expressions of individual genes has been studied by Kyng *et al.* using a cDNA microarray system [175]. They propose a model in which WRN protein, by virtue of its helicase and transcription-activating activities, as well as its protein interactions, is directly or indirectly involved in the transcription of genes upstream in the network of aging pathways [175]. Collectively, these results indicate involvement of WRN in transcription and suggest that decreased transcription rate might be a cause of premature aging phenotypes observed in WS patients.

Importance of WRN for Telomere Maintenance

The DNA polymerases involved in DNA replication process are restricted from continuing DNA synthesis to the end of the chromosome because of their unidirectional nature [176, 177]. While prokaryotes employ a number of diverse mechanisms to deal with this 'end replication problem', eukaryotes have developed a unique solution involving a special structure known as the telomere [178]. Telomeres are nucleoprotein complexes situated at the ends of the linear chromosomes that prevent chromosome termini from being recognized as broken DNA ends (*i.e.*, DSBs). In most of the organisms studied, telomeres consist of long repetitive G-rich and C-rich DNA strands, the ribonucleoprotein telomerase, and telomere binding and associated proteins [179]. Loss of telomeric repeats or loss of protection by telomere-associated proteins triggers telomere dysfunction. Apart from being subject to nucleolytic degradation and undesirable recombination mediated by HR or NHEJ, dysfunctional telomeres are also recognized by many DNA damage response proteins, including ATM, γ -H2AX, 53BP1, MDC1, and NBS1. Association of these proteins can form telomere dysfunction-induced foci and can induce cell cycle arrest, senescence, or apoptosis [180–182]. Aging [183] and premature aging syndromes [11] are also frequently associated with telomere attrition.

The unique sequence and structure of telomeric DNA plays an important role in maintenance of the telomere [184]. In mammals, telomeres are composed of double-stranded tandem repeat sequences followed by a single-stranded short 3'-overhang, which invades the double-stranded region to create a loop structure (Figure 3) [185]. The telomere binding and associated proteins stabilize this so-called "D-loop" (displacement loop) configuration. As the single-stranded region is G-rich, G-quadruplex (G4) structures may also arise during telomere metabolism [186]. Crystal structure analysis of G4 generated from telomeric DNA sequences [187], as well as *in vivo* [188] studies, provided evidence for the existence of G4 structures in the telomeric region. Recently, the formation of G4 structures was also observed within telomeric RNA (TERRA) [189].

Telomerase, which contains both telomerase RNA and a reverse transcriptase (TERT), is another crucial component at telomeres that maintains telomere length [190,191]. Additionally,

telomere-specific binding proteins and their associated proteins protect the telomeric DNA at the end of the chromosomes [192] and are collectively referred to as the 'shelterin' complex [180]. In mammals, this complex includes TRF1 and TRF2, proteins that bind to the double-stranded telomeric DNA, and POT1, a protein that binds to the single-stranded telomeric DNA overhang, as well as their associated proteins Rap1, TIN2, and TPP1.

Apart from the shelterin proteins, a number of DNA repair/damage checkpoint proteins associate with the telomere protein complex. These include ATM, ATR, and DNA helicases and nucleases (WRN, BLM, Apollo, EXOL1), as well as components of HR or NHEJ (KU/DNA-PKcs), DSB (MRE11/NBS1/RAD50 complex), and NER/BER (ERCC1/XPF, PARP1/PARP2, FEN1) [2]. Many of these DNA repair/damage checkpoint proteins are actively involved in telomere maintenance, possibly by assisting in telomeric DNA repair and telomere capping. RecQ helicases, especially WRN, are known to play significant roles in proper maintenance of telomeres. WRN functionally and/or physically interacts with telomeric DNA protecting proteins POT1, TRF1 and TRF2 [193–195]. In addition, it is associated with Mre11, Rad50, and Nbs1, which are also associated with TRF2 [51]. Furthermore, WRN functionally interacts with Ku, which interacts with both TRF1 and TRF2 [156]. Moreover, interactions of WRN with telomeric D-loop and G4 structures were shown by *in vitro* studies [194,196].

Besides biochemical evidence of WRN association with telomere associated proteins, there is cellular evidence of a role for WRN in telomere processing. *WRN* and *TERT* minus double mutant mice have phenotypes resembling human clinical features [197]. *In vivo* gene specific repair studies have shown that the extent and rate of telomeric repair is lower in WS patients [198]. This notion is further supported by accelerated telomere loss displayed in WS cells [199]. A recent study on the effect of the glucose utilization inhibitor 2-deoxy-D-glucose on gene/protein expressions of RecQ helicases and telomerase also indicates that the cooperative interaction between WRN and telomerase is advantageous for proper telomere maintenance in cells [200]. We will discuss the implications of these studies briefly.

Telomere Replication

One of the most important implications of WRN's interaction with shelterin proteins and telomere specific DNA substrates is its involvement in telomere replication. Replication fork terminations at telomeres are particularly damaging due to the absence of any replication origins past the telomeres to account for the unreplicated portions. Early evidence of involvement of WRN in replication comes from the fact that WS patients show prolonged S-phase [67] and WRN colocalizes with RPA in cells arrested in S-phase by HU treatment [201,202]. Although, WRN localizes predominantly to the nucleolus in telomerase-positive human cell lines, a significant fraction of WRN colocalizes with TRF1 and TRF2 at telomeric DNA in human ALT cell lines [193,194]. This is suggestive of a role of WRN in telomeric DNA replication. However, WRN might not participate in the general telomere replication process, as it is associated with only 5% of telomeric S-phase fibroblasts [11]. Rather, it may be recruited to the replicating telomeres in response to replication stress. CO-FISH studies also suggested that WRN is involved in an alternative mechanism to resolve relatively rare, but lethal, events during telomere replication [203].

Dissociation of D-loop structures is required for replication of telomeric DNA replication. *In vitro* studies have shown that WRN unwinds the D-loop structures to release the invading strand, a process that is regulated by TRF1 and TRF2 [194]. WRN helicase activity at telomeric D-loop structures is enhanced by physical interaction with TRF2 [193]. Conversely, TRF1 and TRF2 limit the exonuclease activity of WRN suggesting the cooperation of these telomere binding proteins with WRN in the processing of telomeric DNA. Telomeric single strand binding protein POT1 also enhances D-loop unwinding of WRN [195] by maintaining the

unwound strands in a melted state (Figure 3) [204]. WRN also has a role in restart of stalled replication forks, an important mechanism for continuing the replication process following DNA damage. WRN can efficiently unwind DNA substrates with a one nucleotide gap. More importantly, WRN enhances FEN1 activity to cleave the unwound 5'-strand *in vitro*. WRN and FEN-1 co-localize at PCNA foci after the induction of stalled replication forks *in vivo* [88]. Furthermore, formation of G4 structures is another potential block to the replication of telomeric invading strand. G4 structures are favored substrates for WRN, which can prevent replication stalling at G4 DNA [104]. It was also shown that POT1 can resolve telomeric G4 structures [205]. Thus, WRN and POT1 can cooperate with each other to resolve the G4 structures at telomere (Figure 3).

Telomeric Recombination and Repair

Alternative lengthening of telomeres (ALT) pathways involve multiple telomere binding and recombination proteins. In budding yeast, RecQ helicase Sgs1 acts in resolution of recombination intermediates, when critically short telomeres undergo recombination to restore the telomeric length. A fraction of telomeric DNA from human cell lines maintaining the telomeres by ALT co-localizes with WRN [11], and it can partially substitute the function of Sgs1 in type II ALT [206,207]. *In vivo* study demonstrates that telomerase-positive WS fibroblasts develop an elevated level of telomeric t-circles in the absence of the N-terminal basic domain of TRF2. Reconstitution with WRN reduces this t-circle formation. However, mutant WRN lacking either helicase or exonuclease is not able to reduce the t-circles, indicating roles for both the functions of WRN to suppress the t-circle formations in telomerase-positive cells [208]. WRN interacts physically with the Ku70/80 heterodimer and POT1, which also have crucial roles in protection of telomeres from aberrant HR [209,210]. As WRN interacts with both these proteins, it may be also required to suppress the unusual recombination intermediates at telomeres.

In addition to recombination based repair, WRN has been implicated in oxidative DNA damage repair at telomeres (Figure 3) [11]. *In vitro* analysis has shown that guanine (G) has the lowest oxidation potential among the nucleobases and the GGG sequence has even lower oxidation potential [211]. Repetitive sequence of triple guanines makes telomeric DNA prone to oxidative damage, lesions such as 8-oxodG. The association between oxidative DNA damage and telomere shortening is described in several studies [212,213]. Oxidative DNA damage is repaired by the BER process, and WRN is believed to take part in BER because it physically interacts with several of the proteins involved (see *Multiple Roles of WRN in DNA Repair* section). Recently, we have seen that WRN interacts with the D-loop structures containing 8-oxodG lesions, and it unwinds these more efficiently than the undamaged D-loops [214]. This result also indicates a role of WRN in resolving complex structures at damaged telomeres in order to accelerate the activities of the repair enzymes. However, it is still mostly unclear whether and how BER functions at the telomeres, and a significant amount of research is needed to assess the exact role of WRN in the repair of oxidative damage at telomeres.

Model system for human WS

Although WRN helicase has several activities in human cells, *Wrn*-null mice do not show any phenotypes of human WS [215]. However, studies using mice null with respect to *Wrn* and *mTerc* (encoding telomerase RNA component) revealed that the late generation *mTerc*^{-/-}*Wrn*^{-/-} mouse displays most of the classical clinical features resembling human WS [197,216]. WS human fibroblasts show elevated levels of DNA-damage as detected by γ H2AX and 53BP1 and a profound decline in replicative lifespan, which was also displayed by the *mTerc*^{-/-}*Wrn*^{-/-} mouse embryonic fibroblasts. It is important to note that these features do not represent deterioration of aging phenotypes observed in the telomerase null mouse, but reiterate the specific phenotypes of WS patients. WS patients also exhibit increased incidence of

chromosomal instability and cancer. Studies using the *mTerc*^{-/-}*Wrn*^{-/-} double knockout mice indicate that these may be caused by aberrant recombination at telomeres [10]. Together, the results strongly support the hypothesis that *Wrn*-null mice do not show human WS phenotypes, as they have long telomeres, and to manifest the premature aging symptoms, critically short telomeres are required. These studies also suggest that WRN and telomerase work together to maintain the telomere integrity and WRN is strongly involved in telomere dynamics.

Comparison of WRN with other RecQ Helicases

While most other eukaryotes possess one or two members of the RecQ helicase family, humans have five distinct RecQ helicases. Each of these RecQ1, WRN, BLM, RecQ4, and RecQ5 is involved in multiple pathways to maintain genome stability [1–3,217] (see additional reviews in this issue). Accordingly, questions of redundancy can be raised. Why do humans have multiple RecQ helicases? Do they compete with each other or are their cellular roles unique? To address these questions, it is important to compare the functions of the RecQ helicases and the pathways in which they participate (Figure 4).

The human RecQ helicases are the causative factors of a variety of human premature aging and cancer disposition pathologies. Deficiencies in WRN and BLM are linked to WS and Bloom syndrome (BS), respectively. BS is characterized by short stature, skin lesions, immunodeficiency, low fertility or infertility, diabetes, and cancer predisposition [218,219]. Furthermore, RecQ4 deficiencies are the cause of Baller-Gerold syndrome (BGS), RAPADILINO syndrome, and Rothmund-Thomson syndrome (RTS) [220–224]. BGS patients have short stature, abnormal skull and brain growth (craniosynostosis), and absent development or underdevelopment of the radius (radial aplasia or hypoplasia) [221]. RAPADILINO is typified by radial and patellar hypoplasia, cleft palate, diarrhoea, dislocated joints, little size, limb malformation, slender nose, normal intelligence, and predisposition to lymphoma and osteosarcoma [225]. RTS presents with developmental abnormalities, growth deficiencies, proneness to cancer, predominantly osteosarcomas, and premature aging, including development of cataracts and hair loss [226,227]. To date, no human pathologies have been found with deficiencies in either RecQ1 or RecQ5. Still, while some of the clinical features of each of the RecQ associated diseases overlap, some are unique, suggesting that the RecQ helicases may have differential cellular roles that translate into the distinct symptoms.

Enzymatically all the RecQ helicases are similar, carrying out 3'-5' ATP-dependent unwinding and DNA strand annealing activities [217]. However, the substrate specificity of each of the helicases is varied. WRN, BLM, RecQ1, and RecQ5 are capable of readily unwinding forked substrates with shorter regions of duplex DNA and longer duplexes in the presence of RPA [21,114–116,228–231]. Likewise, it has been shown recently that RecQ4 also shows unwinding on fork substrates [232,233] which is stimulated by RPA (Rossi *et al.*, submitted for publication). In contrast, only WRN and BLM can effectively resolve G4 DNA [34,234, 235], while RecQ1 [236] and RecQ4 cannot (Ghosh and Bohr, unpublished data). Additionally, WRN and BLM readily unwind telomeric D-loops, while RecQ5 displays weak activity on these substrates [214]. RecQ5 displays a similarly weak activity on Holliday junctions [237], which are resolved efficiently by WRN, BLM, and RecQ1 [34,236,238]. However, immobile Holliday junctions are only unwound by RecQ1 not BLM [236]. It is evident from these examples that the RecQ family members are structure specific helicases with some overlapping and some unique substrate specificities.

Moreover, the effectiveness of WRN and BLM in processing complex structures like G4 suggests that they may play a more prominent role in facilitating DNA replication and repair through specialized regions of the genome, such as telomeres. Alternatively, WRN, BLM, and RecQ1 resolution of Holliday junctions could indicate a favored role in homologous

recombination pathways. Still, RecQ4 and RecQ5 preferential unwinding of simpler replication fork substrates could be indicative of a preferred role in replication processes over the other RecQ helicases [1–3,6]. Nevertheless, a comprehensive study comparing the activity of all the RecQ helicases on a panel of substrates representing intermediates of DNA replication and repair processes has not been conducted. Because helicase activity has now been detected in all of the human RecQ family members, such research is merited and may provide insight into which specific pathways each of the RecQ helicases function. It is a remaining challenge to determine whether there is a specific substrate, potentially one that contains DNA damage, on which some of the RecQ helicases can act with synergy.

Furthermore, analysis of the interacting partners of the RecQ helicases provides clues as to the redundancy and uniqueness of functions of the family members. As mentioned above, the activity of the RecQ helicases can be stimulated by RPA [21,114–116,228–231]. Since RPA is involved in both DNA replication and DNA repair [239], and the interaction is conserved among all the RecQ family members, it is not a distinguishing factor of RecQ helicase cellular functions. In addition, another highly conserved physical and functional protein interaction is FEN1. WRN, BLM, RecQ4, and RecQ5 stimulate FEN1 cleavage [109,240,241] (and Speina *et al.*, submitted for publication), implicating these RecQ helicases in lagging strand replication and/or BER.

On the other hand, some protein associations are more restricted to specific RecQ helicases. For example, WRN and BLM both interact with the telomere proteins TRF1 and TRF2 and are stimulated by POT1 [193–195]. Although the interaction between these telomere binding proteins and either RecQ1, RecQ4, or RecQ5 has not been tested, it is conceivable that the associations are limited to WRN and BLM. Such a privileged interaction would suggest that WRN and BLM are uniquely involved in telomere maintenance. Moreover, the associations of WRN with DNA-PK, Ku70/80, and X4L4 imply a direct role of WRN in NHEJ, which has not been detected for the other RecQ helicases [37,153,154,159]. Additionally, WRN and RecQ5 have been linked to transcription [173,242–244]. WRN was shown to regulate RNA pol II transcription efficiency potentially by activation [173]. Conversely, RecQ5 inhibits transcription through a direct physical interaction with RNA pol II [242,243]. These results indicate that even if the pathways in which the RecQ helicases function overlap, each helicase may have differential effects.

Generally, the RecQ helicases participate in many of the same pathways of DNA replication and repair. However, the specific mechanisms of function is not been fully elucidated. While it appears that the RecQ helicases are partly redundant, there are clear examples of distinct function. Additional studies of the biochemical and cellular functions of RecQ1, WRN, BLM, RecQ4, and RecQ5, may reveal more distinct roles of these proteins, distinguishing one from the other.

Perspectives

Although the connection between the WS phenotype and the function of the WRN protein has yet to be made, it is clear that the missing link is related to the function of WRN in DNA replication, DNA repair, transcription, and telomere maintenance. Past studies clearly indicate that WRN is important for protection of genome stability. Yet a complete understanding of all the mechanisms of WRN's function in DNA transactions remains elusive. Future research on the deficiencies in WRN depleted cells and the roles of WRN in DNA metabolism will reveal more complete understanding of its cellular functions. It will be important to determine how the involvement of WRN in a variety of different processes is modulated, whether through post-translational modifications and/or through protein-protein interactions. Moreover, additional study of the function of WRN in comparison with BLM, RecQ1, RecQ4, and RecQ5

may reveal some unique roles, distinguishing it from the other RecQ helicases. Understanding the differences between the RecQ helicases will ultimately be necessary to address the question as to why humans have developed multiple seemingly redundant proteins.

Acknowledgments

We thank Dr. Dharmendra K. Singh and Mr. Joshua A. Sommers for helpful comments on the manuscript. This work was supported entirely by funds from the Intramural Research Program of the National Institutes of Health, National Institute on Aging. We regret that space limitations and the breadth of this field prevented us from citing every relevant reference.

Abbreviations

γ -H2AX	phosphorylated histone H2AX
4-NQO	4-nitroquinoline 1-oxide
5-OHdC	5-hydroxycytidine
5-OHU	5-hydroxyuracil
8-oxodA	8-oxoadenine
8-oxodG	8-oxoguanine
ALT	alternative lengthening of telomeres
APE1	apurinic/apyrimidinic endonuclease 1
ATM	ataxia telangiectasia mutated
ATR	ATM and Rad3 related
BER	base excision repair
BLM	Bloom syndrome protein
CeWRN-1	<i>Caenorhabditis elegans</i> WRN
CPT	camptothecin
D-loop	displacement loop
DmWRN	<i>Drosophila melanogaster</i> Werner syndrome protein
DSB	double strand break
DSBR	double strand break repair
ETO	etoposide
fapy	formamidopyrimidine
FEN1	flap endonuclease 1
FFA-1	focus forming activity 1
G4	G-quadruplex
HR	homologous recombination
HU	hydroxyurea
IR	ionizing radiation
LMNA	lamin A/C
MMR	mismatch repair

MMS	methylmethane sulfonate
NER	nucleotide excision repair
NHEJ	non-homologous end joining
PARP-1	poly (ADP-ribose) polymerase
PCNA	proliferating cell nuclear antigen
Pol β	DNA Polymerase β Pol δ , DNA Polymerase δ
PUVA	psoralen plus UVA
RNA pol I	RNA polymerase I
RNA pol II	RNA polymerase II
ROS	reactive oxygen species
RPA	replication protein A
rRNA	ribosomal RNA
SNPs	single nucleotide polymorphisms
SSB	single strand break
TPT	topotecan
WRN	Werner syndrome protein
WS	Werner syndrome
X4L4	XRCC4/Ligase IV

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Figure 1. Roles of WRN in S-phase at stalled replication forks

A) WRN may participate in repair of double-strand breaks (DSBs) following DNA damage-induced replication fork collapse. Alternatively, WRN may function to regress the fork and allow for synthesis bypass of DNA damage. B) Secondary structures which block DNA polymerases may be resolved by WRN. (See text for details.)

Figure 2. Roles of WRN during DNA repair

Schematic diagram depicting WRN interactions with various repair proteins in multiple pathways following DNA damage. DNA damage leads to post-translational modifications of WRN which may regulate its roles in DNA repair. (See text for details.)

Figure 3. Role of WRN in replication, recombination, and repair processes at telomeres
 (See text for details.)

Figure 4. Involvement of RecQ helicases in maintenance of genomic integrity

Association of different RecQ helicases (RecQ1, WRN, BLM, RecQ4, and RecQ5) with replication, base excision repair (BER), double strand break repair (DSBR), transcription, and telomere maintenance has been indicated by arrows. Dotted line indicates probable involvement. Some major physical and/or functional interacting proteins are also shown with each respective RecQ helicase. (See text for additional details and reviews in [1–3,5,217].)