G-quadruplex nucleic acids and human disease

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Abstract

Alternate DNA structures that deviate from B-form double-stranded DNA such as G-quadruplex (G4) DNA can be formed by sequences that are widely distributed throughout the human genome. G-quadruplex secondary structures, formed by the stacking of planar quartets composed of four guanines that interact by Hoogsteen hydrogen bonding, can affect cellular DNA replication and transcription, and influence genomic stability. The unique metabolism of G-rich chromosomal regions that potentially form quadruplexes may influence a number of biological processes including immunoglobulin gene rearrangements, promoter activation and telomere maintenance. A number of human diseases are characterized by telomere defects, and it is proposed that G-quadruplex structures which form at telomere ends play an important role in telomere stability. Evidence from cellular studies and model organisms suggests that diseases with known defects in G4 DNA helicases are likely to be perturbed in telomere maintenance and cellular DNA replication. In this minireview, we discuss the connections of G-quadruplex nucleic acids to human genetic diseases and cancer based on the recent literature.

Keywords

G-quadruplex DNA structures commonly referred to as G4 DNA have attracted considerable interest in recent years [1–3]. G4 DNA structures deviate from the canonical B-form Watson–Crick double helix by their Hoogsteen hydrogen bonding between four guanines which form a quartet, which stack to form the quadruplex. G-quadruplexes are stabilized by K^+ or Na^+ ions at physiological temperature and pH *in vitro*. The unconventional structure of G4 DNA has led researchers to believe that its unique properties are likely to impact cellular DNA metabolism. Despite evidence that G4 structures form readily *in vitro* under physiological conditions, the existence of G4 nucleic acid in living cells is still controversial. However, evidence suggesting that G-quadruplexes may form *in vivo* in G-rich regions of the human genome has cast a new light on the importance of studying G4 DNA metabolic processes. Several excellent reviews have covered various aspects of G4 structure and metabolism (see [4,5] and the accompanying minireviews in this series [6,7]). Aspects of telomere G-quadruplex structure and metabolism have also been discussed ([8] and the references cited therein), as well targeting telomeres and other DNA structures with G-quadruplex ligands [9,10]. The unique geometry of the G4 structure is thought to allow specific recognition by small molecules through various binding modes in a manner analogous to that of double-helical DNA intercalators. Several reviews have summarized the potential use of G4 DNA as a drug target [11–13], and are not discussed further here. We focus the discussion largely on the importance of G-quadruplexes in human disease and

summarize evidence from the literature for the relevance of these alternate DNA structures in molecular and cellular pathways of DNA metabolism that influence genomic integrity. A distinguishing feature of this minireview is the strong emphasis on helicases that resolve G-quadruplex nucleic acids.

Existence of sequences predicted to form G4 structures in the human genome

Using algorithms to predict the probability of DNA sequences to form G-quadruplex structures, it has been estimated that there are over 300 000 potential quadruplex sequences (PQS) in the human genome [14,15]. However, PQS are not randomly distributed. G-rich sequences are enriched at the 5′-end of human genes (5′-untranslated region, first exon and first intron), but not the coding regions [16], consistent with the notion that PQS contributes to the regulation of gene expression [17]. The abundance of sequences in oncogenes predicted to form G4 structures may influence expression or contribute to genomic instability [18]. Oncogenes that promote cell proliferation and tumorigenesis contain significantly higher PQS than tumor suppressor genes that maintain chromosomal integrity [16].

The G-rich human immunoglobulin heavy-chain switch regions are targeted for class switch recombination to promote antibody diversity [19,20]. Interestingly, the post-transcriptional structure at the immunoglobulin switch region forms a G-loop, as detected by electron microscopy, suggesting a possible role of the G-rich element in DNA rearrangement in switch regions [21]. However, the belief that G-quadruplexes form at single-stranded G-rich loops at switch regions is controversial [22].

Human repeat elements and G-rich triplet repeat disorders

The human genome contains tandem repeat blocks known as microsatellites (1–9 bp) or minisatellites (10–100 bp) that may be prone to expansion or contraction. Such repeat elements contribute to genomic instability and have been implicated in a number of human diseases (for review, see [23]). Expansion of intragenic triplet repeats is observed in human neurodegenerative diseases including ataxias and Fragile X syndrome (FXS). FXS is one of the most common forms of inherited mental retardation [24], and is the result of expansion of a CGG trinucleotide repeat in the 5′-untranslated region of the *FMR1* gene. When the CGG expansion repeats exceed 200 they become hypermethylated, causing transcriptional silencing of the *FMR1* gene, resulting in the loss of its encoded protein FMRP, which is a selective RNA-binding protein implicated in regulating dendritic mRNA transport and local protein synthesis at synapses. Because the unstable trinucleotide repeats can form alternate DNA structures, it is proposed that such structures are responsible for the expanded CGG repeats that interfere with cellular nucleic acid processes.

Fry and Loeb first reported that FXS CGG repeats could form stable G4 DNA in monovalent cation solution [25], and this was further supported by NMR evidence [26] and results from DNA polymerase arrest assays [27]. CGG repeat sequences that can readily form G4 DNA structures *in vitro* can be unwound by specific G4 DNA helicases such as WRN [28] and FANCJ [29] that are mutated in the genetic diseases Werner syndrome (WS) and Fanconi anemia (FA) complementation group J, respectively (see 'Helicase Disorders' section). However, CGG repeats can also form hairpin structures [30], which might be responsible for repeat expansion in genomes [31]. The importance of G4 and other alternate DNA structures in the genomic instability of FXS and other triplet repeat disorders is an active area of investigation [23,32,33]. Further development of antibodies that specifically recognize sequence- or structure-specific G4 and other alternate DNA structures will help to

advance the field's understanding of the mechanism of repeat tract instability. Recently, a single-chain antibody selective for G-quadruplex DNA was developed and found to regulate gene expression in human cells in a manner that correlated with the predicted formation of G-quadruplex structures [34]. However, the existence of G4 structures *in vivo* is still a matter of debate and additional experimental evidence is needed to establish their existence and importance.

Instability of G-rich telomere structures and human disease

Telomeres are specialized nucleoprotein complexes that reside at the physical ends of linear eukaryotic chromosomes and exist in all vertebrates (for review, see [35,36]). In most organisms studied, telomeres contain lengthy stretches of tandem-repeated simple DNA sequences composed of a G-rich strand and a C-rich strand (called 'terminal repeats'). These terminal repeats are highly conserved because all vertebrates appear to have the same telomeric DNA repeat of $(TTAGGG)_n$ for several kilobases.

The single-stranded G-rich 3′ overhang folds back and base pairs with the complementary sequence at the loop junction to form a t-loop structure. The area where the 3′ G-strand extension invades the duplex telomeric repeats is called a 'D loop' (displacement loop).

A growing number of proteins have been discovered that bind to the telomeric tandem DNA repeats, including the telomere protein complex known as shelterin and telomeric-associated factors, some of which have already been shown to play important roles in the maintenance of genomic stability and/or DNA repair [37]. Together, shelterin proteins and telomere-associated factors have important functions in the protection, replication and stabilization of the chromosome ends.

Two mechanisms to maintain telomere length have been described in human tumor cells [38,39]. The first requires a specialized enzyme telomerase which is able to copy as a reverse transcriptase the short TTAGGG motif at the 3′-end of telomeres. Telomerase activity in most human tissues is tightly regulated, leading to gradual telomere shortening with cell divisions [35]. Shortening beyond a critical length causes telomere uncapping, manifested by the activation of a DNA damage response and cell-cycle arrest. The second mechanism is observed in tumors (~ 15%) as well as in immortalized cell lines lacking telomerase activity and involves recombination between telomeres, a mechanism known as alternative lengthening of telomeres (ALT).

Shorter telomere length is a risk factor for the development of cancer [40,41]. Clinical data revealed that telomere length is shorter in subjects with different types of cancer, including cancers of the head, neck, breast, bladder, prostate, lung and kidney [42]. In addition, telomerase is activated in ~ 90% of cancer cells. However, a sizeable fraction of cancerous cells employ the ALT pathway to transfer tandem repeats between telomeres or with extrachromosomal telomere-repeat-containing sequences, resulting in more heterogeneous telomere lengths [43].

Although telomere structure and/or function appears to be defective in certain human diseases, the precise nature of the molecular defects at chromosome ends remains under active investigation. Different forms of anemia, hypertension, coronary heart diseases, chronic human immunodeficiency virus infection, ulcerative colitis and chronic liver disease are among the diseases with defects in telomerase or telomere stability [40,44]. Bone marrow failure syndromes represent a diverse group of diseases with similar phenotypes, including dyskeratosis congenita, aplastic anemia and myelodysplatic syndromes [45]. The fact that patients with bone marrow failure syndromes have shortened telomeres led researchers to screen these patients for mutations in telomerase and components of the

shelterin complex. These efforts resulted in the identification of mutations in hTERT [46], hTERC [47], the telomerase-associated proteins dyskerin [46], NOP10 [48], NHP2 [49] and the shelterin component TINF2 [50,51]. These findings support the hypothesis that dysfunctional telomeres due to mutations in telomere maintenance genes promote exhaustion of the stem cell compartment and defects in cell types that have a high turnover rate such as the hematopoietic system.

Telomeric 3′ G-rich tails play an important role in the protection, replication and stabilization of chromosome ends

Human telomeric DNA can spontaneously assemble into a number of different G-quadruplex conformations, as demonstrated by NMR and X-ray crystallography [52–55] or atomic absorption spectroscopy, confirmed by fluorescence resonance energy transfer and CD spectral analysis [56]. However, the existence of G-quadruplex structures *in vivo* has been debated. Studies on the telomeres of ciliated protozoa have provided the most direct evidence that G4 DNA forms at telomeres *in vivo* [57], consistent with their ability to fold spontaneously into G4 DNA *in vitro* under physiological conditions [58,59]. Owing to the high concentration of telomeric sequences in the transcriptionally active macronucleus, ciliated protozoa were among the first model organisms characterized for telomere composition, structure and behavior. Demonstration that G4 DNA forms *in vivo* has proven difficult, although several studies do provide strong support for its existence at telomeres. The development of antibodies specific for telomeric G4 DNA structures enabled researchers to visualize antiparallel G4 structures by immunostaining in the model organism *Stylonychia* [60]. The staining of *Stylonichia* telomeres with the anti-G4 DNA serum was shown to depend on expression of the natural telomere end-binding proteins (TEBP)-α and TEBP-[61]. TEBP-is required for recruitment of TEBP-β to telomeres, and TEBP-β was observed to catalyze G4 DNA formation *in vitro*. Importantly, this work showed that the staining with the anti-G4 serum was not simply due to stabilization of the G4 fold by the antibody, but rather depended on the natural (i.e. G4) structure of the telomere *in vivo*. Another piece of supporting evidence that a G4 DNA structure can form at the telomere end *in vivo* was provided in a recent article by Zhang *et al*. which showed that the same amino acids found in a proposed divalent cation-binding motif of *Saccharomyces cerevisiae* Est1 protein are required for it to promote G4 DNA formation *in vitro* are required for the protein to stimulate telomere extension by telomerase [62], arguing that G4 DNA also forms at yeast telomeres and can play a positive role in extension of telomeres by telomerase.

If telomeres do indeed form G4 structures *in vivo*, it will be of interest to determine whether special G-quadruplex configurations at telomeres play a role in their stability in mammalian cells. Biochemical studies suggest that G-quadruplexes preferentially form at the 3′-end of telomeric DNA rather than internal positions [63]. The stable G4 structure may pose a challenge to the replication or DNA repair machinery, or the ability of telomerase to elongate the 3′ single-stranded tail assuming a G-quadruplex topology [64]. However, research from the Bryan laboratory has demonstrated that *Tetrahymean thermophila* telomerase can readily extend telomere sequences *in vitro* that are in a G4 configuration [65].

The notion that telomeric overhangs may form G-quadruplexes in cells is supported by indirect experimental strategies which are able to overcome challenges such as reduced telomere concentration and the potentially transient nature of G4 structures. Exposing human cells to G4 DNA-binding compounds such as telomestatin (TMS) induces the dissociation of shelterin proteins (e.g. POT1, TRF2) or telomere-associated proteins (e.g. TOPIII) from their telomeric sites [66–68]. TMS may compete with such proteins for binding to G4 DNA or stabilize a G4 structure that is not favorably bound by the telomere-

interacting protein, leading to telomere uncapping in ALT cells [66,69]. TMS can also effectively reduce proliferation of telomerase positive tumor cells by inhibiting telomerase [68,70]. In future, small molecules that specifically bind G4 DNA structures may be useful for the treatment of cancer and other diseases.

Human proteins that bind G-rich DNA

Certain DNA helicases genetically linked to human diseases characterized by chromosomal instability have been shown to catalytically unwind G4 DNA substrates *in vitro* (Table 1). In addition, a number of human nuclear proteins have been reported to preferentially bind G-rich DNA that can form quadruplexes *in vitro* (Table 2). For example, the nuclear proteins poly[ADP-ribose]polymerase 1 (PARP-1), Ku70/Ku86 and heterogeneous nuclear ribonucleoprotein A1 all are associated with the G-rich promoter of the *KRAS* oncogene which is one of the most frequently mutated oncogenes in human cancer [71,72]. It is proposed that *KRAS*-associated proteins regulate its expression, and may represent targets for anti-cancer strategies to inhibit *KRAS* gene expression. Human nucleolin can bind G4 DNA with high affinity [73,74] and stabilize G4 DNA in the C-MYC promoter, thereby inhibiting *C-MYC* promoter-driven transcription as measured by luciferase assays [75].

An example of a human nuclear DNA repair protein that preferentially binds G-rich repetitive DNA is the MutSα heterodimer (MSH2/MSH6) [21]. Association of MutSα with the G-rich repetitive switch regions was demonstrated by chromatin immunoprecipitation assay [21]. Deletion of Msh2 or Msh6 in mice resulted in decreased switch recombination and reduced heterogeneity of switch junctions [76–78]. MutSα was also shown to bind the G-loop of transcribed S regions, as detected by electron microscopy, further suggesting a potential role of the MSH2/MSH6 protein complex in PQS metabolism of G-rich elements [79].

Understanding the biological importance of preferential G-rich DNA binding by the MutSα complex and other protein factors (Table 2) is an active area of investigation. Whereas some G-rich proteins stabilize the quadruplex structure, other proteins can destabilize certain G4 structures *in vitro* in an ATP-dependent manner (helicases, discussed below) or ATP-independent manner (e.g. replication protein A; RPA) [80]. The human shelterin protein POT1 has been shown to disrupt telomeric G-quadruplexes allowing telomerase extension *in vitro* [81].

Supporting evidence that certain genetic helicase disorders have defects in G-quadruplex DNA metabolism

In the previous sections, we highlighted some diseases with G-rich repeat tract instability or telomere defects that may arise from the instability or improper metabolism of G-quadruplex structures. It is plausible that G4 structures in certain regions of the genome contribute to poor maintenance of genomic stability characteristic of DNA repair disorders or certain cancers. The chromosomal instability disorders WS, Bloom's syndrome (BS) and FACGJ are all characterized by autosomal recessive mutations in genes encoding DNA helicases that unwind G-quadruplex DNA *in vitro* (Table 1). We briefly discuss each of these disorders, highlighting evidence that defects in G4 metabolism may contribute to the cellular phenotypes of individuals with these genetic helicase diseases.

Fanconi anemia complementation group J

FA is a genetic disease characterized by bone marrow failure and a predisposition to cancer [82–84]. Some FA patients also show congenital abnormalities, growth and endocrine

abnormalities, infertility and hematologic manifestations [82]. The incidence of FA has been estimated at 1–5 per million, with a carrier frequency of 1 in 300. The cellular characteristics of FA are chromosome instability and hypersensitivity to genotoxic stress by DNA interstrand cross-linking (ICL) agents such as cisplatin and mitomycin C.

FA results from mutations in one or more of 13 complementation genes (FA-A, -B, -C, -D1, -D2, -E, -F, -G, -I, -J, -L, -M and -N). One of the more recently identified FA genes implicated in FA complementation group J is *FANCJ* [85–87]. FANCJ was originally designated BACH1 (BRCA1 associated C-terminal helicase), a protein that binds to the BRCT repeats of the tumor suppressor BRCA1 [88]. Like other FA mutant cell lines, FANCJ-deficient cells are sensitive to ICL agents as evidenced by their reduced cell viability and accumulation of 4N DNA content, representing cells arrested in either late S or G_2/M [85,87]. Following cellular exposure to ICL agents, FA-J mutant cells are characterized by elevated chromosomal aberrations such as chromosome breaks, quadriradials and triradials [87,89].

Although FANCJ is proposed to function downstream of FANCD2 monoubiquitination in the FA pathway, the precise role(s) of FANCJ in cross-link repair or the replicational stress response remains poorly understood. The demonstration that two *FANCJ* mutations detected in women with early-onset breast cancer result in the synthesis of enzymatically defective proteins suggests that *FANCJ* is a breast cancer tumor suppressor gene [90]. Notably, the 2533C→T nonsense mutation in exon 17, resulting in a premature stop codon (R798X) has been reported in a high percentage of FA-J patients, as well as in breast cancer patients [91]. Several missense mutations have been reported in FANCJ that may be associated with inherited breast cancer cases [91–97].

Evidence from genetic studies of *Caenorhabditis elegans* suggests that a FANCJ ortholog prevents genomic instability in G-rich tracts [98]. We summarize some of the key findings from this and related work, which set the stage for recent studies suggesting that human FANCJ resolves PQS to maintain chromosomal stability.

Biology of FANCJ-like proteins suggest a role in the metabolism of G-rich DNA structures

Convincing evidence for a defect in the metabolism of G-rich sequences *in vivo* was provided by the Lansdorp group who characterized the genomic instability of the *C. elegans dog-1* mutant, an ortholog of mammalian FANCJ. Nematodes mutated in *dog-1* showed germline as well as somatic deletions in genes containing polyguanine tracts [99]. In 2008, the Boulton laboratory further characterized the genomic instability of *dog-1* mutants and reported that mutations in other FA genes of the worm do not interfere with G/C tract maintenance [100]. In the same year, the Tijsterman laboratory also reported massive genome rearrangements at G4 DNA sites in *dog-1* animals [101]. Taken together, the results in *C. elegans* strongly suggest that DOG-1 plays an important role in maintaining genetic integrity by preventing the accumulation of aberrant DNA structures that form in G-rich tracts. However, there is as yet no biochemical evidence that DOG-1 is a helicase or has the ability to resolve G-quadruplex structures.

Evidence that DOG-1 plays a critical role in genome maintenance through its action at G-rich loci led researchers to search for mammalian orthologs of DOG-1. A gene named *rtel* that encodes a protein sharing sequence homology with DOG-1 was first identified by genomic mapping of loci that control telomere length differences between *Mus musculus* and *Mus spretus* [102]. *rtel* knockout mice were embryonic lethal and cells derived from these mice exhibited a rapid reduction in proliferative capacity upon differentiation,

accompanied by an increased incidence of chromosomal abnormalities and telomere loss [102]. Interestingly, *rtel* mutant worms do not display G-rich sequence deletions, suggesting that RTEL and DOG-1 have distinct functions and operate in different pathways [103]. *rtel-1* mutant worms and RTEL1-depleted human cells share phenotypes characteristic of yeast *srs2* mutants: lethality upon deletion of the *sgs1/BLM* homolog, hyperrecombination, and DNA damage sensitivity [103].

In vitro, purified human RTEL1 antagonizes homologous recombination by promoting the disassembly of D loop recombination intermediates in a reaction dependent upon ATP hydrolysis [103]. However, RTEL1 does not dismantle RAD51 protein filaments. Taken together, these results suggest that RTEL not only regulates telomere metabolic events at the chromosome end, but may also act as an anti-recombinase to dismantle recombination intermediates and allow completion of repair by homologous recombination. Although RTEL shares sequence homology with FANCJ, whether mammalian RTEL could resolve G-quadruplex structures is not known. Clearly, biochemical studies of DOG-1, RTEL and other proteins sharing sequence homology with FANCJ [98] should be informative to understand their mechanism(s) of action.

Evidence that human FANCJ unwinds G4 DNA *in vitro* and has a role in the metabolism of G-rich sequences in cells

Human FANCJ was demonstrated to efficiently unwind G-quadruplex DNA substrates flanked by a 5′ single-stranded (ss)DNA tail in an ATP hydrolysis-dependent manner *in vitro* [29], and shown to resolve a variety of G4 DNA structures composed of human genomic DNA sequence such as those observed in telomeres and Fragile X triplet repeats [29,104]. Unwinding of G4 DNA by FANCJ *in vitro* is stimulated by the human single-stranded DNA binding protein replication protein A (RPA), but is inhibited by MutSα, a mismatch repair protein complex that binds G-rich repetitive DNA with high affinity [29]. FANCJ unwinding of G4 DNA structures but not duplex DNA is also inhibited by TMS, a G4-specific DNA binding compound [29]. FANCJ-depleted human cells are sensitive to TMS, and show elevated DNA damage and apoptosis upon exposure to the drug [29]. Double-strand breaks marked by γ-H2AX foci were observed to accumulate in FANCJ-depleted cells exposed to TMS, suggesting that FANCJ prevents replication-associated DNA damage by removing G-quadruplex structures which can no longer be bound with high affinity by TMS. The role of FANCJ in the metabolism of PQS appears to be unique among the FA proteins because neither FA-D2 nor FA-A mutant cells are hypersensitive to TMS [29]. The fact that FA-D2 and FA-A cells are not sensitive to TMS suggests that FANCJ is operative in at least these other FA mutant cell lines, and so G4 DNA would not be predicted to be increased. The strong co-localization of FANCJ with RPA after nucleotide depletion by hydroxyurea exposure [105] suggests that the two proteins collaborate in a situation of replicational stress that might arise when the replication fork is impeded by an obstacle such as a PQS.

It has been reported that FANCJ helicase activity is manifest in the S phase and is required for normal S-phase progression [106], suggesting that FANCJ removes structural barriers to replication. Indeed, cells that are deficient in FANCJ accumulate deletions at genomic sequences with a G4 DNA signature [104]. Comparative genome hybridization was performed on genomic DNA extracted from FA-J patient cell lines encoding a truncated FANCJ protein, and human FA-D2 cell line or normal human fetal fibroblast cells. From the genomic deletions identified as unique to FA-J cells, a bias toward breakpoint regions adjacent to DNA sequences with a G4 DNA signature was identified. This bias was most pronounced among larger deletions (~ 36 kb or more), where nearly 75% possess sequences with the G4 signature in the vicinity of the breakpoint [104]. The results suggest that

FANCJ-deficient cells accumulate genomic deletions in the vicinity of sequences with the G4 signature. A model is suggested that in FANCJ-deficient cells the replication fork stalls at a G-rich region that has a propensity to form G4 DNA. Conceptually, because FANCJ is not present to unwind the G4 DNA, the accumulation of stalled replication forks lead to chromosomal instability.

RecQ helicase disorders Werner syndrome and Bloom's syndrome

WS and BS are autosomal recessive diseases with mutations in the RecQ helicase genes *WRN* and *BLM*, respectively [107,108]. Both diseases are characterized by chromosomal instability. WS is a premature aging disorder that displays many clinical symptoms of age-associated diseases such as cancer, atherosclerotic cardiovascular disease, diabetes mellitus (type II) and osteoporosis [109]. Unlike normal individuals, WS patients are highly susceptible to early onset of sarcomas and mesenchymal tumors. The genomic instability of WS has been described as variegated translocation mosaicism punctuated by chromosomal deletions and rearrangements as well as elevated spontaneous mutations [110,111].

In contrast to WS, the hallmark of BS is elevated sister-chromatid exchange [112]. BS is associated with a very high incidence of different types of cancers, solid tumors and leukemia, as well as sarcoma (e.g. osteosarcomas). BS is characterized by skin disorders, proportional dwarfism, immunodeficiency and male sterility [113]. Both BS and WS cells are hypersensitive to various agents that induce DNA damage or interfere with replication, including topoisomerase I inhibitors or DNA ICL agents. BS and WS cells also progress more slowly through the S phase than wild-type cells, suggesting that cell-cycle or replication checkpoints may be activated in these cells, even in the absence of cellular stress. Although both WRN and BLM encode DNA helicases, the precise roles of these enzymes to confer genomic stability and insure proper cellular DNA replication are still not well understood.

Preferential unwinding of G-quadruplex DNA structures *in vitro* by the WRN and BLM helicases

Only a limited number of DNA helicases have been shown to unwind G4 structures (Table 1). Among these, WRN and BLM helicases have been found to efficiently unwind a variety of G4 DNA structures *in vitro*, including those composed of G-rich sequences found in the human genome [114,115]. The ability of BLM to preferentially unwind G4 DNA relative to HJ substrates reflects an increased G4 binding affinity that is mediated by the RecQ C-terminal (RQC) domain located just after the helicase core domain [116]. However, an RQC motif is not universally required for G4 unwinding activity because the Superfamily 2 helicase FANCJ lacks a recognizable RQC domain but efficiently unwinds G-quadruplex structures [29,104]. Moreover, human RECQ1, which contains a sequence resembling the RQC motif in WRN and BLM, fails to unwind G-quadruplexes despite its efficient ability to unwind duplex and three-stranded D-loop structures [29,117]. This suggests that not all RQC domains are the same. Some (like those in Sgs1, WRN and BLM) can bind G-rich DNA that forms G4 DNA structures *in vitro*, whereas the RQC domain in RECQ1 may not be able to. This view is consistent with the fact that the RQC domain from any given protein generally binds more than one nucleic acid structure [116,118].

Directionality of unwinding distinguishes the action of WRN and BLM helicases from FANCJ on G-quadruplex DNA structures

One distinction between FANCJ and the human RecQ helicases (BLM, WRN) that unwind G4 structures is the requirement by FANCJ for a 5′ ssDNA tail in the G4 substrate [29,104], whereas WRN and BLM require a 3′ ssDNA tail to unwind at least some G4 substrates [114,115] (Fig. 1), with the possible exception of a d(CGG) Fragile X triplet repeat G4 DNA substrate in which a structure with a 5′ tail was unwound by WRN [28]. The tail requirements likely reflect the directionality of DNA unwinding because FANCJ has been shown to unwind duplex DNA substrates 5′ to 3′ with respect to the strand that it is presumed to translocate, whereas WRN and BLM unwind with a 3′ to 5′ directionality.

The interaction between WRN helicase and DNA polymerase δ specifically facilitates DNA synthesis through quadruplex and hairpin structures of the d(CGG)$_n$ trinucleotide repeat sequence [119], suggesting a role of WRN helicase for enabling replication progression through genomic DNA roadblocks such as secondary structure. In this regard, WRN (and BLM) seem better suited to operate in concert with a DNA polymerase synthesizing nascent DNA complementary to the template when a single-stranded loading site 3′ to the G-quadruplex is provided. The same directionality of helicase and polymerase translocation may enable DNA synthesis through G-rich tracts prone to form quadruplexes during cellular processes of replication or DNA repair.

As suggested in a review by Maizels [64], leading strand synthesis arrested by the G4 structure may result in a duplex state or stalled DNA polymerase and associated factors immediately adjacent to the G-quadruplex structure that would not permit loading of the WRN or BLM helicases. Single-stranded DNA that accumulates from unwinding at the replication fork catalyzed by the replication fork leading helicase (MCM) would be coated by RPA. The demonstration that RPA stimulates FANCJ unwinding of G4 structures [29] suggests that FANCJ may be able to load onto RPA-coated tails and unwind the adjacent quadruplex structure. Perhaps FANCJ helicase enables smooth replication of G4 structures by 'turning its shoulder' as it unwinds the G4 structure to enable the DNA polymerase enzyme advancing from the opposite direction to proceed unimpeded past the FANCJ helicase as it unwinds the G4 obstacle (Fig. 2). Presumably, this would involve a conformational change enabling the polymerase and helicase to pass one another in a coordinate fashion. Shoulder turning by FANCJ and/or the DNA polymerase could be useful in lagging strand synthesis because FANCJ could then move along to the next Okazaki fragment where it might also be needed. For leading strand synthesis, FANCJ might work at gaps on the leading strand template left far behind the advancing fork. Because the unwound G4 structure may form again relatively quickly, the efficiency of polymerase synthesis using the unwound G-rich region as a template might be important. In such a scenario, FANCJ helicase may have to unwind G4, turn its shoulder, and allow polymerase to move past it and catalyze DNA synthesis.

A reconstituted system that measures DNA synthesis through a G-quadruplex forming sequence with G4 DNA helicases and replication-associated factors would help to address how these replication roadblocks are tolerated. Investigation of protein interactions between helicases that resolve G-quadruplexes and cellular factors such as those that directly bind G-rich sequences (Table 1) may help to better understand the metabolism of G4 structures during cellular DNA replication or transcription, processes that are likely to be affected by G4 DNA.

The yeast RecQ helicase Sgs1 was suggested to have a role in gene expression through its ability to unwind G4 DNA based on a recent study showing that genes with altered

expression are preferentially those with the potential to form G4 structures [120]. Very recently, it was also reported that WS and BS cells have preferentially altered expression of genes with PQS [121]. It will be of interest to analyze transcription initiation and elongation of G-rich sequence elements in a reconstituted system to evaluate the importance of G4 binding factors and helicases for transcription.

Mouse studies suggest that premature aging and chromosomal instability of Werner syndrome reflects a role of WRN at telomeres

Although biochemical experiments demonstrate that the WRN and BLM helicases can efficiently unwind G4 structures *in vitro*, biological studies have only begun to elucidate the importance of WRN or BLM unwinding G-rich sequence elements *in vivo*. Model systems such as the WRN knockout mouse and defined human cell lines have been helpful in showing that WRN plays an important role in the metabolism of a G-rich DNA structure, the telomere. Although a disease phenotype in WRN-deficient mice is absent, late-generation telomerase and WRN-deficient mice ($mTerc^{-/-}$ $WRN^{-/-}$) have dysfunctional telomeres and exhibit many of the clinical symptoms of premature aging and the types of tumors (osteocarcinomas, soft tissue sarcomas) typically observed in WS patients [122]. $mTerc^{-/-}$ $WRN^{-/-}$ cells displayed replicative senescence, chromosomal instability, accelerated telomere shortening and elevated recombination rates between the telomeres of sister chromatids [123]. Normal telomere sister-chromatid exchange was restored by the expression of helicase-active WRN protein. *WRN*-deficient telomere dysfunctional cells escaped senescence by the ALT pathway, suggesting that the chromosomal instability and cancer of WS is a consequence of aberrant telomere sister-chromatid exchange that activates the ALT pathway (for a review, see [124]).

$mTerc^{-/-}$ $BLM^{-/-}$ later-generation mice also showed an acceleration of phenotypes characteristic of later-generation *Terc* mutants and showed some characteristics such as bone loss reminiscent of BS [125]. $mTerc^{-/-}$ $WRN^{-/-}$ $BLM^{-/-}$ triple mutants showed even more severe phenotypes suggesting a synergistic relationship between the WRN and BLM helicases in telomere maintenance [125]. Overall, the mouse studies provided evidence that the complex pleiotropic phenotypes of *WRN* or *BLM* deficiency in mice relate to telomere shortening.

WRN confers telomeric stability by its helicase action on the G-rich lagging strand

The first evidence to suggest a role of human WRN in the maintenance of telomeres was that the cells cultured from WS patients senesce prematurely and display elevated telomere shortening rates [126,127]. Partial localization of WRN to telomeres and its interaction with telomere-associated factors further suggested a function of WRN at telomeres [128]. Cells deficient in the WRN protein display deletion of telomeres from sister chromatids [129]. Curiously, only telomeres that underwent lagging strand synthesis exhibited sister chromatid deletions, and the telomere loss was prevented by WRN helicase activity. Expression of telomerase activity could circumvent the lagging strand telomere deletions. From these elegant studies, the Karlseder laboratory concluded that cells lacking WRN helicase activity are defective in telomere lagging strand synthesis. This work is very significant in the field because it provided a signature form of genomic instability that characterized WS, a model disease for understanding aging. Demonstration that WRN-deficient cells were characterized by a telomere defect in the G-rich strand suggested that the ability of WRN to resolve G-quadruplex structures may be relevant to the peculiar form of telomeric DNA instability characteristic of the disease.

The Karlseder laboratory went on to further dissect the cause of genomic instability in telomeres of WRN-deficient cells by showing that replication-associated telomere loss is responsible for the chromosome fusions in WS fibroblasts [130]. Telomere extension by telomerase could prevent the accumulation of new chromosomal aberrations in WRN-deficient cells [130]. Altogether, the characterization of a telomere lagging strand defect in WS and the requirement of WRN helicase activity to prevent telomere deletion from sister chromatids point toward a specialized role of WRN to unwind an alternate DNA structure that forms in the G-rich strand of the telomere end. The ability of WRN to preferentially unwind G-quadruplex structures may be important for replication and telomere stability.

The stochastic loss of telomere length in WS cell lines may directly contribute to their premature senescence. The observation that the premature senescence and genomic instability prevalent in WS cells can be overcome by forced telomerase expression further suggests a role of WRN in telomere maintenance [131]. WRN and BLM interactions with shelterin proteins [132–134] and other telomere-associated factors [38] are consistent with a model that these helicases act directly on telomere-associated DNA structures. Based on the cellular evidence that WRN is necessary for efficient replication of G-rich telomeric DNA, and telomere lagging strand synthesis is defective in cells lacking WRN helicase activity, it is reasonable to propose that WRN action on telomeric G4 DNA structures is important for telomere maintenance.

Telomere-associated proteins and fragile-telomere phenotype

Using a procedure called proteomics of isolated chromatin segments, Dejardin and Kingston purified human telomeric chromatin and identified the majority of known telomere-associated proteins as well as novel factors [135]. Interestingly, the BLM and FANCJ helicases, but not WRN, were found specifically bound to ALT telomeres. Given the evidence that WRN is found at telomeres of ALT cells [132], it is conceivable that the WRN interaction is transient and was disrupted by the purification procedure. The FANCJ localization at telomeres of ALT, but not telomerase-positive cells, was verified by co-immunostaining with TRF2 [135].

Recent evidence implicates the shelterin protein TRF1 in suppression of telomere fragility as well as degenerative pathologies and cancer in mice [136,137]. Telomeres resemble aphidicolin-induced fragile sites that require TRF1 for stability and their efficient replication. TRF1 promotes efficient replication of TTAGGG repeats and prevents their stalling. To explore the hypothesis that TRF1 might alleviate telomere replication problems by recruiting G4 DNA helicases, various helicase-deficient mouse cells were examined for spontaneous fragile telomeres [137]. Both BLM and RTEL, but not WRN, were required to repress the fragile-telomere phenotype. Future work in this area will likely address the importance of TRF1–helicase interactions to prevent fork stalling and fragility at telomeres in mammalian cells.

Other G4 DNA helicases with roles in nucleic acid metabolism

DHX36

A novel G4 DNA resolvase activity from human placental tissue was isolated ~ 13 years ago [138]. The G4 resolvase was able to catalytically unwind four-stranded G4 DNA to single-stranded DNA in an ATP- and Mg^{2+}-dependent manner, but failed to unwind duplex or Hoogsteen-bonded triplex DNA. More recently, the same group purified this major non-RecQ, NTP-dependent G4 DNA-resolving enzyme from human cell lysates using G4 DNA affinity chromatography [139]. The protein was identified as the DEXH box helicase product of gene *DHX36* (also known as *RHAU*) by mass spectrometry. The gene encoding

DHX36 was cloned and the protein overexpressed in *Escherichia coli* from which it was purified to apparent homogeneity. The recombinant DHX36 protein displayed robust, highly specific G4 DNA-resolving activity. This enzyme preferred G4 DNA structures over conventional duplex molecules, and its NTPase activity and Mg^{2+} dependence characteristics were consistent with the properties of the native protein. Immunodepletion experiments using human cell lysates demonstrated that DHX36 protein was responsible for the majority of the detectable G4 DNA resolvase activity.

G4 Resolvase 1 (G4R1) encoded by DHX36 was further characterized and shown to bind and unwind both DNA and RNA quadruplexes with high affinity *in vitro*. RNA interference experiments demonstrated that G4R1 is the major source of G4 DNA and G4 RNA unwinding activity in HeLa cell lysates [140]. G4R1/RHAU is an RNA helicase associated with the AU-rich sequence of mRNAs that accelerates their degradation [141]. Upon transcription arrest, G4R1/RHAU was observed to colocalize in nucleolar caps and become associated with the RNA-processing helicases p68 and p72 [142]. Taken together, it is proposed that G4R1/RHAU may have an important role in RNA processing in mammalian cells, presumably mediated by its interaction with mRNA transcripts and robust G4 RNA-resolving activity.

It is notable that DHX36 shares sequence homology within the helicase core domain with *C. elegans* DOG-1 which is important for G-rich tract stability [99,101] and mouse Rtel that is important for maintaining telomere length [102]. Based on the biochemical studies of DHX36 (G4R1) from the Akman and Vaughn laboratories, it will be important to determine the biological consequences of DHX36 deficiency since the protein represents the major G4 DNA- and G4 RNA-resolving activity in human cells.

Dna2

Dna2 is a multifunctional enzyme with 5′–3′ DNA helicase, 3′ and 5′ exo/endonuclease, single-strand annealing and strand exchange activities [143]. Biochemical and genetic evidence (largely from yeast) suggest that Dna2 plays an important role in conjunction with Flap endonuclease 1 (FEN1) for Okazaki fragment maturation during cellular DNA replication [144,145] and in DNA repair [146]. Yeast Dna2 is localized to telomeres in both the G_1 and G_2 phases of the cell cycle [147]. Genetic evidence in yeast suggests that Dna2 is involved in the generation of G-rich overhangs, suggesting a role of Dna2 in processing of telomeric ends [148].

Recently, Masuda-Sasa *et al.* found that human and yeast Dna2 unwind G4 DNA structures in an ATP-dependent manner *in vitro* [149]. It was proposed that Dna2 resolves G4 DNA on G-rich flaps through its helicase and/or nuclease activity in conjunction with RPA, to prepare a substrate for FEN1. Dna2 may act upon G4 structures that form in ribosomal DNA because yeast Dna2 has been shown to be important for its stability [149]. Alternatively, the localization of Dna2 at telomeres and genetic evidence that Dna2 plays a role in telomere maintenance suggests that the enzyme may process telomeric G4 structures [147]. Although genetic and biochemical evidence demonstrates that Dna2 is important for DNA replication, a human disease genetically linked to mutation in Dna2 is yet to be identified, possibly because it is an essential gene.

Pif1

Pif1 is an evolutionarily conserved helicase involved in the maintenance of both nuclear and mitochondrial genomes [150]. Using yeast as a model organism, Ribeyre *et al.* determined that Pif1 prevents genomic instability of a G-quadruplex forming human minisatellite CEB1 sequence that was inserted into the genome of *S. cerevisiae* [151]. CEB1 repeats were found

to be unstable in *pif1* deletion cells only if the synthetic CEB1 minisatellites were able to form G4 structures *in vitro*. Mutations in the yeast helicase genes *sgs1*, *dna2* or *rrm3* did not destabilize the CEB1 locus. Consistent with the *in vivo* results, purified recombinant yeast Pif1 efficiently unwound G4 structures derived from the synthetic CEB1 arrays *in vitro*. It was proposed that the defective mitochondrial DNA maintenance of a *pif1* mutant reflects defective processing of G4 structures that are prone to undergo recombination at the sites of breaks or at the G4 structures themselves. Nuclear Pif1 is implicated in telomere metabolism and was previously shown to inhibit telomerase [152]. Ribeyre *et al*. propose that Pif1 may also unwind G4 structures in telomeric DNA to insure its proper architecture [151]. The action of Pif1 on G4 DNA may also play a role in the replication or repair of nuclear DNA outside telomeres, based on its genetic interaction with Dna2 [153] and its importance in maintaining the replication fork barrier in G-rich ribosomal DNA that can also potentially form G-quadruplexes [149].

The specificity of Pif1 in conferring CEB1 stability suggests that other yeast G4 helicases may recognize G4 structures distinct from the human CEB1 G4 structure acted upon by Pif1. Applying the knowledge gained from the yeast Pif1 studies to mammalian systems will be a high priority to determine how human Pif1 is important for genomic stability through its action on alternate DNA structures such as G4. Human G4 DNA helicases genetically linked to human disease may act upon specific DNA structures that arise in distinct pathways of nucleic acid metabolism. It is interesting to note the number of different helicases that can process G4 structures (Table 1). Perhaps it relates to the fact that G4 species represent a large family of structures, or that the particular subgenomic location or phase of the cell cycle in which G4 structures form or persist require different activities.

Conclusions

The evidence that G-rich sequences can form G4 DNA *in vitro* and that G-rich sequences influence genomic stability raises challenges to researchers in the field. More elegant methods to detect G-quadruplexes would be extremely helpful to assess their abundance, enabling researchers to study the effects of genetic mutations that are linked to human disease on the prevalence of G4 nucleic acids. Genetic and cell biological systems to carefully evaluate the metabolism of G-rich DNA sequences should be further developed. Small molecules that specifically bind distinct G4 DNA structures will continue to be useful not only as tools to probe G4 dynamics but also potentially as anti-cancer drugs that target the regulation of oncogene expression, telomere maintenance and ultimately the nucleic acid structures and pathways in cancerous cells. Clear demonstration that G-quadruplexes exist *in vivo* is a high priority for the field. If this is accomplished, then researchers can focus on understanding how G4 nucleic acid structures are important in human disease.

Abbreviations

ALT	alternative lengthening of telomeres
BS	Bloom's syndrome
FA	Fanconi anemia
FXS	Fragile X syndrome
G4	G-quadruplex
ICL	interstrand cross-linking
PQS	potential quadruplex sequence

RPA	replication protein A
RQC	RecQ C-terminal
TEBP	telomere end-binding proteins
TMS	telomestatin
WS	Werner syndrome

Acknowledgments

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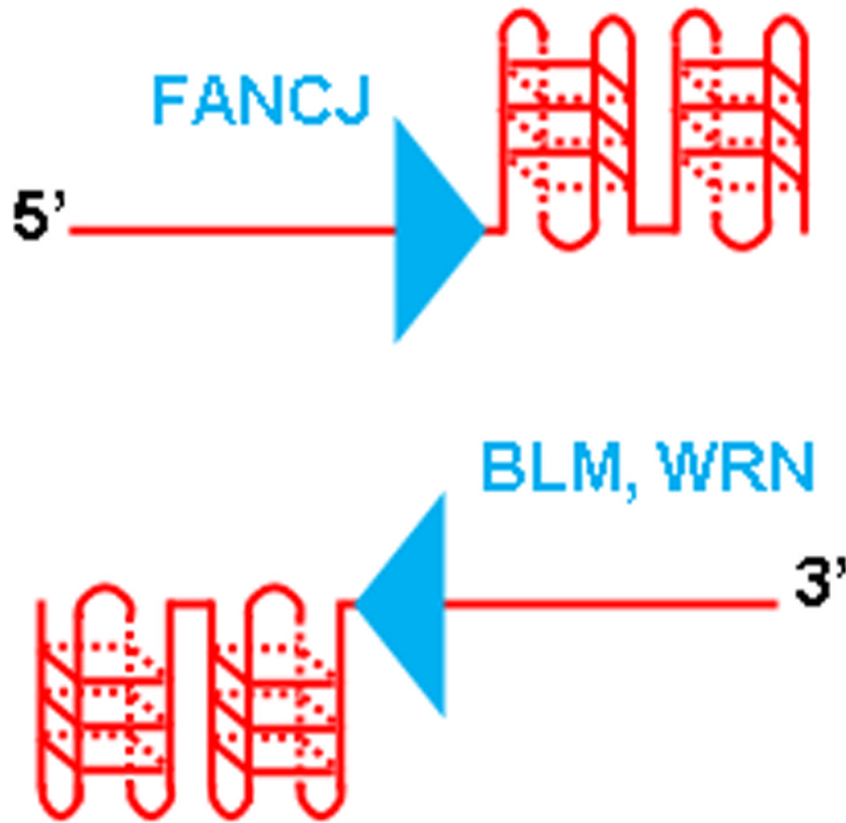
Fig. 1.
Disease-causing mutations in the FANCJ or WRN and BLM helicases affect G4 DNA
unwinding enzymes with opposite polarities of action. The FANCJ helicase mutated in FA
complementation group J requires a 5′ ssDNA tail to unwind the adjacent G4 structure. The
WRN and BLM helicases mutated in Werner syndrome and Bloom's syndrome,
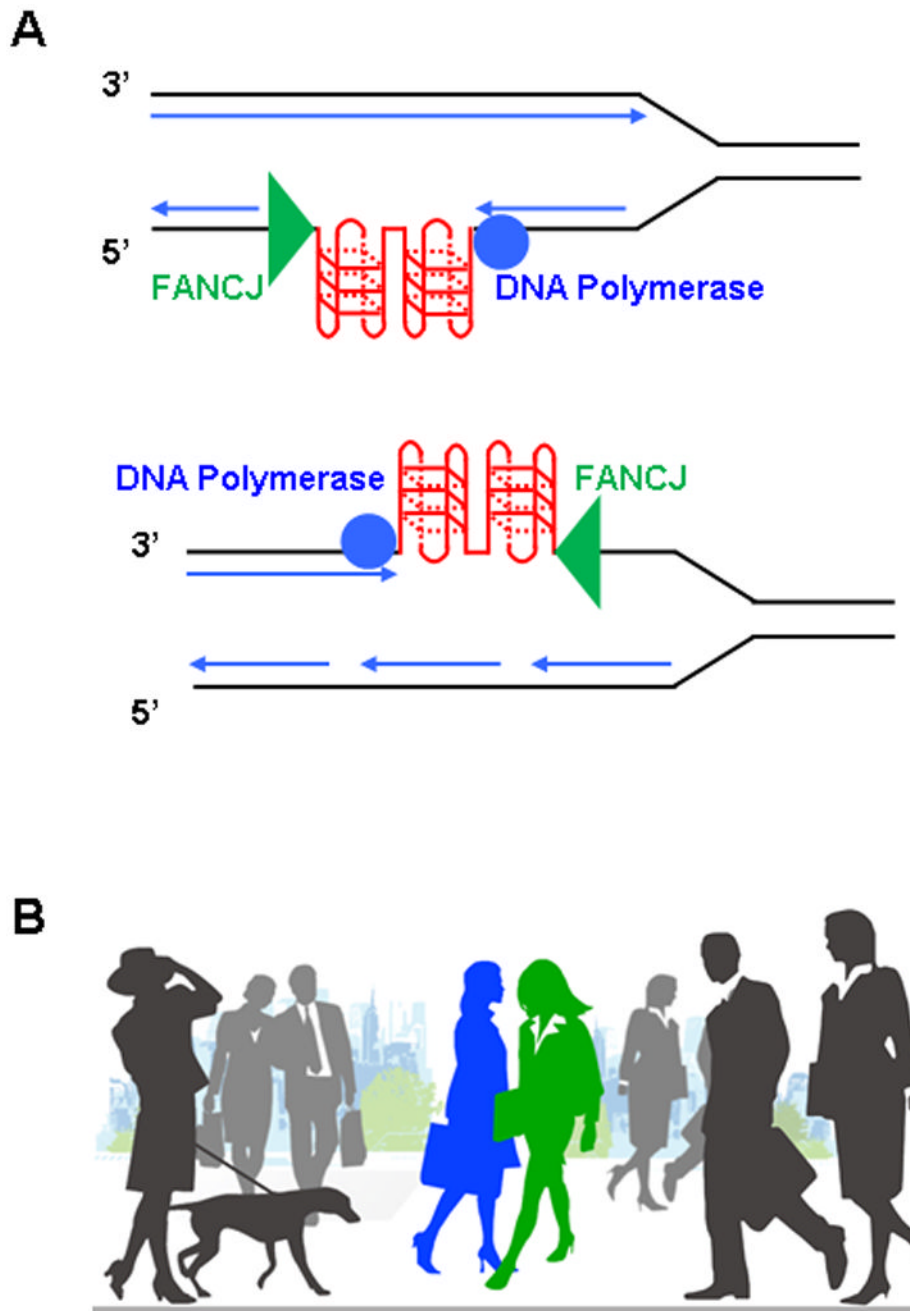respectively, require a 3′ ssDNA tail to unwind the adjacent G4 structure.

Fig. 2.
Coordinate action of FANCJ helicase and DNA polymerase during replication. (A) Lagging or leading strand synthesis during replication can be inhibited by G-quadruplex DNA structures. The action of FANCJ helicase is proposed to unwind the G4 block, enabling the DNA polymerase to synthesize the complementary strand. Because FANCJ is a 5′ to 3′ helicase with respect to the strand that it is presumed to translocate, it would have to coordinate its action with the DNA polymerase that is advancing from the opposite direction. (B) Two pedestrians on a busy city sidewalk may proceed to pass each other by turning their shoulders. This analogy may apply to FANCJ and a DNA polymerase that undergo conformational changes to get by one another in an efficient manner.

Table 1

Biochemical properties of G4 helicases and phenotypes of helicase deficiencies

Protein	Organism	G4 DNA	G4 RNA	Phenotype	Ref.
WRN	Human	$3' \rightarrow 5'$ [a]	ND [b]	Premature aging, genomic instability	[28,107,115]
BLM	Human	$3' \rightarrow 5'$	ND	Cancer, elevated sister-chromatid exchanges, genomic instability	[114,154]
FANCJ	Human	$5' \rightarrow 3'$	ND	Fanconi anemia, breast cancer, defective interstrand cross-link repair and slow S phase progression	[29,98,104]
G4R1/RHAU	Human	Yes [c]	Yes [c]	Abnormal mRNA deadenylation and decay	[139–141]
Pif1	Human	ND	ND	Possible telomere defects	[152,155]
	Yeast	$5' \rightarrow 3'$	ND	Mitochondrial DNA and G-rich DNA instability, longer telomeres, defects in Okazaki fragment metabolism	[151,153,156,157]
Dna2	Human	$5' \rightarrow 3'$	ND	Cell-cycle delay and aberrant cell division, genomic and mitochondrial DNA instability	[158,159]
	Yeast	$5' \rightarrow 3'$	ND	Defects in Okazaki fragment metabolism	[149,160,161]
Sgs1	Yeast	$3' \rightarrow 5'$	ND	Defective double-strand break repair, defective restart of stalled replication forks, inappropriate processing of meiotic recombination intermediates, telomere instability	[162,163]

[a] Although WRN is reported to require a 3′ single stranded (ss)DNA tail to unwind a number of G4 DNA substrates [115], WRN was found to unwind a d(CGG)n G4 DNA substrate that contained a 5′ ssDNA tail [28]

[b] ND, not determined.

[c] Polarity not determined

Table 2

Human proteins that interact with G-quadruplex DNA *in vitro*. hnRNP, heterogeneous nuclear ribonucleoprotein A1

Protein	Function	Ref.
Topo I	Promotes G-quadruplex formation and binds G-quadruplex	[164]
GQN1 (Nuclease)	Cleaves G-quadruplex specifically	[165]
hnRNP A1/UP1	Binds and unfolds G-quadruplex (e.g. G-quadruplex in KRAS promoter)	[166–169]
hnRNP D/BD2	Unfolds human telomeric G-quadruplex	[170]
Pot1	Disrupts telomeric G-quadruplex	[81]
Insulin and insulin-like growth factor-2 (IGF-2)	Binds G-quadruplex formed in human insulin gene promoter	[171,172]
Nucleolin	Binds and stabilizes G-quadruplex formed in c-MYC promoter	[75]
MutSα (MSH2/MSH6)	Binds G-quadruplex	[29,79]
RPA	Binds and unfolds human telomeric intramolecular G-quadruplex, but not intermolecular G-quadruplex	[173,174]