# CHAPTER 2

# DNA Repair Aspects for RecQ Helicase Disorders

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## Abstract

RecQ family DNA helicases are defined by amino acid sequence similarities to *Escherichia coli* RecQ which has been known to act in homologous recombination and to suppress illegitimate recombination, particularly during the repair of DNA double strand breaks. Five *RecQ* family genes have been identified in humans, and three (*BLM*, *WRN*, and *RECQL4*) have been identified as defective in the human genetic disorders; Bloom syndrome, Werner syndrome, and a subset of Rothmund-Thomson syndrome. Despite strong homology in the helicase domains, human *RecQ* family genes differ markedly outside these domains. Indeed, each syndrome presents different phenotypes. however, all are characterized by an increased predisposition to cancer, which is consistent with increased chromosomal aberrations and hypermutability observed in cultured cells. These data suggest that each of these helicases contributes to maintaining genomic stability and that an important function of these helicases appears to be the resolution of recombination intermediates.

## Introduction

In a wide range of organisms from the prokaryotes to eukaryotes, DNA helicases, with their ability to unwind DNA structures, are involved in many basic cellular processes such as replication, recombination, and repair. The RecQ family of helicases is a subfamily of the DExH-box-containing DNA helicases, which include *Escherichia coli* RecQ,[1] *Saccharomyces cerevisiae* Sgs1,[2,3] and *Schizosaccharomyces pombe* Rqh1.[4] In *E. coli* lacking a functional RecBCD helicase, *RecQ* mutations increase UV sensitivity.[1] RecQ was originally identified as a component of the RecF pathway of conjugational recombination operating in the absence of a functional RecBCD helicase.[1,5] Subsequent work showed that *E. coli* RecQ protein disrupts recombination intermediates to suppress the formation of large chromosomal deletions mediated by illegitimate recombination using short homologous sequences.[6]

Similar to bacteria, budding and fission yeasts have a single RecQ-like helicase. *SGS1* was initially found because its deficiency resulted in suppression of slow growth in *Top3* mutants of the budding yeast *S. cerevisiae*. In fact, SGS1 protein was found to interact with Top3 and to act as a 3'-to5' helicase.[2,7] In the fission yeast *S. pombe*, a *Rqh1* mutation was identified in radiaition-sensitive (*rad*) and hydroxyurea-sensitive (*hus*) mutants.[4] Genetic analysis demonstrated that *Rqh1* mutants are defective in the recombination bypass of UV-induced DNA damage during the S phase.[8] Like SGS1 protein, Rqh1 protein has a 3'-to5' DNA-unwinding activity and exists with Top3 in a high-molecular-weight complex in fission yeasts.[9]

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In humans, five homologues of the bacterial RecQ helicase have been identified. Disruption of their functions may reduce genomic stability and thus contribute to tumorigenesis.[10,11] Mutations in three different human RecQ helicase family members, the *WRN* gene, the *BLM* gene and the *RECQL4* gene, give rise to three distinct human disorders, Werner syndrome (WS), Bloom syndrome (BS), and Rothmund-Thomson syndrome (RTS), which lead to cancer predisposition and/or segmental premature aging.[12-14] Defining in vivo functions of RecQ family helicases is necessary for understanding the mechanisms of cancer predisposition and segmental premature aging in the *RecQ*-deficient disorders. The hyper-recombination phenotype of *RecQ*-deficient cells suggests that these helicases seem to maintain replication forks to ensure processive DNA replication, and/or carry out conventional recombination or recombination-mediated DNA repair. Much information obtained from previous studies on RecQ family helicases may illuminate the mechanism of action of these important molecules.

## Bloom Syndrome Gene (*BLM*)

BS is characterized by severe pre- and post-natal growth retardation, immunodeficiency, sun-sensitive facial erythema, male infertility, genomic instability and predisposition to cancer in many types of tissue.[15] The varieties of cancer are almost identical to that seen in the general population, and also include Wilms tumor and osteosarcomas, which are considered more rare. Cultured cells from BS patients exhibit hypermutalism and chromosomal and DNA instability characterized by an excessive number of locus-specific mutations, including insertions, deletions, and loss of heterozygousity.[16] In additon, BS cells show a high frequency of microscopically visible chromaid gaps, breaks, and rearrangements, quadriradials, and associations at telomeres.[17]

The *BLM* gene is located on chromosome 15 at 15q26.1 and encodes a protein of 1417 a.a.[12] The *BLM* gene is not essential in humans, however, the murine homolog is essential in mice since the homozygous disruption of *BLM* results in embryonic lethality by E13.5.[18] Mice heterozygous for a targeted null mutation of *BLM* demonstrated that *BLM* haploinsufficiency enhances T cell tumorigenesis in response to murine leukemia virus infection and intestinal tumorigenesis when crossed with mice carrying a mutation in the *APC* tumor suppressor gene.[19]

Biochemical studies have shown that BLM has an ATP- and $Mg^{2+}$- dependent 3'-to-5' DNA-unwinding activity.[20] Like the SGS1 protein, the human BLM protein is a hexameric helicase.[21,22] Among the human RecQ homologues, BLM is most similar to two yeast RecQ-type helicases, SGS1 and Rqh1, both of which regulate genetic exchange and maintain genomic stability by preventing inappropriate recombination during interrupted S phase.[4,23,24] These three proteins contain a helicase domain of similar size and position as well as two acidic amino acid clusters in their amino termini.[3,4,12] In *S. cerevisiae*, *SGS1* mutants are hypersensitive to hydroxyurea (HU),[25] and they exhibit mitotic hyper-recombination, resulting in increased frequencies of ectopic, interchromosomal homologous exchange and intrachromosomal excision as well as poor sporulation.[23,24] *BLM* can suppress increased homologous and illegitimate recombination, and restore increased sensitivity to HU.[25] Also, *BLM*, but not *WRN*, can prevent premature aging and increased homologous recombination at the rDNA loci caused by *SGS1* mutation.[26]

Like the yeast *SGS1* mutants, an abnormally high incidence of spontaneous sister chromatid exchanges (SCEs) are observed in BS cells.[15,27,28] The increase in frequency of SCEs in BS cells is further facilitated by exposure to UVC light and DNA damaging agents such as ethyl methansulfonate, N-ethyl-N-nitrosourea and 5-bromodeoxyuridine (BrdU).[29-31] Although thymine dimers on single stranded DNA formed by UVC irradiation and base damage to genomic DNA caused by hydrolysis, oxidation and nonenzymatic methylation are usually repaired by the nucleotide-excision repair (NER) pathway prior to replication,[32] large populations of such DNA adducts may be overlooked by the NER pathway. When a replication

fork is stalled at a DNA adduct, it may produce a single-strand gap between the damaged base and a new Okazaki fragment being synthesized downstream, possibly resulting in a chromatid break.[33,34] Such strand discontinuities can be repaired post-replicationally by homologous recombination (HR) with the sister chromatid, i.e. by SCE.[35] In fact, BS cells exhibit hypersensitivities to DNA damaging agents such as N-ethyl-N-nitrosourea, ethyl methanesulfonate, methyl methanesulfonate (MMS) and 4-nitroquinoline 1-oxide (4-NQO), as well as to irradiation with UVC.[36-38] In addition to BS cells, chicken *BLM* mutant DT40 cells exhibit hypersensitivity to UVC irradiation and genotoxic agents such as bleomycin, etoposide, MMS and 4-NQO.[39] They also show chromosomal aberrations that ultimately lead to cell death. Karyotypic analysis demonstrated that UVC irradiation during $G_1$ to early S phase causes chromosomal aberration prior to cell death in *BLM* mutant DT40 cells, suggesting that BLM is involved in early S phase-specific surveillance of damaged sites on DNA.

On the other hand, BLM has also been shown to possess the ability to selectively recognize Holliday junctions and efficiently promotes ATP-dependent branch migration of Holliday junctions during S phase in vitro, resulting in the prevention of promiscuous recombination events.[40] The level of SCE in *BLM* mutant DT40 cells is considerably reduced in the absence of *RAD54*, indicating that a large proportion of the SCE occurs via HR.[41] In addition, *BLM* mutant DT40 cells exhibit hyper-recombination as indicated by the increased frequency of targeted genome integrations in addition to that of SCE.[39,41] Thus, a *BLM* defect would lead to the initiation of double-strand break (DSB) repair by HR, resulting in an increase in frequency of SCE. DNA crosslinking within GC-rich regions exposed by recombination or replication in mammalian chromosomes can create quadriradials which are well described as one of the representative phenotypes seen in BS cells.[42] Previous work has shown that BLM is a general helicase with preference for a quadriradial formed in a single stranded G-rich region exposed by recombination.[43] The fact that the number of chromatid quadriradials observed in the *BLM* mutant DT40 cells are elevated after UVC irradiation supports this speculation.[39]

BLM localizes to promyelocytic leukemia protein (PML) nuclear bodies,[44] and its expression is cell cycle-regulated, peaking in late S phase and $G_2$.[45,46] Both the recombination/repair proteins hRAD51 and replication protein (RP)-A assemble with BLM in PML nuclear bodies during late S/$G_2$ phase. BS cells have been reported to be more sensitive than wild-type cells to radiation, particularly in late S and $G_2$ phase.[47,48] BLM protein assembles into foci specifically in response to agents that cause DNA DSBs in normal cells, but not in cells with defective PML.[44] HR repair is carried out by the RAD52 complex, which includes hRAD51 and RP-A.[49,50] These studies suggest that BLM is part of a nuclear matrix-based complex that requires PML and functions in HR repair after DNA damage. Additionally, BLM also has been identified as a member of a group of proteins that associate with BRCA1 to form a large complex that includes tumor suppressors and DNA damage repair proteins MSH2, MSH6, MLH1, ATM, and the RAD50-MRE11-NBS1 protein complex.[51] Recently, It was observed that BLM is required for correct nuclear localization of RAD50-MRE11-NBS1 complexes after replication fork arrest.[52] The γ-irradiation-induced BLM phosphorylation requires functional ATM.[53] Both UVC radiation and HU-mediated DNA synthesis inhibition induced BLM phosphorylation via an ATM-independent pathway.[54] Among the assembly components of the BRCA1-associated large complex, both BRCA1 and NBS1 are phosphorylated via an ATM-dependent pathway in response to ionizing radiation, and via an ATM-independent pathway in response to UVC and HU.[55,56] It has been demonstrated that ATM responds exclusively to DNA DSBs, whreas the ATM-related kinase, ATR, exhibits substrate specificity similar to ATM and also reacts to UV damage and replication arrest.[57,58] The BRCA1 phosphorylation induced by UVC or HU has been shown to be controlled by ATR.[56] These findings suggest that ATR phosphorylates BLM upon UV damage and replication arrest. Damage during S phase causes BLM to rapidly form

nuclear foci at replication forks that develop DNA breaks. These BLM foci then recruit BRCA1 and NBS1. Assembly of BRCA1 and NBS1 repair complxes at stalled replication forks was markedly delayed in damaged BS cells.[59] It has been suggested that BLM plays a role in repair by recognizing DNA adducts and recruiting BRCA1 to the damage-induced nuclear matrix, which results in assembly of the BRCA-associated repair complex at a stalled replication fork. Concurrently or alternatively, BLM functions in HR repair after DNA damage as part of a RAD52 complex, and/or by resolving DNA structures such as Holliday junctions at stalled replication forks in addition to quadriradial structures prior to HR repair.

## Werner Syndrome Gene (WRN)

Individuals afflicted with WS exhibit at an early age, features associated with the normal advanced aging process such as bilateral cataracts, diabetes mellitus, atrophy of the skin, graying and loss of hair, osteoporosis, atherosclerosis and increased predisposition to development of cancer.[60] The most prevalent types of cancer are soft tissue sarcomas, however, thyroid carcinomas, osteosarcomas, meningiomas, and melanomas are also seen. In vitro studies of fibroblast growth characteristics suggest that WS may be related to normal aging. The life span of WS fibroblasts as measured by in vitro population doubling levels is much shorter than that of normal fibroblasts, and they have a prolonged S phase.[61,62] WS cells are also characterized by genomic instability in the form of variegated translocation mosaicism and extensive deletions.[63,64] WS cells display a defect in the $G_2$-phase decatenation checkpoint which is known to inhibit progression into mitosis until chromatids are correctly decatenated by topoisomerase II.[65] In WS cells, failure to phosphorylate BRCA1 in an ATR-dependent manner in response to decatenation checkpoint activation results in enhanced chromosomal damage and apoptosis.[65] It has been shown that the suppression of RAD51-dependent recombination leads to significantly improved survival of WS cells following DNA damage, suggesting defective recombination resolution in WS cells.[66] Like *BLM*, *WRN* can suppress increased homologous and illegitimate recombination in the *SGS1* mutant.[25]

The *WRN* gene is located on chromosome 8 at 8p11-12 and encodes a protein of 1432 a.a..[13,67] Subcellular localization studies revealed that WRN protein is localized both in the nucleoplasm and the nucleoli.[68] Transcriptional activation facilitates translocation of WRN protein from the nucleoplasm to the nucleoli,[69] whereas DNA damages lead to extensive translocation of WRN from the nucleolus to nucleoplasmic foci.[70] In addition, WRN is acetylated in vivo, which is markedly stimulated by the acetyltransferase p300, which results in the augmentation of WRN translocation into nucleoplasmic foci.[70] WRN has DNA-dependent ATPase activities and a 3'-to-5' unwinding activity for not only duplex DNA but also RNA-DNA heteroduplexes.[71] A region near the N-terminus of WRN contains three conserved motifs that resemble the conserved motifs in the proofreading exonuclease domain of *E. coli* DNA polymerase I and in *E. coli* RNaseD.[72] In fact, WRN protein has an exonuclease activity although there is some disagreement on its directionality and dependency on helicase activity.[73-76] WRN exonuclease preferentially hydrolyzes alternative DNA structures that contain bubbles, extra-helical loops, or 3-way or 4-way junctions.[77,78] Like BLM, WRN can resolve aberrant DNA structures such as G-quadruplex and G-triplex DNAs.[43,79]

*WRN*-null mouse mutants are born at the expected Mendelian frequency and are healthy and fertile, showing no signs of premature organismic aging or increased rates of tumor formation. Cells from these animals do not show elevated susceptibility to genotoxins. Thus, the knockout of the *WRN* gene in mice does not recapitulate many of the phenotypes of human WS. It is possible that defective nucleolar localization of murine WRN protein may be related to this discrepancy of phenotypes.[68] However, mice lacking *WRN* display a shorter life span in a $p53^{-/-}$ background.[80]

WS cells exhibit a delayed and attenuated accumulation of p53 after exposure to UV irradiation.[81] Notably, WRN contributes to the induction of p53 by various DNA damaging agents. WRN and p53 can form specific protein-protein interactions through their respective C-terminal domains.[82] In addition, WRN has been shown to bind to and/or functionally interact with RPA,[83,84] proliferating cell nuclear antigen (PCNA), and DNA topoisomerase I.[85] Each of these interacting proteins is involved in DNA manipulations including those that resolve alternative DNA structures. WRN also interacts functionally with polymerase δ which is a eukaryotic polymerase required for DNA replication and repair.[86] WRN is phosphorylated in vitro by DNA-PK and requires DNA-PK for its phosphorylation in vivo.[87] WRN interacts directly with the catalytic subunit of DNA-PK (DNA-$PK_{CS}$), which inhibits both helicase and exonuclease activities of WRN. In addition, WRN forms a stable complex on DNA with DNA-$PK_{CS}$ and Ku 86/70.[87] Although Ku proteins have no effect on ATPase or helicase activity, they strongly stimulate the specific exonuclease activity of WRN.[88] When WRN is assembled with DNA-$PK_{CS}$ and Ku proteins, WRN enzymatic inhibition by DNA-$PK_{CS}$ is reversed. In response to replication blockage, WRN is phosphorylated in an ATR/ATM-dependent manner and colocalizes with ATR, suggesting that WRN and ATR kinase collaborate to prevent genomic instability during S phase.[89] WRN also forms a complex with RAD52 in vivo that colocalizes in foci associated with arrested replication forks.[90] WRN has been known to promote the ATP-dependent translocation of Holliday junctions, an activity that is also exhibited by BLM.[84] WRN increases the efficiency of RAD52-mediated strand annealing between non-duplex DNA and homologous sequences, whereas RAD52 can both inhibit and enhance WRN helicase activity in a DNA structure-dependent manner.[90]

Hypersensitivities of B-lymphoblastoid cells from WS patients to 4-nitroquinoline 1-oxide (4-NQO), etoposide and camptothecin (CPT) have been reported.[91-93] However, WS B-lymphoblastoid cells do not exhibit a hypersensitivity to other DNA-damaging agents such as most alkylating agents, and X-rays, bleomycin, or $H_2O_2$ that produce reactive oxygen species,[91,94,95] as well as to UV irradiation.[96] In chicken *WRN* mutant DT40 cells irradiated with UV light, the frequency of chromatid breaks are identical to that observed in wild-type cells.[97] In *WRN*/*BLM*-double knockout DT40 cells, UV irradiation in the late S to $G_2$ phase synergistically enhances the increase in the number of chromatid breaks.[97] *WRN*-mutant DT40 cells also exhibit an increased incidence of spontaneous HR. However, disruption of *WRN* partially diminishes the SCE frequency which is increased in *BLM*-mutant DT40 cells despite the fact that the SCE frequency does not change in *WRN*-mutant cells compared with wild-type cells.[97] These results imply that WRN may contribute to accelerate the post-replicational HR repair which occurs due to the absence of BLM.

## Rothmund-Thomson Syndrome Gene (RT)

Rothmund-Thomson syndrome (RTS) patients exhibit a heterogeneous clinical profile that includes a characteristic skin rash (poikiloderma), small stature, sparse hair, bony abnormalities, juvenile cataracts, and an increased risk of cancer, particularly osteosarcoma, a malignant primary bone tumor.[98] Several cases of RTS patients with frequent infections, impaired lymphocyte function, and decreased T lymphocyte and leukocyte counts have been reported, although distinct immunological dysfunction has not been observed in these patients.[99] A pure candidate gene approach showed that mutations in the *RECQL4* gene, which is located on human chromosome 8q24.3, occurred in at least some cases of RTS.[14] Neither complementation nor linkage studies have been reported, suggesting that mutations in more than one gene are responsible for RTS. In addition, the absence of detectable mutations in the *RECQL4* gene in approximately one-third of the patients has been reported in previous studies.[14,100] Thus, the finding of genetic heterogeneity in RTS contrasts with BS and WS.

*RECQL4*-knockout mice in which exons 5-8 were disrupted, die between embryonic day 3.5-6.5.[101] The growth rate of both the inner cell mass and trophoblast cells of the blastocysts from the *RECQL4*-deficient mice markedly decrease. Recent studies demonstrated that the exon 13-deleted *RECQL4*-deficient mice are viable, but exhibit severe growth retardation and abnormalities in several tissues, and that embryonic fibroblasts show a defect in cell proliferation.[102] These abnormalities in the *RECQL4*-deficient mice are similar to those exhibited in RTS patients. Exon 13 is one of the coding exons of the consensus RecQ-helicase domain. This domain is the primary site of mutations that have been identified in RTS patients.

In addition to WS cells and BS cells, RTS cells show genomic instability, including trisomy, aneuploidy and chromosomal rearrangements. However, in some cases of RTS, chromosomal breakage in lymphocytes is not notably greater than that of unaffected controls.[103-105] Conflicting studies report that cells from RTS patients exhibit both increased[106,107] or no change[108,109] in sensitivity to UV and γ irradiation. Embryonic fibroblasts derived from exon 13-deleted *RECQL4*-deficient mice do not exhibit a statistically significant difference in sensitivity to UV and ionizing radiation in comparison with wild type cells.[102] RTS cells exhibit normal sister chromatid exchange rates and a normal response to genotoxin-induced DNA breakage in vitro.[105] There has been comparatively little information available on the RECQL4 protein and proteins interacting with it, thus, functions of RECQL4 protein concerning DNA repair remain unclear.

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