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REVIEW

G-quadruplex DNA: a novel target for drug design

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Abstract

G-quadruplex (G4) DNA is a type of quadruple helix structure formed by a continuous guanine-rich DNA sequence. Emerging evidence in recent years authenticated that G4 DNA structures exist both in cell-free and cellular systems, and function in different diseases, especially in various cancers, aging, neurological diseases, and have been considered novel promising targets for drug design. In this review, we summarize the detection method and the structure of G4, highlighting some non-canonical G4 DNA structures, such as G4 with a bulge, a vacancy, or a hairpin. Subsequently, the functions of G4 DNA in physiological processes are discussed, especially their regulation of DNA replication, transcription of disease-related genes (*c-MYC*, *BCL*-2, *KRAS*, *c-KIT* et al.), telomere maintenance, and epigenetic regulation. Typical G4 ligands that target promoters and telomeres for drug design are also reviewed, including ellipticine derivatives, quinoxaline analogs, telomestatin analogs, berberine derivatives, and CX-5461, which is currently in advanced phase I/II clinical trials for patients with hematologic cancer and BRCA1/2-deficient tumors. Furthermore, since the long-term stable existence of G4 DNA structures could result in genomic instability, we summarized the G4 unfolding mechanisms emerged recently by multiple G4-specific DNA helicases, such as Pif1, RecQ family helicases, FANCJ, and DHX36. This review aims to present a general overview of the field of G-quadruplex DNA that has progressed in recent years and provides potential strategies for drug design and disease treatment.

Keywords Cancer · Aging · Telomere · G-quadruplex · G4 ligand · DNA replication · Epigenetic · Helicase

Detection of G-quadruplex DNA

Since the discovery of the right-handed B-type double helix DNA by Watson and Crick in 1953, many high-level DNA structures have been discovered, such as Z-DNA, Holliday junction DNA, and triplex DNA. In 1962, Gellert et al. first discovered that four guanosine 5′-monophosphate (GMP) molecules can form a planar helical unit, thus forming a relatively stable helical aggregate, which laid an important foundation for the subsequent discovery of G-quadruplex structures [1]. In 1988, Sen et al. found that DNA sequences similar to telomeres containing multiple guanine sequences could spontaneously fold to form a four-stranded DNA structure under certain conditions, which were called g-quadruplexes or g-tetraplexes [2]. Since then, increasing studies have discovered a series of specific DNA sequences that can

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form G4 structures, especially sequences located in the regulatory regions of certain oncogenes and in the telomeres.

Potential G4 sequences (PGSs) in the genome can be analyzed and identified using bioinformatics methods. Algorithms were developed to predict the putative G4 structures in the genome, with the typical G4 sequence motif being $G_{\geq 3}N_{L1}G_{\geq 3}N_{L2}G_{\geq 3}N_{L3}G_{\geq 3}$, where N_{L1}, N_{L2}, and N_{L3} represent the loop sequences of G4, with lengths limited to 1–7 nt [3, 4]. Through bioinformatics prediction, as many as 376,000 potential G4 sequences were found in the human genome [4]. Meanwhile, PGSs in *Escherichia coli* [5–7], *Saccharomyces cerevisiae* [8, 9], and other species have also been analyzed and located, and the potential functions of these sequences have also been speculated. Currently, depending on different strategies, multiple algorithms are available to determine and analyze PGSs in the indicated DNA sequences or genomes, such as the regular expression matching tools ImGQfinder [10] and AllQuads [11], the scoring and sliding window tools G4Hunter [12–14], pqsfinder [15] and QGRS Mapper [16], and the machine learning tool Quadron [17].

Apart from bioinformatics methods, next-generation sequencing has also been adopted to map the G4 structures in the genome. Currently, two strategies based on next-generation sequencing are mainly exploited: G-quadruplex sequencing (G4-seq) and G4 chromatin immunoprecipitation sequencing (ChIP-seq). G4-seq is a combination of the genome-wide DNA polymerase-stop assay and high-throughput sequencing, and it has identified 716,310 G4 DNA sequences in the human genome [18], nearly twice that predicted using bioinformatics methods. The G4s identified via G4-seq include many non-canonical G4 structures with long loops and/or bulges, which are difficult to predict using bioinformatics methods. Subsequently, whole-genome G4 maps of 12 important model organisms and pathogens of clinical relevance, including *S. cerevisiae*, *Arabidopsis thaliana*, *Mus musculus,* and *Homo sapiens*, were generated using an improved version of G4-seq, which provided the key sequence features that determine different patterns of G4 formation and the relevance of G4 localization across genomes [19]. It should be noted that some of the G4 sites mapped via G4-seq might not form G4 structures in vivo, as the technique was performed using cell-free systems, and the effect of proteins that may alter the stability of G4 structures cannot be excluded, especially for some long-loop G4s with low stability.

G4 ChIP-seq is dependent on chromatin immunoprecipitation using G4-specific antibodies and high-throughput sequencing [20, 21]. Using the G4 structure-specific antibody BG4, it was found that G4 structures are enriched in the promoters and 5′-UTRs of highly transcribed genes, particularly in genes related to cancer, such as *c-MYC* [20]. In addition, some endogenous proteins, such as human XPB/XPD1 [22], ATRX [23], yeast Rif [24], and Pif1 [25], which can bind to folded G4 structures, have also been mapped to G4 structures in the human genome via ChIP-seq. G4 sites mapped through G4 ChIP-seq can represent the actual G4 structures in the cell; however, the number of G4 sites can be affected by antibody specificity and sample treatment.

Structure of G-quadruplex DNA

G-quadruplexes are stabilized by Hoogsteen hydrogen bonding; four guanines form a G-tetrad (Fig. 1A), then, two or more G-tetrads stack on top of each other, forming a quadruple helical structure driven by cations. According to the number of DNA strands, folded G4 can be grouped into intermolecular G4 (two or four DNA strands, Fig. 1B, C) or intramolecular G4 (one DNA strand, Fig. 1D–F) [26, 27]. Intramolecular G4 can display diverse topologies depending on the sequence difference, loop length, and ionic environment [28]. According to the orientation of the sequence in the G4 spatial structure, it can be divided into parallel, antiparallel, or hybrid structures (Fig. 1D–F). The spatial configuration of G4 has been widely studied using biophysical methods, such as NMR, X-ray, and circular dichroism spectra. As for the human telomeric G4 sequence $d(AGGG(TTAGGG)_3)$, it exhibits a canonical antiparallel state in a Na^+ solution (Fig. 1E) [29]; however, in a K^+ solution, it presents a mixture of parallel, antiparallel, and hybrid structures (Figs. 1D–F) [30, 31], which indicates that the same G4 sequence may have multiple complex structures in different environments. However, the effect of different topologies on the function of G4 in cells requires further study.

In addition, an increasing number of studies regarding G4 structures with unique features, such as a G-vacancy, a bulge, or a long loop with duplex, have surfaced recently, expanding the repertoire of G-quadruplexes. The G-vacancy-bearing G-quadruplex (GVBQ) was recently excavated, which is formed by one G2 and three G3 tracts [32] (Fig. 1G). By accepting a guanine derivative, such as dGMP, GMP, or GTP, the G-vacancy can be filled up to form an intact G-tetrad, thereby forming an enhanced G4 [33, 34]. Using the bioinformatics method, approximately 220,000 potential GVBQ-forming sequences emerged in the human genome, most of which are preferentially located at the 5′-end of genes and may potentially respond to the environment in specific cellular processes [32]. The formation of GVBQ in promoters can regulate gene expression and has emerged as a novel therapeutic target. One typical example of this is the GVBQ from the promoter of the platelet-derived growth factor receptor beta (PDGFR-β) gene, which is a cell surface receptor tyrosine kinase that is closely associated with various pathologies, including atherosclerosis, fibrotic

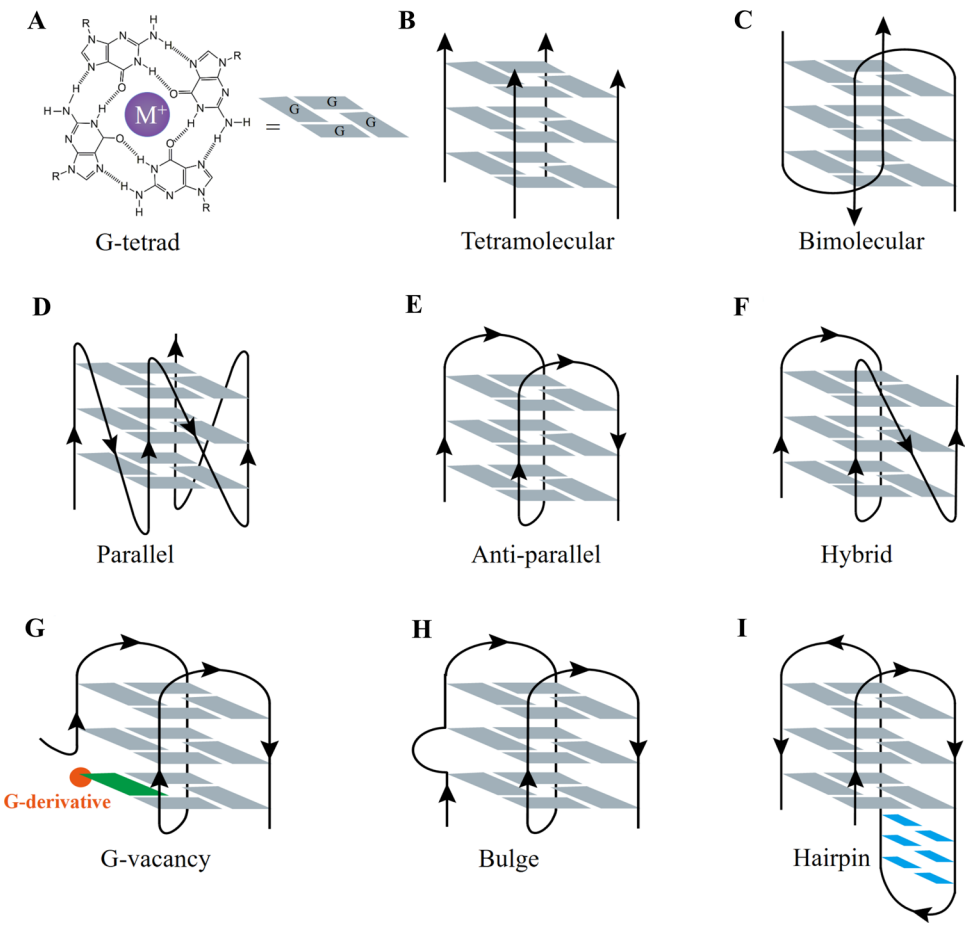

Fig. 1 Structures and topologies of G-quadruplex DNA. **A** The chemical structure of the G-quartet. The G-quartet is formed by four guanines and is stabilized via Hoogsteen hydrogen bonding in the presence of a central cation. **B**, **C** Representative topologies of intermolecular G-quadruplex structures. **D–F** Schematic representation of canonical intramolecular G-quadruplex structures. According to the orientation of the G4 sequences, these can be divided into parallel, antiparallel, and hybrid G4 structures. **G–I** Schematic representation of non-canonical intramolecular G-quadruplex structures

disorders, rheumatoid diseases, and cancers [35–37]. The G4 sequence in the nuclease hypersensitivity element (NHE) of the PDGFR-β promoter can form GVBQs. Intriguingly, 3′- and 5′-end G-vacancies are both present in the PDGFR-β promoter G4 sequences; also, guanine metabolites and drugs showed a conserved selectivity for the 5′-vacancy, and cGMP preferred to bind both the 3′- and 5′-end vacancies and formed two fill-in G4s with similar populations [33, 38]. Recently, a bi-functional guanine-RHAU23 peptide conjugate composed of a guanine moiety and a 23-aa G4-binding domain from the RHAU helicase has been reported to target and stabilize the GVBQs in DNA with superior specificity, providing a novel and promising alternative targeting strategy to a distinctive panel of G4s [39].

Meanwhile, bulge occurrence among G-tetrads in G4 has also been discovered in a series of G4 topologies (Fig. 1H). High-throughput sequencing of G4s in the human genome indicates that G4s with bulges are widespread, accounting for 21.6% and 30% of the total observed G4 sequences in solutions containing K^+ and the G4 ligand pyridostatin, respectively [18]. The bulge varies with the base identity, size, and number per structure [40–42]. In single projection G4, the pyrimidine bulge is more common and more stable than the adenine bulge [40–42], while the guanine bulge has not yet been reported. In polynucleotide projection G4, the size of the bulge can increase to 7 nt [43]. Recently, a G4 structure containing a duplex bulge of up to 33 nt was reported, and that G4 stability slightly increased with increasing duplex bulge size, broadening the diversity of G4 topologies [44]. G4s containing bulges are widespread in gene promoters; for example, a thymine bulge G4 has been reported in the NHE of the *KRAS* promoter and plays a critical role in regulating KRAS signaling [45], which is a driver of many cancers by activating multiple pathways [46], especially PI3K, MAPK, PLCe, and RalGDS signaling.

The loop size of canonical G4 in bioinformatics algorithms has been defined as 1–7 nt. However, in the human genome, G4 sequences with long loops (> 7 nt) account for 21.5% of the total observed G4 in K^+ and 24% in pyridostatin solutions [18]. Increasing studies have revealed the existence of hairpins or duplexes in the long loop of G4s [47, 48], forming quadruplex–duplex hybrids (Fig. 1I), which are also known as stem-loop-containing quadruplex sequences (SLQS) [49]. In the human genome, 80,307 SLQS embedded within 60,172 unique clusters were identified, most of which were strand-specifically located in promoter regions

and were closely related to hundreds of brain tissue-related and cancer-associated genes [49]. Usually, the loop size has a significant impact on the stability and formation of G4 both in cell-free and cellular systems [50], but a stem loop can reinforce the stability of SLQS compared to a non-structured loop [51], thereby regulating the expression of key genes. Recently, an SLQS was reported and presented two distinct solution conformations that could co-exist in the human oncogene *PIM1*: form 1, containing a (3 + 1) G-tetrad core with a propeller loop, a co-axially stacked hairpin stem-loop, and a lateral loop; and form 2, containing a chair-type G-tetrad core and an adjoining G · C · G · C tetrad, with two lateral loops and a co-axially stacked hairpin stem loop [52]. The existence of a long loop can diversify the spatial configuration of G4 and provide sequence-specific (duplex binding) and scaffold-specific (G4 binding) structural elements, which can be targeted and selectively modulate gene expression.

Intracellular functions of G-quadruplex DNA

The general existence of G4s in specific regions of the genome, such as in DNA replication origins, chromosome ends, promoters, and gene transcriptional regulatory regions, determines their pivotal functions in various biological processes, including DNA replication, telomere maintenance, transcription, homologous recombination (HR), and epigenetics.

G-quadruplexes and DNA replication

G-quadruplexes affect DNA replication and genome stability

G4 DNA plays a dual role in DNA replication, as a critical component of metazoan replication origins and as an obstacle during DNA replication, leading to genome instability.

In mammalian genomes, ~ 100,000 potential DNA replication origins have been predicted, and 80%–90% of these origins contain GC-rich regions, forming origin G-rich repeated elements (OGRE), which can potentially form G-quadruplexes [53]. A previous experiment showed that G4 motifs in DNA replication origins are required for replication initiation and that affecting G4 stability also impairs the origin function (Fig. 2A) [54]. Furthermore, a recent study proclaimed that the deletion of G4 motifs in OGRE can strongly reduce the origin activity in mouse cells, and a G4 sequence from OGRE introduced into an ectopic origin-free region can establish a new functional origin [55]. The mechanism of G4-induced replication initiation may be due to the recruitment of replication initiation factors to replication origin sites, such as the origin recognition complex [56], treslin–MTBP complex [57], and the replication timing regulation protein Rif1 [58], which were reported to bind to G4 specifically both in cell-free and in cellular systems.

After initiation, DNA replication proceeds in a semi-discontinuous manner, during which the lagging strand remains single-stranded for a while and is intrinsically more prone to G4 formation. There is mounting evidence that G4 formation can obstruct the DNA replication process in both cell-free and cellular systems (Fig. 2B). Mutation of the dog-1 helicase in *C. elegans* was first reported for deletions in genes containing G-rich regions [59], which indicates that a deficiency in G4 unfolding may give rise to DNA synthesis disorders and genome instability. Simultaneously, mutations or deficiencies in other G4 unfolding-related proteins in the cell were authenticated to obstruct the DNA replication process, such as the helicases FANCJ [60], BLM [61], WRN [62], Pif1 [63], and DDX11[64]. The effect of G4s on DNA replication was also detected directly using cell-free systems. In a DNA polymerase-stop assay, G4s from telomeres were reported to arrest Taq DNA polymerase [65, 66] and yeast polymerase δ [67], leading to replication blockage. Furthermore, the G4 replication process by *E. coli* Pol I depends on the stability of G4 and the concentration of Pol I [6]; when high-stability G4s, such as four-layer G4s, occur on the template, *E. coli* Pol I will similarly be blocked.

Replication of G-quadruplexes

There are several pathways that can resolve the process of impeded G4 replication. A major method by which this happens is mediated by polymerase and G4-specific helicase to collaboratively replicate G4. G4-specific helicases, including FANCJ, BLM, WRN, Pif1, and RHAU, will be reviewed in the following sections. Some nucleases, such as DNA2 [68] and EXO1 [69], also facilitate the replication of G4 structures to avoid genome collapse and fork stagnation at G4 sites.

In addition, some polymerases that specifically recognize and replicate G4 structures have been observed in both cell-free and cellular systems. In recent years, increasing evidence has demonstrated that some translesion DNA synthesis (TLS) polymerases, such as Rev1, Pol κ, and Pol η, are implicated in G4 processing to promote DNA fork progression. A cell-free study showed that yeast Rev1 could replicate G-tract DNA in a template-specific manner, indicating that Rev1 is a G template-specific DNA polymerase [70] and can incorporate dCMP into the 3′ end of DNA primers and function as a scaffold for proteins during TLS or base excision repair. Interacting with FANCJ helicase, REV1 can be directly recruited to G4s or through interactions mediated by PCNA to remodel oxidatively damaged G4 DNA [71]. Furthermore, human Rev1 has been shown to specifically bind G4 DNA substrates, dislodge tetrad guanines to unfold

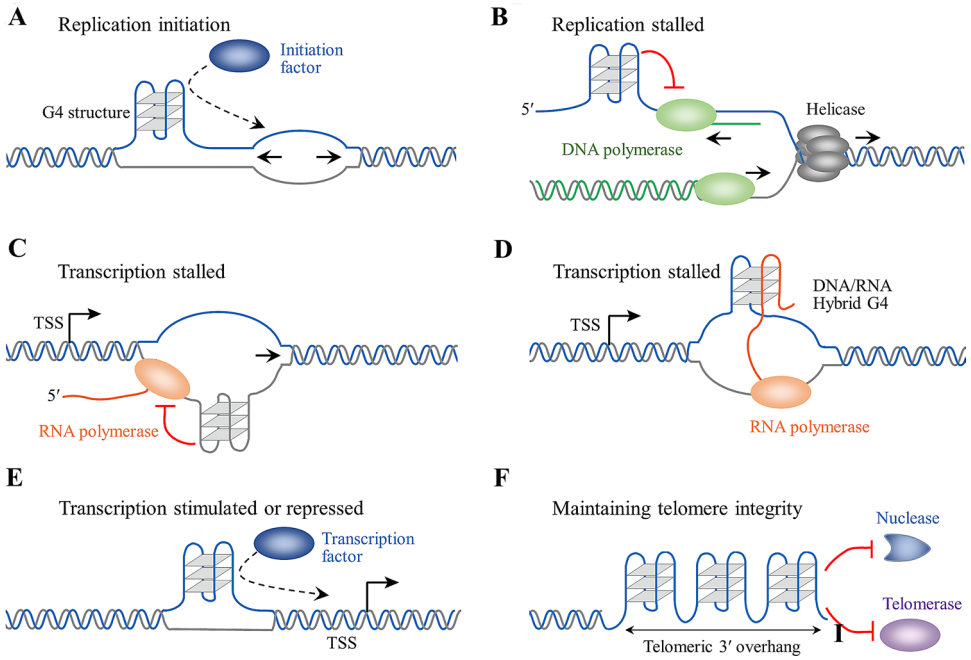

Fig. 2 The biological function of G-quadruplex DNA in DNA replication, transcription, and telomere maintenance. **A** G4 can initiate DNA replication via the recruitment of replication initiation factors, such as the origin recognition complex, treslin-MTBP complex, and Rif1. **B** G4 can stall DNA polymerase during the DNA replication process, causing DNA replication disorder and genome instability. The replication of G4 can be resolved through the participation of G4-specific helicases such as Pif1, FANCJ, BLM and WRN, and TLS polymerases such as Rev1, Pol κ, Pol η, and Primpol. **C** G4 on the template strand can directly impede RNA polymerase during the transcription process. **D** G4 sequences on the non-template strand can form hybrid G4 with the newly synthesized RNA, inducing transcription termination. **E** The formation of G4 upstream of the TSS will usually inhibit transcription. G4 can also recruit certain transcription factors, which may either facilitate or restrain transcription. **F** A string of G-quadruplexes in the telomeric 3′-overhang involves telomere metabolism and telomere integrity maintenance. The G-quadruplex can protect telomeres from nuclease and interfere with telomerase activity

G4 DNA, and prevent G4 structures from refolding, thereby promoting fork progression [72]. It is worth noting that a loss of human Rev1 increased G4 mutation frequency > 200-fold compared to a control sequence and that hRev1 exhibited a strong affinity for parallel G4 rather than antiparallel or hybrid G4, whereas insert-2 in Rev1, a motif conserved in vertebrates but not yeast or plants, is responsible for the selective binding and accurate replication of G4 [73]. Apart from Rev1, other TLS polymerases, such as Pol κ, Pol η, and Dpo4, also participate in G4 replication [74–79]. Interestingly, the C-terminal of Rev1 may interact with Pol κ or Pol η to collaboratively replicate G4s.

Meanwhile, a novel eukaryotic DNA primase and DNA-directed polymerase, named PrimPol, has emerged in recent years and may be involved in G4 replication. PrimPol is a critical factor in DNA damage tolerance and mitochondrial DNA replication, particularly in invertebrate and human cells [80–82]. Recently, it was reported that PrimPol bound to and suppressed the formation of G4 structures, promoting the restart of DNA synthesis closely coupled to G4 replication impediments in DT40 cells [83]. PrimPol can also suppress R-loop formation in genes containing G4 and H-DNA motifs across the genome in both avian and human cells [84]. However, a recent study indicated that DNA replication by PrimPol is strongly blocked by representative stable G4 structures from human mitochondrial DNA, and that it could be overcome by the presence of Pif1 helicase in an error-prone DNA synthesis manner [85]. Therefore, cooperation between multiple proteins is indispensable for maintaining genome stability.

G-quadruplexes and transcription

G-quadruplexes affect gene transcription

The effect of G4 on DNA transcription depends on multiple factors, especially its orientation (on the template or non-template) and its location relative to the transcription start site (TSS). During DNA transcription, the presence of G4 on the DNA template strand blocks the movement of RNA polymerase (Fig. 2C), resulting in the arrest or even termination of transcription, directly decreasing the expression of the target genes [86, 87]. Also, G4 on the DNA non-template strand has also been reported to block the

transcription process, which forms an unusually stable RNA/DNA hybrid between the G4 sequence on the non-template and the nascent RNA [88–90] (Fig. 2D). In contrast, recent studies discovered that G4 on the non-template strand can couple with and stabilize the R-loop [91], and the stabilized R-loop facilitates transcription through a mechanism involving successive rounds of R-loop formation [92]. It is worth noting that R-loop spreading caused by multiple stabilized G4 ligands can trigger the accumulation of DNA double-strand breaks (DSBs) in human cancer cells, thereby causing genome instability and activating cell apoptosis [93].

G4s have been mapped using a machine learning approach and high-throughput sequencing and were found to be enriched in cancer breakpoints and the TSSs of highly transcribed genes in human cells, particularly in genes related to cancer, such as *c-MYC*, *BCL-2*, *KRAS*, *c-KIT,* and *VEGF* (Table 1), further verifying the correlation among G4, transcription initiation, and the emergence of certain diseases.

G4s in TSSs play diverse roles during gene transcription (Fig. 2E). One classical example is the G4 in the promoter of the *BCL-2* gene, which encodes a mitochondrial membrane protein and functions by inhibiting apoptosis. *BCL-2* is aberrantly overexpressed in a wide range of tumors and other diseases, including Alzheimer's disease, Parkinson's disease, and stroke [94]. Three promoters have been reported in *BCL-2* [95]: P1, P2, and M. P1 is located 1489 to 1451 bp upstream of the TSS and is responsible for most of the expression of *BCL-2*; P2 and M are located between P1 and the TSS, and mainly modulate P1 activity. As a TATA-less and GC-rich promoter, P1 and its vicinity have been reported to have four disparate G4 DNA sequences (Table 1), including Pu39, bcl2G4-2, P1G4, and P32. Pu39 harbors six runs of guanine tracts and has the potential to form multiple overlapping quadruplexes with more than 15 possible combinations [96]. Pu39 functions as a transcriptional silencing element during *BCL-2* expression, and the deletion or mutation of the Pu39 site in lymphoma DHL-4 cells has been shown to increase P1 activity by 2.1-fold or 2.6-fold, respectively [97]. Multiple transcription factors can bind to Pu39 and relieve its inhibitory effect on *BCL-2* expression, such as specificity protein 1 (SP1) [98], Wilms' tumor 1 (WT1) [97], and E2F [99]. Bcl2G4-2 was the second G4 sequence found near the P1 promoter, which is located 1653 to 1618 bp upstream of the TSS and can form a parallel G4 in the presence of K^+; however, the stability of bcl2G4-2 appears to be much lower than that of other *BCL-2* G4s, as its T_m is approximately 60 °C [100]. The potential bcl2G4-2-targeting transcription factors and their relevance in terms of *BCL-2* transcription are yet to be explored. Located 1439 to 1412 bp upstream of the TSS with five runs of guanine tracts, P1G4 also functions as a *BCL-2* transcription repressor, whereas in the extended *BCL-2* P1 promoter region containing Pu39 and P1G4, P1G4 seems to play a more dominant role in inhibiting *BCL-2* transcription in human breast cancer MCF-7 cell lines [101]. P32 is located 1906 to 1875 bp upstream of the TSS and occupies four runs of guanine tracts. Under physiological conditions with K^+, P32 was identified to have an intramolecular hybrid G4 topology with high thermal stability ($T_m = 82$ °C) [102]. However, the inhibition of P32 via base mutations or the induction of a conformational transition with a phenanthroline derivative could cause obvious downregulation of *BCL-2* expression in human lung A549 cancer cells [102], indicating that P32 may play an activating role in regulating *BCL-2* expression in cellular systems. The regulation of G4s near the P1 promoter on *BCL-2* expression mainly depends on the transcription factors recruited by G4, which is worthy of further study in vivo, especially for bcl2G4-2, P1G4, and P32. Also, whether there is cross talk between different G4s also deserves exploration, especially for Pu39 and P1G4, which are only 11 nt apart.

Similarly, the NHE of the oncogene *KRAS* also contains three putative G4 sequences, which are separated by 12 and 17 nucleotides, respectively, and are designated as $G4_{far}$, $G4_{middle}$, and $G4_{near}$ relative to the TSS (Table 1). $G4_{near}$, also known as *KRAS* 32R, was shown to form two topologies in the 100 mM KCl solution, a hybrid G4 with a $T_m \sim 72$ °C, and a parallel G4 with a thymidine bulge in one strand, with a $T_m \sim 55$ °C [103, 104]. Pull-down assays and LC–MS/MS identified three DNA protein complexes that have an affinity for the $G4_{near}$ structure in human pancreatic cancer Panc-1 cells, including hnRNPA1, PARP-1, and Ku70 [103]. The hnRNPA1 was shown to bind to and destabilize the *KRAS* promoter G4, resulting in the upregulation of *KRAS* expression [105]; the same phenotype was also discovered in CCHC-type zinc-finger nucleic acid-binding protein (CNBP) [106] and myc-associated zinc finger (MAZ) [107] in later studies. In contrast, HMGB1, a ubiquitous non-histone protein involved in DNA repair, transcription, and telomere maintenance, was shown to bind to and stabilize $G4_{near}$ to suppress *KRAS* expression in Panc-1 cells [108]. In addition, the *KRAS* promoter G4 was associated with epigenetic mechanisms, as guanine has the lowest redox potential and can be oxidized to 8-oxoguanine (8OG) upon interaction with reactive oxygen species (ROS), impairing DNA, which will be discussed in the following section. However, based on the luciferase assay, a previous study claimed that $G4_{middle}$ might be a stronger repressor of promoter activity than $G4_{near}$ in HEK-293 cells [109], but more evidence is needed to support that claim. Furthermore, it was reported that $G4_{far}$ could not form an inducible and stable structure under a variety of buffer conditions in cell-free systems, while $G4_{middle}$ and $G4_{near}$ formed strong G4 structures [109], indicating that $G4_{far}$ may be inactive during

Table 1 Disease-related genes containing potential G-quadruplex structures in their promoters

Gene	Sequence (5′–3′)	Position from TSS	Function	Disease	References
BCL-2	P1G4: CGGGCGGGAGCGCGGCGGGCGGGCGGGC[a]	− 1439 to − 1412	Apoptosis, programmed cell death	Various cancers, Alzheimer's disease, Parkinson's disease, stroke, and spinal cord injuries	[96, 97, 100-102]
	Pu39: AGGGGCGGGCGCGGGAGGAAGGGGGCGGGAGC GGGGCTG	− 1489 to − 1451			
	P32: TGGGGTCCGCGACGGGGTGGGGGCTCCCGGGG	− 1906 to − 1875			
	bcl2G4-2: CCGGGCCAGGGAGCGGGGCGGAGGGGGCGGTC GGGT	− 1653 to − 1618			
KRAS	$G4_{near}$: AGGGCGGTGTGGGAAGAGGGAAGAGGGGGAGG	− 160 to − 129	Signal transduction, cell proliferation, and cell survival	Various cancers	[45, 130]
	$G4_{middle}$: CGGGGAGAAGGAGGGGGCCGGGCCGGGCCGGC GGGGGAGGAGCGGGGGCCGGGC	− 228 to − 175			
	$G4_{far}$: AGGGGTGGCTGGGGCGGTCTAGGGTGGCGAGCC GGGC	− 275 to − 239			
c-MYC	GCGCTTATGGGGAGGGTGGGGAGGGTGGGGAAGGT GGGGAGGAGAC	− 148 to − 103	Cell proliferation	Over 70% of cancers	[110, 111]
c-MYB	S1: AGGGGCGCCAGATTTGGCGGGAGGGGGAGTGT	− 80 to − 49	Cell proliferation, cell differentiation	Colorectal tumors, leukemia, breast tumors	[253]
	S2: CGGAGGCGGCGGGCAGGGC	− 114 to − 94			
	S3: AGGGAGGAGGGGAGGCGGCGGGACTGGGCGCGGGT	− 352 to − 318			
	S4: TGGGCCGGGCGGGGCGGGGTGGGTGGGGCCCGGGT	− 809 to − 775			
hTERT	GGGGAGGGGCTGGGAGGGCCCGGAGGGGGCTGGG CCGGGGACCCGGGAGGGGTCGGGACGGGCGGGG	− 90 to − 22	Telomere maintenance	85%–90% of cancers	[254]
c-KIT	*c-KIT1*: AGGGAGGGCGCTGGGAGGAGGGG	− 109 to − 87	Cell growth, proliferation, migration, and survival	Gastrointestinal stromal tumors, pancreatic cancer, melanoma, and hematological neoplastic diseases	[255]
	c-KIT2: GGGCGGGCGCGAGGGGAGGGG	− 160 to − 140			
VEGF	GGGGCGGGCCGGGGGCGGGGTCCCGGCGGGGCGGAG	− 85 to − 50	Angiogenesis, cell proliferation	Age-related macular degeneration, diabetes, rheumatoid arthritis, cancers	[256, 257]
HIF-1α	GGGCGGGGGAGAGGGGAGGGG	− 85 to − 65	Immune response, cellular responses to hypoxia	Diabetes, cancers, vascular disease	[258]
PDGF	GGGGGGGGGGGGCGGGGGCGGGGGCGGGGGAGGGG	− 82 to − 47	Major mitogen, embryonic development, cellular differentiation, wound healing	Gliomas, sarcomas, astrocytoma, atherosclerosis, various fibrotic, neurological conditions	[35-37]
ILPR	$(A(C/T)AGGGGT(G/C)TGGGG)_{40–160}$	− 363 bp upstream of TSS	Insulin synthesis	Diabetes	[259]
C9orf72	$(GGGGCC)_{2–1600}$	–	Autophagy	Devastating neurological diseases, including amyotrophic lateral sclerosis, frontotemporal dementia	[260, 261]
MET	GCGGGCGGGCGGGGCGCTGGGCT	− 48 to − 26	Cell proliferation, anti-apoptosis, angiogenesis	Cancers of kidney, liver, stomach, breast, and brain	[262]
RET	AGCGGGTAGGGGCGGGGCGGGGCGGGGGCGGTCC	− 59 to − 26	Tyrosine kinase receptor, activating MEK/ERK and PI3K/Akt	Medullary thyroid carcinomas	[135, 184]

Table 1 (continued)

Gene	Sequence (5′–3′)	Position from TSS	Function	Disease	References
DUX4	DME1 GQ: CAGGGGATGGTGGGGCTGGGGTTGAGTGAT GGGC	− 10,281 to − 10,252	Embryonic genome activation, cell death	Facioscapulohumeral muscular dystrophy, various sarcomas, and hematological malignancies	[185]
	D4P GQ: CGGGGTGGGGCGGGCTGTCCCAGGGGGGC	− 223 to − 195			
PIM1	GGGAGGGCGCGCCAGCGGGGTCGGG	–	Cooperation with *c-MYC*	The triple-negative breast cancer, lymphomas, hematopoietic and prostate cancers	[52]

[a]The sequences with underlines represent G-tracts in the G4s.

KRAS transcription, but this needs further verification using in vivo experiments.

Another classical example is the G4 sequence in the promoter of *c-MYC*, which is the dominant carcinogenic driver in many cancers [110]. Located upstream of the *c-MYC* P1 promoter, the nuclease-hypersensitive element III1 (NHE III1) contributes to 80%–90% of *c-MYC* transcription and contains a 46-bp G4 sequence (named as Pu46), which occupies six G-tracts (Table 1) and forms stable G4s with multiple topologies in a cell-free system [110, 111]. Pu46 itself is critical for suppressing *c-MYC* expression; moreover, Pu46 can also recruit other transcription factors to regulate *c-MYC* transcription, including nucleoside diphosphate kinase B (NM23-H2), nucleolin, SP1, and CNBP [112]. NM23-H2 was found to maintain the single-stranded form of Pu46 to activate *c-MYC* transcription [113–115] and interact with CNBP, which can modulate the topological structure of G4 and unfold it [106], thereby increasing the transcription of *c-MYC* [116]. In contrast, nucleolin can contribute to G4 formation and stabilize Pu46 G4 to inhibit *c-MYC* expression [113, 117], as well as repress *c-MYC* transcription promoted by SP1 [118], which can recognize and bind both canonical dsDNA and G4s in the double-stranded NHE III1 [119, 120]. However, nucleolin may also act as a transcription activator in other promoters, such as *VEGF* [121] and *ZEB1* [122]. The folding and structure of *c-MYC* G4 containing the G-tracts 1-2-4-5 [112], 1-2-3-4 [123], and 2-3-4-5 [124] have been reported in cell-free systems. However, the predominant G4 structure in living cells may be G4 involving G-tracts 2-3-4-5, which was verified in cells using multiple Pu22-specific small molecular probes, including a fluorescent probe named 9CI [125], a cyanine dye fluorescent probe named Cy-1 [126], and a self-assembled quinazoline–quinazolinone derivative named 4b [127]. The G-tracts 2-3-4-5 of Pu46, also known as Pu22, can fold into a parallel structure with a 1:2:1 nt loop arrangement; at present, most of the small molecule ligands targeting G4 to suppress *c-MYC* expression are based on this Pu22 structure.

G4 ligands targeting promoters for drug design

The structural diversity and cross talk between protein–G4 interactions originating from the G4s formed in the promoters make them attractive targets for drug design. Currently, the G4-interacting proteins database is available, providing information about proteins interacting with G4 structures [128], where more than 1000 G4 ligands have been discovered and shown in the G4 ligands database for drug discovery [129], providing a platform for the discovery and development of novel anticancer therapeutics. G4 ligands have been discovered or designed to suppress the expression of oncogenes by stabilizing G4 structures in the promoters, including porphyrin derivatives, acridine derivatives,

pyridine derivatives, fluoroquinolone derivatives, among others (Table 2). Further, some natural alkaloids have been found to also target G4s and can be potential antitumor drugs, such as berberine, cryptolepine, and ellipticines.

Taking *c-MYC* as an example, we have summarized potential G4 ligands with physiological effects as reported in recent years. The earliest reported *c-MYC* G4 ligands include TMPyP4, BRACO-19, and pyridostatin, which possess the ability to suppress the growth and proliferation of multiple tumor cells including breast cancer cells, retinoblastoma cells, and melanoma cells. However, they were unable to distinguish different G4 topologies; for example, TMPyP4 has a high affinity for multiple G4s and inhibits the expression of multiple oncogenes, such as *KRAS* [130] and *BCL-2* [131].

Ellipticine analogs, including GQC-05, NSC311153, and EPED3, have been reported to target proto-oncogenes and decrease tumor cell viability. GQC-05 was first reported as a high affinity and selective stabilizer of the *c-MYC* G4 [132]. In Burkitt's lymphoma cell line CA46, GQC-05 induced cytotoxicity by altering the process of protein binding to the NHE III region of *c-MYC*, resulting in a corresponding decrease in *c-MYC* mRNA expression. Also, GQC-05 could also reduce cell viability and result in increased DNA damage and apoptosis in the acute myeloid leukemia cell lines KG-1a, CMK, and TF-1 [133]. Furthermore, GQC-05 has been shown to combine with navitoclax, a Bcl-2/Bcl-X_L inhibitor, increasing its cytotoxic activity, which was more significant than either navitoclax or GQC-05 alone and more significant than navitoclax combined with cytarabine and adriamycin; however, its biological efficacy needs further verification in vivo. In addition to *c-MYC* G4, GQC-05 also targets the RNA G4 near the 5′ splice site in Bcl-X pre-mRNA to induce apoptosis in HeLa cells [134]. NSC311153 incorporates a piperidine ring into ellipticine and achieves better G4 binding activity and stability than ellipticine [135]. In the medullary thyroid carcinoma (MTC)-derived TT cell line and a mouse MTC xenograft model, NSC311153 could target the promoter G4 and interfere with the transcription of the proto-oncogene rearranged during transfection (RET), which encodes a receptor tyrosine kinase and is related to MTC [135]. EPED3 is a highly stable and hydrophilic ellipticine analog, which initiates apoptosis at nanomolar concentrations in multiple myeloma cell lines while leaving stromal cells unharmed [136]. However, whether EPED3 targets G4s and the specific mechanism remains to be explored.

Recently, a difluorosubstituted quinoxaline analog, named QN-1, was designed and assessed for triple-negative breast cancer (TNBC) treatment [137]. QN-1 exhibited distinctive binding to the 5′-end G-tetrad of *c-MYC* G4, with weaker binding to other G4s (including G4s from the telomeres, *BCL*-2, *VEGF*, *HRAS*, and *c-KIT*1), which can be distinguished from most of the reported G4 ligands. In 4T1 breast cancer cells, QN-1 selectively downregulated *c-MYC* transcription, resulting in cell cycle arrest and apoptosis. In a 4T1 tumor-bearing mouse model, QN-1 exhibited good in vivo anti-TNBC activity with fewer side effects [137]. Although QN-1 can target *c-MYC* G4 and show excellent efficacy in TNBC, it also has some inherent disadvantages, especially its high molecular weight and poor solubility in water. Therefore, depending on the properties of QN-1 and a benzothiazole-based derivative named 4I [138], another group designed a better drug-like imidazole-benzothiazole conjugate, named IZTZ-1 [139]. In a cell-free system, water-soluble IZTZ-1 showed high affinity to *c-MYC* Pu22 G4 ($K_D = 2.0$ μM) by stacking on both terminal G-quartets of Pu22. IZTZ-1 exhibited the same specific stabilizing ability on *c-MYC* G4 as with QN-1. Intracellular assays showed that IZTZ-1 could induce cell cycle arrest, apoptosis, and inhibit cell proliferation, whereas in a melanoma mouse model, IZTZ-1 can effectively inhibit tumor growth by downregulating *c-MYC* expression [139].

In addition, many other *c-MYC* G4 target ligands have been synthesized and assessed in vitro and in vivo, including DC-34 for myeloma [140], the quindoline derivative SYUIO-05 for Ramos and CA46 lymphoma cells [141], the SYUIO-05 based quindoline derivative 7a4 for Burkitt's lymphoma [142], and the aryl-substituted imidazole/carbazole conjugate IZCZ-3 for squamous cell carcinoma [143], which may provide many promising anticancer candidates in future clinical trials.

Currently, two G4 ligands have already entered human clinical trials: CX-3543 and CX-5461, both of which were derived from fluoroquinolones. CX-3543, also known as quarfloxin, can selectively disrupt nucleolin/G4 complexes in the nucleus, thereby suppressing the transcription of RNA polymerase I and inducing apoptosis in cancer cells [144, 145]. CX-3543 was the first G-quadruplex target drug used in human clinical trials and has progressed to phase II clinical trials for carcinoid/neuroendocrine tumors, but it was withdrawn from further trials because of bioavailability issues [146].

CX-5461, known as Pidnarulex, was reported to be highly selective for human telomeric, *c-KIT*1, and *c-MYC* G4s [147], and is a first-in-class selective rDNA transcription inhibitor [148]. CX-5461 can block replication forks and induce ssDNA gaps or breaks [149], and exerts cytotoxicity primarily via topoisomerase II poisoning [150] and the activation of the ubiquitination pathway [151]. An increasing number of studies have indicated that CX-5461 effectively targets multiple tumors. CX-5461 exhibited specific toxicity against BRCA deficiencies in cancer cells and polyclonal patient-derived xenograft models, including tumors resistant to PARP inhibition [149]. Meanwhile, CX-5461 was also reported to effectively treat aggressive acute myeloid leukemia by targeting a leukemia-initiating cell population [152] and the specific inhibition of the translation of certain

Table 2 G4 ligands and their therapeutic effects

G4 ligand	Target G4	Model	Effect	References
BRACO-19	*c-MYC*, Telomere, *KRAS*	Breast cancer cells MCF-7, human epidermoid carcinoma A431 cells, human uterus carcinoma UXF1138L cells, etc	Uncapping 3′ telomere ends, inhibiting the activity of BLM and WRN, T-loop disassembly, apoptosis induction	[189–191]
TMPyP4	Telomere, *c-MYC*, *KRAS*, *BCL-2*	Breast cancer cells MCF7, MDA-MB-231, retinoblastoma cell, melanoma cell	Suppressing the growth and proliferation of tumor cells	[130, 131, 262–265]
Pyridostatin	Telomere, *BRCA1*, *SRC*	Human breast cell line MDA-MB-231, human MRC5 fibroblasts, cortical neurons, astrocytes,	Promoting growth arrest, inducing neurotoxicity, promoting DSBs	[266, 267]
RHPS4	Telomere, mitochondrial G4	SAOS-2 osteosarcoma, U251MG glioma cells, etc	Inhibition of cell proliferation, promoting DNA damage and replicative stress, promotes recombination	[194]
GQC-05	*c-MYC*	Burkitt's lymphoma cell line CA46, acute myeloid leukemia cell lines KG-1a, CMK and TF-1	DNA damage, apoptosis	[132–134]
NSC311153	*RET*	Medullary thyroid carcinoma-derived TT cell line	Reduce cell viability	[136]
QN-1	*c-MYC*	Mouse breast carcinoma 4T1 cells, TNBC mouse model	Cell cycle arrest and apoptosis	[137]
IZTZ-1	*c-MYC,*	B16 melanoma cell, melanoma mouse model	Induce cell cycle arrest, apoptosis, and inhibit cell proliferation	[139]
DC-34	*c-MYC*	Human multiple myeloma cell lines L363, KMS12PE, JIM1, etc	Cell cycle arrest	[140]
SYUIO-05	*c-MYC*	Ramos and CA46 lymphoma cells	Dissociating the binding of NM23-H2 to the promoter	[141]
7a4	*c-MYC*	RAJI, CA46, CCRF-CEM, and U266B1 cells human Burkitt's lymphoma xenograft	Disrupting the NM23-H2/c-MYC interaction, cell cycle arrest, and apoptosis and suppress tumor growth	[142]
IZCZ-3	*c-MYC*	SiHa cells, human cervical squamous cancer xenograft	Provoking cell cycle arrest and apoptosis and inhibit cancer cell growth	[143]
CX-3543	*c-MYC*	HT29 cell lines, lung carcinoma A549 cells, colorectal adenocarcinoma HCT-116 cells, breast adenocarcinoma MDA-MB-231 cells and MCF7 cells, pancreatic carcinoma MIA paca-2 cells, and acute promyelocytic leukemia HL-60, etc	Disrupting nucleolin/G4 complexes, suppressing RNA transcription, inducing apoptosis	[144, 145]
CX-5461	Telomere, *c-KIT1*, *c-MYC*	Colorectal cancer DLD1 cells, ovarian cancer PEO1 cell, glioblastomas, oligodendrogliomas, oligoastrocytomas, etc	Blocking replication forks, inducing ssDNA gaps or breaks, disrupting topoisomerase II, activating the DNA damage response	[147–150, 152–155, 158]
Telomestatin	Telomere, *c-MYB*	Human lymphoma U937, glioma stem cell, etc	Dissociating telomere-binding proteins, causing telomere dysfunction in cancer cells	[172, 173, 175–177]
6OTD	Telomere	Human glioblastoma U251 cells, GSC lines GBM146 and GBM157, glioblastoma U251 mouse xenografts	DNA damage, G1 cell cycle arrest, and apoptosis	[179]
Berberine	Telomere, *RET, DUX4*	Medullary thyroid carcinomas TT cells, Rhabdomyosarcoma TE671 cells	Cell cycle arrest and apoptosis activation; inhibit muscle fibrosis, and consequently rescue muscle function	[183–185]

Table 2 (continued)

G4 ligand	Target G4	Model	Effect	References
Ber8	Telomere	Human cervical cancer cell Siha, human lung cancer cell A549, and human promyelocytic leukemia cell HL-60	Inhibition of cell cycle arrest, cell senescence, and accelerating profound DNA damage at telomere regions	[186]
Ber-360A and Ber-PDS	Telomere	HeLa and HepG2 cells	Telomerase inhibition	[187]
EPI	Telomere	–	–	[188]

metabolic regulators [153]. In combination with a single-dose X-ray, CX-5461 can enhance tumor cell killing effects in ovarian cancer cells and the CaSki cervical cancer line [154]. Besides, recent studies reported that CX-5461 was a promising therapy in the treatment of high-grade serous ovarian cancer (HGSOC), and it was shown that CX-5461 can arrest the G2/M cell cycle checkpoint in multiple HR-proficient HGSOC cell lines in combination with the clinically used TOP1 inhibitor topotecan [148], and activate the DNA damage response in combination with PARP inhibitors in HR-deficient HGSOC [155]. CX-5461 was also reported to significantly hamper the proliferation of TNBC cells and synergistically enhance the efficacy of the p53 activator APR-246 by cleaving PARP and caspase 3, while also maintaining Annexin V positivity [156]. In addition to cancers, a study corroborated that CX-5461 could prevent the development of pulmonary arterial remodeling, perivascular inflammation, pulmonary hypertension, and improved survival. CX-5461 could also partially reverse established pulmonary hypertension by inducing cell cycle arrest in human pulmonary arterial smooth muscle cells and enhancing the activity of p53 [157], highlighting its potential applications in chronic diseases.

CX-5461 is currently in advanced phase I/II clinical trials for patients with hematologic cancers (Trial ID: ACTRN12613001061729) and BRCA1/2-deficient tumors (Canadian Cancer Trials Group ID: NCT02719997) [149]. Meanwhile, the phase I dose-escalation clinical trial of CX-5461 in advanced hematologic cancers was completed in 2019, indicating that it is safe at doses associated with clinical benefit, and can attain prolonged partial response in patients with anaplastic large cell lymphoma and achieve stable disease as the best response in patients with myeloma and diffuse large B-cell lymphoma [158].

G-quadruplexes in the telomeres

G-quadruplexes affect telomere metabolism

Telomeric DNA are repetitive DNA sequences found at the ends of the chromosomes, and their lengths are closely related to cancers, aging-related diseases, type 2 diabetes, cardiovascular disease, and the human life span [159]. Telomeric DNA in humans usually contains a double-stranded repeat region 2–50 kb long that has approximately 300–8000 precise CCCTAA/TTAGGG repeats, as well as the 3′-tail single-stranded repeat region with 10–50 TTAGGG repeats, which can form G4 structures under certain conditions, thereby regulating telomere metabolism [160]. G4s in telomeres can perform multiple functions. First, G4s can act as a telomeric capping structure [161] and avoid the hydrolysis of the 3′-tail single-stranded region by nuclease (Fig. 2F), thereby guaranteeing the integrity of the telomere. However,

G4 is also a potential risk factor for telomere integrity, as large-scale G4s in the double-stranded repeat region of the telomere may hinder DNA replication by DNA polymerase. When G4-specific proteins such as helicase WRN [62], Pif1 [63], and nuclease DNA2 [68], EXO1 [69] are deficient, the replication forks at telomeres may stagnate and ultimately collapse. Therefore, G4 has played a dual role in maintaining telomere integrity.

In addition, G4 affects the activity of telomerase (Fig. 2F). As a unique reverse transcriptase, telomerase consists of the telomerase reverse transcriptase and telomerase RNA template and can catalyze telomeric DNA repeats onto the 3′-ends of the linear chromosome, generating new 3′-tail single-stranded repeat regions [162]. An early study reported that the folding of telomeric DNA into G4 impedes the elongation of the ciliate *Oxytricha nova* telomere [163]. Multiple studies have indicated that parallel intermolecular G4 DNA can serve as a substrate for telomerases from ciliates [164] and humans [165, 166]; however, the antiparallel and hybrid G4 structures formed by telomeric overhangs impede telomerase elongation [164–166]. It is worth mentioning that the POT1-TPP1 complex can bind to and unfold all telomeric G4 topologies (parallel, antiparallel, hybrid, or two contiguous quadruplexes) through an obligatory unfolding mechanism [167, 168]. Replication protein A (RPA), located at the telomere, acts as a telomere end-binding protein and mediates the unfolding of G4s [169, 170]. After the POT1-TPP1 complex or RPA disrupts telomeric G4s, telomerase can achieve the proper elongation of the telomere.

In most normal cells, telomerase activity is almost silenced, except in some T cells or stem-like cells that need to activate reverse transcriptase activity transiently during cell proliferation. Telomerase upregulation and activation are the general characteristics of various cancers, indicating that strategies targeting telomerase activity by telomeric G4 ligands could have significant anticancer effects [171].

G4 ligands targeting telomeres for drug design

Multiple telomeric G4-targeting ligands can inhibit telomerase activity and block tumor growth and metastasis. Telomestatin is a natural macrocyclic compound isolated from *Streptomyces anulatus* [172] and has been reported to interact specifically with the human telomeric intramolecular G4 in the absence of monovalent cations [173, 174]. Telomestatin was demonstrated to inhibit telomerase activity and shorten telomere length, exerting pro-apoptotic and antiproliferative effects in acute leukemia [175] and multiple myeloma [176]. Telomestatin can also segregate the telomere-capping protein TRF2 from the telomere, resulting in telomeric DNA damage and effectively activating the replication stress response pathway in glioma stem cells [177]. In addition to the disruption of telomeric G4, telomestatin also targets the promoter G4 of the proto-oncogene *c-Myb* and reduces *c-Myb* expression in glioma stem cells [178]. Based on the structure of telomestatin, a new series of macrocyclic hexaoxazole-type G4 ligands (6OTD) was synthesized. Ligands with the 6OTD structure caused DNA damage, G1 cell cycle arrest, and apoptosis in human glioblastoma cells, GSC cell lines, and glioblastoma U251 mouse xenografts, and these DNA damage foci were co-localized with telomeres, indicating that 6OTD limited the growth of GSCs by targeting telomeres [179]. Compared with temozolomide, a clinical antiglioma DNA alkylating agent, 6OTD needs a lower concentration to exert its anticancer effects, preferentially affecting GSCs and telomeres, verifying that 6OTD may be a potential therapeutic against glioblastoma [179]. Meanwhile, several other 6OTD derivatives have been synthesized, including L2H2-6OTD (1a), which was reported to target RNA G4s and show cytotoxicity towards cancer cells [180], as well as the 6OTD analog 5b, which preferentially stabilizes telomeric G4s over the promoter G4s of *c-KIT* and *KRAS* [181], their effects in vivo remain to be further identified.

Berberine, a natural isoquinoline alkaloid isolated from the Chinese traditional herb *Coptis chinensis* and other berberis plants, has shown antiinflammatory activity in a variety of chronic diseases and anticancer potential against various human cancer cells [182]. Berberine was reported to bind to telomeric G4 and was verified as a 2:1 ligand to the G-tetrad structure model, in which two berberines were in the two binding sites and directly interacted with each tetrad [183]. However, apart from telomeric G4s, berberine can also target other non-telomeric structures. In the medullary thyroid carcinoma TT cells, berberine inhibited RET expression by more than 90%, inhibiting cell proliferation via cell cycle arrest and apoptosis activation [184]. A recent study confirmed that berberine could target the promoter G4 of the *DUX4* gene, reduce *DUX4* expression, inhibit muscle fibrosis, and consequently rescue muscle function in facioscapulohumeral muscular dystrophy [185]. To date, multiple berberine derivatives have been designed to have improved efficacy. Ber8, a 9-substituted berberine derivative, exhibited strong interaction with telomeric G4s and could effectively inhibit cell cycle arrest, cell senescence, and accelerate DNA damage at telomeric regions in multiple cancer cells [186]. A further study revealed that Ber8 could not only facilitate the formation of endogenous telomeric G4s in cancer cells but also disaggregate TRF1 and POT1 from the telomere and induce telomere uncapping [186]. Some berberine derivatives have been shown to exhibit telomeric G4 specificity; for example, two berberine-bisquinolinium conjugates with fluorescence response, named Ber-360A and Ber-PDS, were able to distinguish telomere double G4s from other types of G4s and dsDNA, and displayed strong telomerase inhibition and anti-tumor activity, especially in HeLa and HepG2

cells [187]. In addition, a berberine derivative named epiberberine (EPI), was first reported to specifically bind to the hybrid-2 telomeric G4, which is the major form of G4 in wild-type human telomeric DNA, and induce an unprecedented extensive four-layer binding pocket specific to the hybrid-2 G4 [188] Furthermore, EPI could convert other telomeric G4 forms, such as hybrid-1 and basket-type, to hybrid-2, which was the first such example reported [188]. However, EPI still binds weakly to dsDNA, and its antitumor activity remains to be identified in cellular systems.

In addition, several other G4 ligands which can target G4 in the promoters of oncogenes, also can stabilize telomeric G4 and exert antitumor activity, such as BRACO-19 [189–191], TMPyP4 [192], CX-5461 [193], and RHPS4 [194] (Table 2). The tight relationship between cancer, telomerase, and telomeres makes antitumor therapeutic strategies involving G4 ligands more attractive.

G-quadruplexes affect epigenetic modifications

The relationship between G4 and epigenetic modifications is multifaceted. Under endogenous oxidative stress, reactive oxygen species (ROS) in cells cause DNA damage and focus on G4 sites, and different feedback mechanisms can upregulate or downregulate the expression of a large number of redox genes, resulting in oxidative DNA damage with epigenetic characteristics [195]. G4s, therefore, become epigenetic modification sites when the levels of oxidative stress become significantly increased during the inflammatory response and in cancer cells [196], as ROS can enhance the level of 7,8-dihydro-8-oxoguanine (8OG) more in G4 motifs than in non-G4 motif G-rich regions. The presence of 8OG in the G-quadruplex may lower the stability of the G4 depending on where the damage is located on its structure. The $G4_{near}$ of the *KRAS* promoter is a classical example of this. When 8OG occurs within the 11-nt loop of the $G4_{near}$ structure, its T_m and stability are almost unaffected; however, when 8OG is situated in the G-tetrad, both T_m and stability are greatly reduced [197]. It has been reported that the presence of 8OG on promoter G4s leads to an approximately 300% increase in gene expression [198]. Moreover, oxidized G4s that have been suggested to form unique, looped structures could also stimulate PARP-1 activity [199]. Furthermore, 8OG could enhance the binding of PARP-1 to $G4_{near}$ (Fig. 3A) and promote the recruitment of MAZ and hnRNPA1 to the promoter, thereby stimulating the transcription of *KRAS* in Panc-1 cells [200]. Certainly, 8OG on G4 can be removed through the DNA base excision repair (BER) pathway, which is initiated by 8-oxoG DNA glycosylase (OGG1) and subsequently recruits AP endonuclease 1 (APE1) to the G4 sequences and stabilize G4 folding [201]. However, a recent study indicated that OGG1 efficiently excised 8OG from oxidized $G4_{near}$ near the duplex but not within the G4 conformation; instead, endonuclease VIII-like 1 (Neil1) showed higher 8OG-excising activity in the G4 than the duplex DNA in Panc-1 cells [202].

G4 stability, position, and chromatin accessibility are closely related to CpG island methylation, where low- or non-methylated CpG islands prefer to form highly stable G4 [203]. The formation of G4 on CpG islands directly affects the activity of DNA methyltransferase (Fig. 3B) [204]. A typical example of this is DNA (cytosine-5)-methyltransferase (DNMT), which predominantly stalls and maintains cytosine methylation at CpG islands in mammals. It has been reported that human DNMT1, DNMT3A, and DNMT3B possess strong binding activity to G4 [205, 206]. It is vital to mention that the formation of the G4 structure can inhibit the methylation activity of DNMT1, thereby protecting certain CpG islands from methylation and suppressing local methylation [207].

G4 could also affect histone epigenetics by recruiting proteins with histone-modifying activity. A classic example of this is the G4-dependent recruitment of the RE1-silencing transcription factor (REST)-lysine-specific histone demethylase 1A (LSD1) repressor complex (Fig. 3C), which can remove the gene-activating monomethylation and demethylation of histone H3K4 [204]. G4 is indispensable for the occupancy of non-metastatic 2 (NME2) at the *hTERT* promoter as the REST-LSD1 repressor complex maintains repressive chromatin at the *hTERT* promoter and is dependent on NME2 [208]. In addition, telomere repeat-binding factor 2 (TRF2), which functions both inside and outside the telomere, was found to also interact with G4s in promoters [209, 210]. After recruitment in the promoter G4, TRF2 can promote the formation of the REST-coREST-LSD1-repressor complex at the p21 promoter and alter histone marks, resulting in the downregulation of p21 transcription and a reduction in the DNA damage response activation upon treatment with doxorubicin and G4-ligand 360A in cancer cells [210]. In contrast, four G4 motifs are formed in the promoter of Zinc-finger E-box binding homeobox 1 (*ZEB1*), which is frequently associated with cancer aggressiveness. After binding to the P1 G4, nucleolin could remodel the local genomic region, facilitate the binding of SP1, recruit P300 acetyl transferase, and enrich acetyl-histone H3 at the promoter, thereby inducing transcription and oncogenic progression (Fig. 3D) [122].

G4 causes local epigenetic reprogramming after stalling the replication fork [204, 211]. In REV1-deficient cells, DNA replication stagnation induced by G4 DNA can result in the uncoupling of DNA synthesis from histone recycling, leading to the localized loss of repressive chromatin through the preferential incorporation of newly synthesized unmodified histones during gap filling [212, 213] (Fig. 3E). G4 ligands can also hinder DNA replication and trigger local epigenetic plasticity via H3K4me3

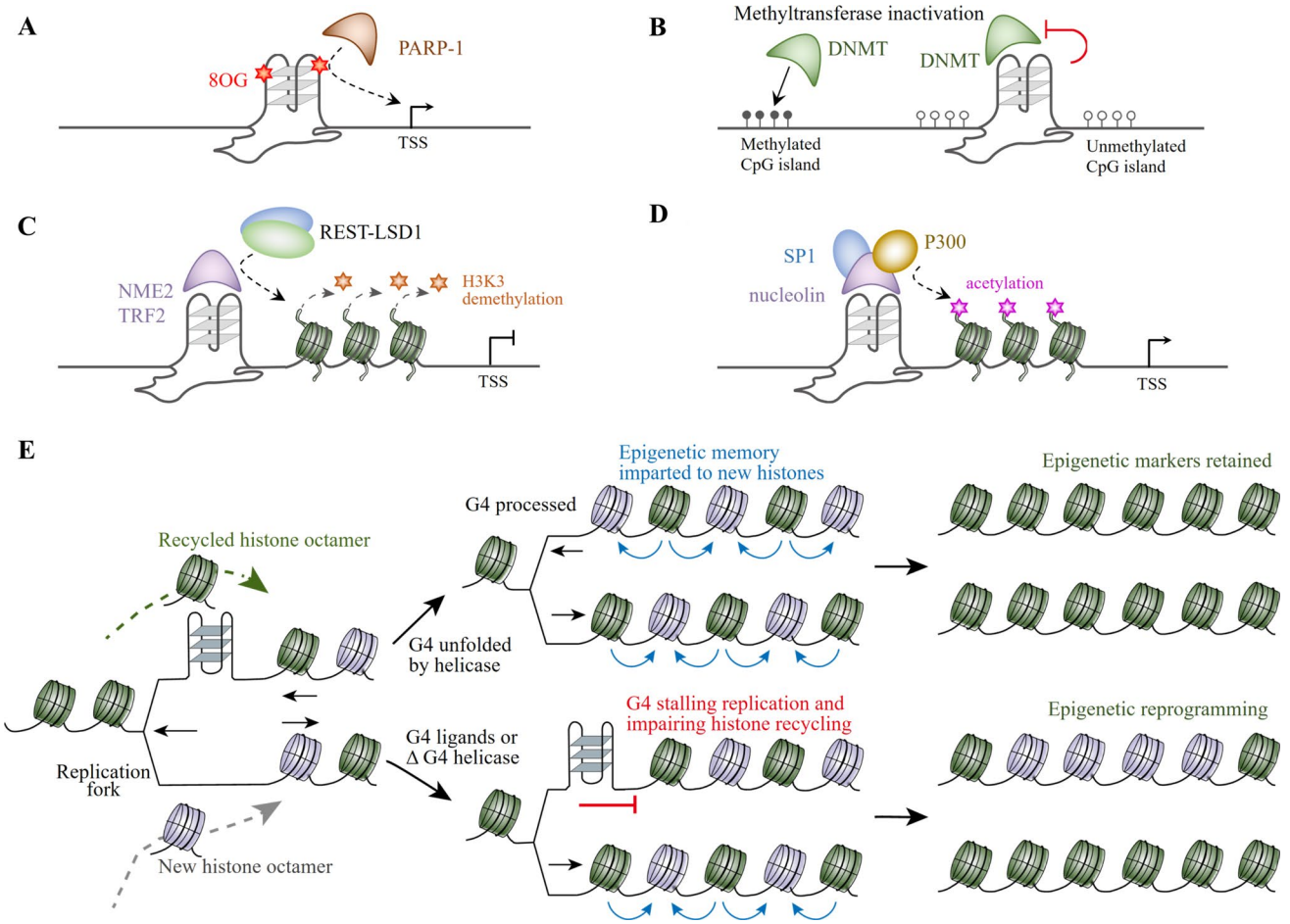

Fig. 3 G-quadruplexes can affect epigenetic modifications. **A** G4 can become epigenetic modification sites when the levels of oxidative stress increase. The presence of 8-oxoguanine (8OG) on the G-tetrad decreases the stability of the promoter G4, leading to an increase in gene expression. Meanwhile, 8OG could stimulate the binding of PARP-1 to the promoter G4 and promote the recruitment of MAZ and hnRNPA1 to the promoter, thereby stimulating transcription. **B** The formation of G4 at CpG islands can directly inhibit the activity of DNA methyltransferase after G4-preferred methyltransferase binding to the G4 structure. A typical example of this involves the DNA (cytosine-5)-methyltransferase DNMT1, DNMT3A, and DNMT3B. **C** G4 can affect histone epigenetics through the recruitment of proteins with histone-modifying activity, such as NME2 or TRF2, which can selectively bind to G4 and recruit the REST-LSD1 complex to remove the methylation of histone H3 Lys4 (H3K4) and inhibit gene expression. **D** In the promoter of *ZEB1*, nucleolin could bind to the G4 motif, remodel the local genomic region, facilitate the binding of SP1, and recruit P300 acetyltransferase, leading to enriched acetylhistone H3 at the promoter, inducing gene transcription and oncogenic progression. **E** G4 can cause epigenetic reprogramming after the replication fork stalls at the G4 site in the presence of G4 ligands or the deficiency of G4-specific helicases. G4 structures may also impair histone recycling and lead to the localized loss of repressive chromatin, causing epigenetic reprogramming

loss and DNA cytosine methylation at the BU-1 locus of chicken DT40 cells [214]. Furthermore, a depletion of the nucleotide pool and a deficiency of G4-processing helicases, such as FANCJ, WRN, and BLM, will also induce epigenetic reprogramming and change in gene expression [215, 216]. Therefore, the replication fork stalling caused by unresolved G4 not only affects genome stability and telomere length but also impacts histone recycling and epigenetic reprogramming.

G4-interacting DNA helicases

Helicases are molecular motors that hydrolyze nucleoside triphosphates to execute critical functions in various DNA metabolic processes, including DNA replication, transcription, HR, repair, and telomeric metabolism. As the formation of stable G4 during DNA replication and transcription may cause genomic instability and gene expression

regulation, G4 structures must be disrupted or unfolded in a regulated manner. Therefore, G4-specific unwinding helicases have been studied in-depth through various methods in recent years. The DNA G-quadruplex unwinding helicases are listed in Table 3, among which Pif1, RecQ family helicases, FANCJ, and DHX36 are known to be closely related to human diseases and have recently been studied in depth.

Pif1

As the founding member of superfamily 1 DNA helicase, Pif1 is evolutionarily conserved from bacteria to humans and plays a vital role in maintaining genome stability in both the nucleus and mitochondria [217, 218]. Pif1 was first found to affect the activity of telomerase by displacing telomerase from telomeric DNA in both cell-free and cellular systems [219] and promote the proliferation and suppress the apoptosis of cervical cancer cells [220]. It was then found that Pif1 can remove telomerase from the telomere ends and accelerate the cycling of telomerase for additional telomere length-dependent extensions, thereby maintaining telomere length [221]. Furthermore, some proteins were found to contribute to telomere length homeostasis in cooperation with Pif1, such as Hrq1 [222] and RPA [223].

Pif1 directly promotes DNA replication through G4 motifs. The G4 stabilizer PhenDC3 was found to cause single-strand DNA lesions and impede DNA replication at G4 sites in the cell, and this stalled DNA synthesis machinery can be resolved by the members of the Pif1 family of helicases [224]. In Pif1-deficient cells, the DNA replication fork becomes stalled, inducing DNA breakage at the G4 motif sites [25]. Furthermore, the absence of Pif1 can cause genomic instability when G4s occur at the leading strand template [225]; however, another study concluded that stalled DNA replication occurred at the lagging strand G4 sites in Pif1-deficient cells, rather than on the leading strand [226]. The differences between the two studies may stem from the differences in the G4 sequences studies, assays for characterization, and genomic location. Meanwhile, it was affirmed that the coupling between Pif1 and PCNA is indispensable for the optimal progression of the replisome through G4 motifs [226].

Pif1 preferentially binds G4 DNA relative to ssDNA, dsDNA, and a partially single-stranded duplex DNA helicase substrate, highlighting its G4 interaction specificity [227]. In

Table 3 Summary of G4 unfolding helicases

Superfamily	Subfamily	Name	Polarity	Function	Genetic disease	References
SF1		Pif1	5′–3′	Telomere maintenance, DNA replication, mitochondrial genome maintenance, DSB repair, epigenetic regulation		[229, 268]
		DNA2	5′–3′	DNA replication, telomere maintenance		[68, 269]
		UvrD	3′–5′	Nucleotide excision repair, mismatch repair, HR		[270, 271]
		Rep	3′–5′	DNA replication		[272]
		RecD	5′–3′	Double-strand-break repair		[273]
SF2	Fe-S	FANCJ	5′–3′	DNA replication, DNA crosslink repair, epigenetic regulation	Fanconi anemia, breast cancer	[215, 232, 274]
		XPD	5′–3′	Nucleotide excision repair, transcription regulation	Cockayne syndrome, COFS syndrome, Xeroderma Pigmentosum	[22]
		DDX11	5′–3′	Sister chromatid cohesion, post-replicative repair, DNA replication	Warsaw Breakage Syndrome	[64, 275, 276]
		RTEL1	5′–3′	Telomere maintenance, DNA replication, DNA transcription	Dyskeratosis congenita	[234, 277, 278]
	RecQ	RecQ	3′–5′	DNA repair, HR, DNA replication		[243]
		BLM	3′–5′	DNA repair, HR, DNA replication	Bloom syndrome	[238, 279]
		WRN	3′–5′	DNA repair, HR, DNA replication, epigenetic regulation	Werner syndrome	[279, 280]
		RecQ5	3′–5′	HR, DNA replication		[244]
	DEAH	DHX9	3′–5′	R-loop generation, DNA replication		[281]
SF3		SV40 T-ag	3′–5′	DNA replication, transcription		[282, 283]
SF4		Twinkle	5′–3′	Mitochondrial DNA replication		[284]
SF5		DHX36	5′–3′	Telomere maintenance, transcription regulation		[250, 251]

a cell-free system, simulated DNA replication of an ongoing synthesis of lagging strands stalled by G4 was established and detected using single-molecule fluorescence assays, verifying that Pif1 can unfold the G4 structure sequentially in two large steps, then halt at the ss/dsDNA junction, followed by a rapid reformation of G4 and the "acrobatic" re-initiation of G4 unfolding, exhibiting its periodic patrolling activity at the stalled DNA replication sites [228, 229]. In addition, G4 unfolding mediated by Pif1 is sensitive to G4 stability, and it was found that G4 structures with short loops can be barely unfolded by Pif1 and that Pif1 preferentially unfolded antiparallel G4 rather than parallel G4 of similar stability [230, 231]. Therefore, Pif1 is a G4-specific helicase with strong G4 unwinding activity, but cooperation with other helicases or proteins may be needed when interacting with highly stable G4s.

FANCJ

FANCJ was initially ascertained as a direct interaction partner of breast cancer type 1 susceptibility protein (BRCA1); therefore, it was also termed BRCA1-associated C-terminal helicase 1 (BACH1) or BRCA1-interacting protein 1 (BRIP1) [232]. FANCJ functions in the Fanconi anemia (FA) pathway that promotes inter-strand crosslink (ICL) repair through the interplay of lesion excision, translesion synthesis, and HR; a FANCJ mutation was found to be a cause of the bone marrow failure syndrome FA. Currently, FANCJ mutations are associated with an increased risk of multiple cancers, such as breast cancer, ovarian cancer, melanoma, prostate, and hereditary colon cancers [233].

In addition to ICL repair, FANCJ was reported to dominate G4 unwinding activity in both cell-free and cellular systems, laying the foundation for its function in DNA replication stalling and epigenetic reprogramming caused by G4s. The combination of a fluorescent probe and fluorescence lifetime imaging microscopy confirmed that a decrease in FANCJ expression can increase the number of G4s in mammalian cells, implying that FANCJ plays a role in unfolding G4 structures in cells [234]. In *C. elegans*, a deficiency in the FANCJ ortholog dog-1 can cause exclusive deletions of G4 DNA and eventual genome instability [235], and that depletion of FANCJ caused persistent DNA replication stalling at G4 sites [60], demonstrating the vital role of FANCJ in G4 replication. FANCJ was reported to coordinate two pathways for the efficient replication of G4s in the cell, in concert with either TLS polymerase REV1 or the WRN and BLM helicases, to initiate G4 unfolding and replication [215]. FANCJ occupies an iron–sulfur (FeS) cluster, wherein mutations in this cluster have been associated with cancer predisposition. A recent study reported that FANCJ required an intact FeS cluster to unfold G4 structures on the DNA template as observed via a primer extension assay using the lagging strand DNA polymerase δ [232], and that FANCJ knockout cells expressing FeS cluster-deficient variants displayed a similar enhanced sensitivity to the G4 ligands pyridostatin and CX-5461 [232], highlighting the remarkable role of the FeS domain in G4 metabolism as executed by FANCJ. Several other G4 ligands have also been reported to affect the G4 unfolding activity by FANCJ; telomestatin, Phen-DC3, and Phen-DC6 inhibited FANCJ helicase in unimolecular G4 and in bi- or tetramolecular G4 DNA [236].

RecQ family helicases

The RecQ family helicases, including *E. coli* RecQ, *S. cerevisiae* sgs1, *H. sapiens* RecQ1, BLM, WRN, RecQ4, and RecQ5, widely participate in various DNA metabolic processes, including DNA repair, HE, and telomerase maintenance. Importantly, mutations in *H. sapiens* BLM, WRN, and RecQ4 cause Bloom, Werner, and Rothmund–Thompson syndromes, respectively, which are linked to premature aging, profound developmental abnormalities, and an increased risk of cancers [237]. RecQ family members *E. coli* RecQ, *S. cerevisiae* sgs1, and *H. sapiens* BLM, WRN, and RecQ5 were reported to dominate G4 unfolding activity (Table 3). As for BLM, three mechanisms have been authenticated to be involved in G4 unfolding [238]: (1) BLM unfolds G4 with a 3-ssDNA tail in three discontinuous steps via unidirectional translocation, (2) the unfolded G4 gets connected to dsDNA via ssDNA in a repetitive manner in which the same helicase remains anchored at the ss/dsDNA junction, and (3) the G4 unfolds by reeling in 5′-ssDNA. Mechanistically, the structure of a bacterial RecQ helicase bound to unfolded G4 DNA has been reported, revealing a guanine-flipping and sequestration mechanism for G4 unfolding by RecQ helicases or other G4 unwinding helicases [239].

The helicase and RNaseD C-terminal (HRDC) domain [240], which are connected to the RecQ core by a flexible linker and are unique to *E. coli* RecQ, *S. cerevisiae* sgs1, and *H. sapiens* BLM and WRN, have been reported to perform vital functions in G4 unfolding. Using a stopped-flow assay, the HRDC domain was reported to improve G4 unfolding in *Neisseria gonorrhoeae* RecQ [241]. The cooperation between the RecQ core and the HRDC domains of BLM was demonstrated during G4 binding and unfolding, where the RQC domain interaction with G4 can be stabilized via HRDC binding to ssDNA [242]. Recently, using single-molecule assays, the HRDC domain was reported to be essential for the unfolding of the G4 structure by *E. coli* RecQ, while another model different from BLM was proposed, in which HRDC can reinforce the association of RecQ on the DNA by interacting with the RecA core, leading to a complete and longer-lasting G4 unfolding [243]. Interestingly, *H. sapiens* RecQ1 and RecQ4, which do not occupy the HRDC domain

intrinsically, cannot unfold G4, and RecQ5, which does not possess the HRDC domain, had G4 unfolding activity that was an order of magnitude weaker than that of BLM or WRN [244]. Therefore, the HRDC of RecQ family helicases plays a unique role in the G4 unfolding process, but its function in cells requires further exploration.

DHX36

DHX36, also known as G4 resolvase (G4R1), MLE-like protein 1 (MLEL1), or RNA helicase associated with AU-rich element (RHAU), is involved in multiple cell differentiation processes, including spermatogenesis, heart development during embryogenesis, hematopoiesis, as well as the dendritic localization of neuronal precursor microRNA [245], and functions in the regulation of RNA structures. DHX36 has extreme affinity and efficiency in binding and unfolding both RNA G4 and DNA G4 [246], regulating the expression of cancer-related genes, such as *p53*, *PITX*, *YY1*, *VEGF*, and *ESR1* [247, 248], and function in breast cancer, lung cancer, and colon cancer, among others [249].

The G4 unfolding mechanism of DHX36 was revealed through its crystal structure. The G4 DNA-resolving model was proposed, stating that the negatively charged G4 DNA was tightly bound to and was partially destabilized by a positively charged structural pocket in the RecA2 and OB-like domains of *Drosophila* DHX36; subsequently, the G4 structure was thoroughly unfolded by the translocation activity of DHX36 [250]. Another study shed light on a parallel G4 unfolding model by DHX36 in an ATP-independent manner, in which the N-terminal specific DNA binding-induced α-helix of DHX36 together with the OB-fold-like subdomain selectively binds to parallel G4, then the G4 binding process induces the rearrangement of the helicase core, finally actuating G-quadruplex unfolding one residue at a time by pulling on the ssDNA tail [251]. Depending on the structure of the DHX36 binding to G4, a bi-functional guanine–RHAU23 peptide conjugate composed of a guanine moiety and a 23-aa G4-binding domain from the N-terminus of the RHAU helicase was designed to target and stabilize G-vacancy-bearing G4s with superior specificity, providing a novel and promising strategy to target some non-classical G4s [39].

Conclusions

The existence, structure, and function of G-quadruplex DNA structures have expanded exponentially in the last decade. With the development of detection strategies, especially through the application of high-throughput sequencing, G4 detection in cellular systems is becoming increasingly accurate. However, the topology of inherent G4 structures is highly diverse, dependent on their sequence specificity, existing ionic environment, and the accompanying molecular chaperones. Multiple G4 topologies may co-exist for the same sequence, so the investigation of the potential topologies for G4 sequences that play key roles in biological processes requires further in-depth studies. The biological effects of different G4 topologies and their transformations also merit a thorough exploration, particularly regarding their regulation of disease-related gene expression. In addition, G4-specific helicases are indispensable in organisms; apart from G4 unfolding, the biological function of helicase-mediated G4 remodeling also needs to be uncovered.

The diversity in distribution and the vital regulatory functions of G-quadruplex structures in the genome show that they are promising targets in drug design. Currently, large numbers of G4 ligands have been discovered or synthesized, but many of these do not exhibit specificity or clear structure–activity relationships in vivo, so ligands capable of targeting specific G4 structures and their therapeutic potential still require further study, especially in vivo.

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