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Existence and consequences of G-quadruplex structures in DNA

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While the discovery of B-form DNA 60 years ago has defined our molecular view of the genetic code, other postulated DNA secondary structures, such as A-DNA, Z-DNA, H-DNA, cruciform and slipped structures have provoked consideration of DNA as a more dynamic structure. Four-stranded G-quadruplex DNA does not use Watson-Crick base pairing and has been subject of considerable speculation and investigation during the past decade, particularly with regard to its potential relevance to genome integrity and gene expression. Here, we discuss recent data that collectively support the formation of G-quadruplexes in genomic DNA and the consequences of formation of this structural motif in biological processes.

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Introduction

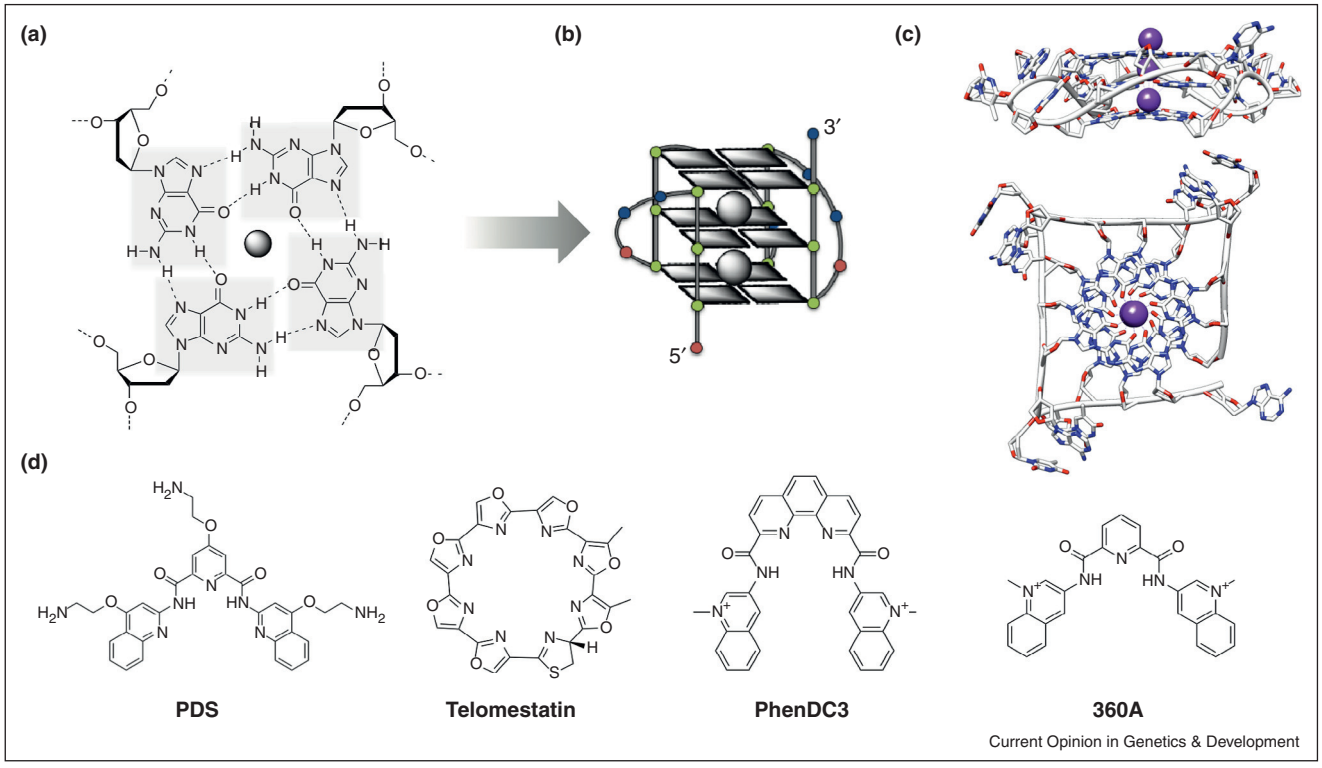
The self-assembly of guanylic acid derivatives has been known for more than a century [1] and the structural basis for this phenomenon was elucidated in the 1960s [2]. Guanine-tetrad formation (Figure 1a) drives the assembly of four-stranded helixes by guanine-rich oligonucleotides (G-quadruplexes, Figure 1b). Seminal studies by Sen and Gilbert, and by others [3–7], showed that these cation-dependent, G-quadruplex structures are thermodynamically stable under physiological conditions, and subsequently such structures were proposed to be involved in telomere association, recombination and replication. Biophysical methods have provided extensive *in vitro* data on the structure(s) and thermodynamics of DNA G-quadruplexes formed from oligonucleotides derived from genomic sequences that have included the human telomere (Figure 1c) [8] and promoter regions of oncogenes (e.g. *MYC*) [9]. Structural data has facilitated the design and synthesis of G-quadruplex-specific small molecules [10] (see Figure 1d for example of G-quadruplex ligands), several of which trigger cellular mechanisms proposed to be linked with G-quadruplexes. Notable examples of chemical biological studies include the small molecule inhibition of telomerase action via G-quadruplex stabilization [11], and also transcriptional suppression of *MYC* by a G-quadruplex ligand [12]. There are indeed many more examples in the literature of cell-based studies that provide supportive correlations. However, some such studies have not addressed whether the key G-quadruplex in question actually exists in the genomic DNA and if so whether it is responsible for causation of the observed effects.

Visualization and mapping of genomic G-quadruplex DNA

Biophysical studies on G-quadruplex structures formed by oligonucleotides *in vitro* have allowed the formulation of quadruplex-prediction algorithms on the basis of sequence motifs such as $G_{\geq 3}N_XG_{\geq 3}N_XG_{\geq 3}N_XG_{\geq 3}$. The use of such algorithms, for example, QuadParser [13] and G4P Calculator [14], has shown that quadruplex motifs prevalent in genomes are enriched within regions associated with regulation including promoters, introns and UTRs [13–16] (Figure 2a). The great potential for G-quadruplex formation in cellular genomic DNA has stimulated the need to experimentally confirm the presence of these structures in cells.

G-quadruplex DNA-recognizing antibodies have been exploited to visualize these structures within genomic DNA. In a landmark paper, Schaffitzel *et al.* described use of high-affinity single-chain antibodies, generated by ribosome display, to visualize quadruplex structures at the telomeres of the ciliate *Stylonychia lemnae* [17]. Immunofluorescence studies show that one of the selected antibodies, Sty49, reacts specifically with the macronucleus but not the micronucleus of the ciliate (Figure 2b). Of particular note is the observation that, the replication band is not stained suggesting that G-quadruplex DNA is resolved during replication in ciliates. Using the same antibody, Paeschke *et al.* showed that the telomere end-bing proteins (TEBPα and TEBPβ) co-operate to control the formation of anti-parallel G-quadruplex structures at telomeres *in vivo* in *S. lemnae* [18] via a mechanism biochemically linked to a cell cycle-dependent

Figure 1

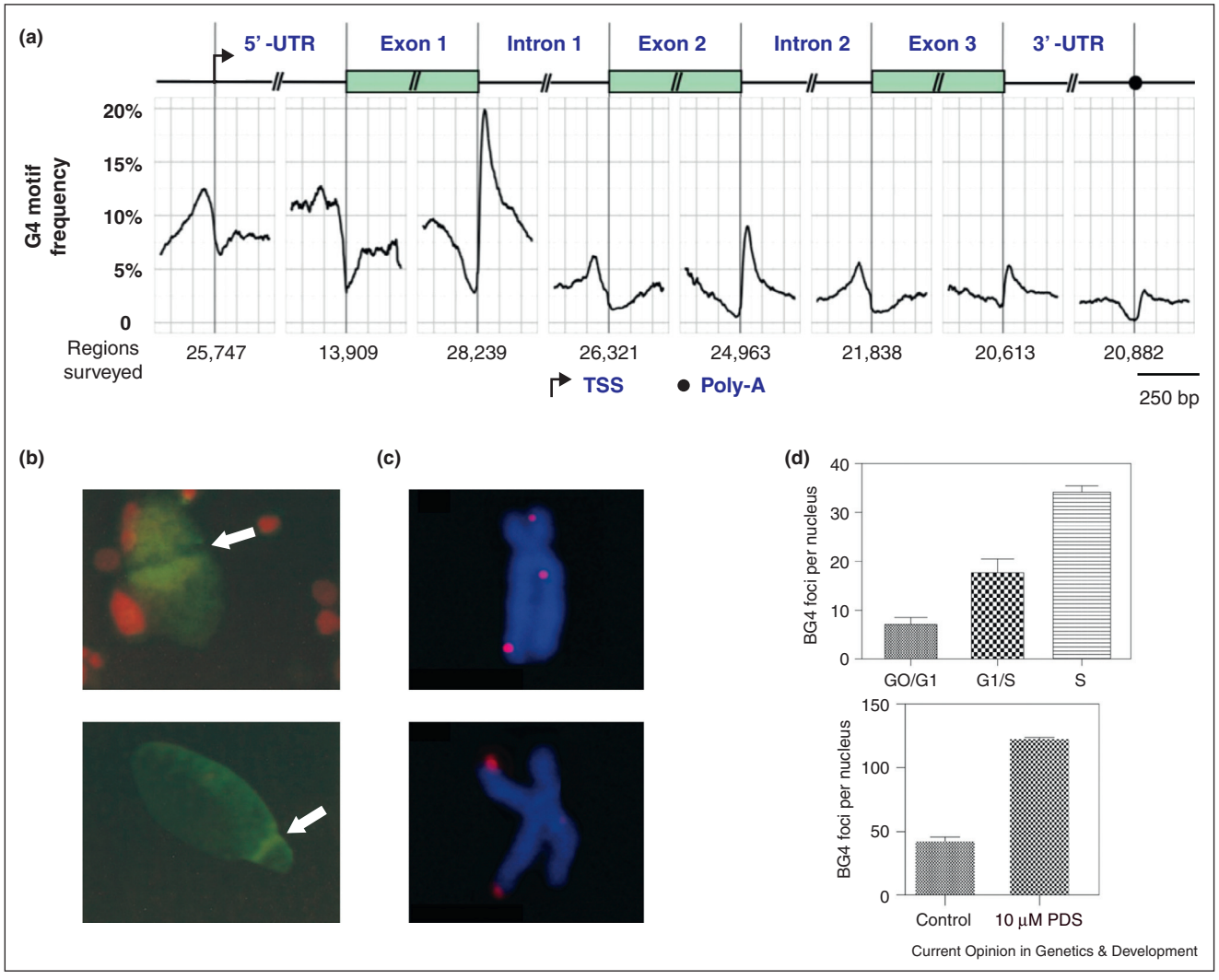


(a) Structure of guanine tetrads formed by the coplanar arrangement of four guanines held by Hoogsteen bonds and stabilised by monovalent cations (usually K^+). **(b)** Schematic representation of a quadruplex motif formed by guanine rich DNA sequences and stabilized by the stacking of guanine tetrads. **(c)** Side and top view of the crystal structure of the human telomeric quadruplex (PDB: 1KF1). **(d)** Small molecule G-quadruplex ligands used to study G-quadruplex DNA in human cells and yeast.

phosphorylation of TEBPβ [19]. Recent work reported by Biffi *et al.* described a monoclonal single chain antibody, BG4, generated by phage display with high affinity and specificity for intramolecular G-quadruplex structures [20••]. Immunostaining of a range of human cells shows the presence of G-quadruplex structures in cellular genomic DNA. Interestingly, positional analysis of foci either by metaphase chromosome spreads or by analysis of co-localization with antibodies to the telomere binding protein, TRF2, indicated quadruplex formation in telomeres and outside telomeres with a higher proportion at non-telomeric sites (Figure 2c). Quantitation of the immunofluorescent foci in synchronized cells showed that: (a) some quadruplex formation was evident during all phases of the cell cycle; and (b) that overall quadruplex levels are modulated during cell-cycle progression with a maximal number of foci observed during the S phase, consistent with replication-dependent formation of G-quadruplex structures (Figure 2d) [20••]. Treatment of live cells with the G-quadruplex-trapping small molecule pyridostatin (PDS), before immunostaining, increases the number of foci, providing substantive evidence that a small molecule can trap quadruplex structures in cellular DNA (Figure 2d). Indeed, complementary studies previously carried out using the radioactively labeled G-quadruplex ligand [^{3}H]-360A, showed selective binding at the telomeres of chromosomes of both human normal (peripheral blood lymphocytes) and tumor (T98G and CEM1301) cells [21]. Collectively, such studies have provided insights into the formation of G-quadruplex structures in the DNA in a cellular context. It cannot be ruled out that the process of fixing cells or the binding probe influences the formation of G-quadruplex-structures. However, the dynamic changes in apparent quadruplex sites coupled to changes in cellular states suggest the observations reflect structures that are intrinsic to genomic DNA.

The emergence of practical high-throughput DNA sequencing has transformed our ability to characterize genomic features at scale with precision [22]. Earlier this year, Lam *et al.* reported the use of another single-chain G-quadruplex specific antibody, hf2, to enrich for genomic DNA fragments containing folded G-quadruplex structures from mechanically fragmented DNA derived from MCF7 breast cancer cells [23]. Deep sequencing of libraries generated from the enriched DNA was used to identify technically reproducible peaks that correlated

Figure 2



(a) G-quadruplex frequency in a generic human RefSeq gene (extracted from Ref [16]). **(b)** Top: Immunostaining of a replicating macronucleus of *Stylonychia lemnae* using the single chain antibody, Sty49, directed against telomeric quadruplex. Bottom: hybridization of an FITC-labeled telomeric probe to a replicating macronucleus; arrow points to the replication band (extracted from Ref [17]). **(c)** Immunofluorescence for BG4 on metaphase chromosomes isolated from Hela cervical cancer cells. Discrete BG4 foci (red) were observed both within the non-telomeric regions (top) and at the telomeres (bottom) (extracted from Ref [20••]). **(d)** Top: Quantification of BG4 foci per nucleus for staining of MCF-7 cells. Bottom: Quantification of BG4 foci number per nucleus (U2OS cells) with or without PDS treatment.

with computationally predicted G-quadruplex motifs. Stable quadruplex structures were experimentally mapped in regions that included sub-telomeres, gene bodies and gene regulatory sites. This approach allowed the identification of several genes with associated promoter G-quadruplexes, including *PVT1* and *STARD8*, whose expression could be modulated by addition of the quadruplex ligand PDS to cells [23]. Rodriguez *et al.* used deep sequencing to map the sites of the DNA damage marker γH2AX induced by the treatment of human cancer cells with the quadruplex binding small molecule PDS [24••]. Chromatin immunoprecipitation with an antibody against the DNA damage marker γH2AX followed by sequencing of the enriched DNA (ChIP-Seq) identified regions that were enriched for computationally predicted G-quadruplex motifs. Cell cycle analysis and the use of chemical inhibitors confirmed that PDS induces double strand breaks which are replication and transcription dependent.

Natural G-quadruplex binding proteins have provided important insights into the location of G-quadruplex structures in genomic DNA. For example, the binding sites of the *Saccharomyces cerevisiae* Pif1 DNA helicase, a

Table 1

Selection of proteins that have been identified to interact with G-quadruplex DNA *in vitro* and that have been associated to G-quadruplex biology.

Protein	Species	Type	Proposed function	Ref
ATRX	Human	Nucleosome remodeling complex, possess homology with helicases	Modify epigenetic state at G-quadruplex sites and/or resolve G-quadruplexes.	[36,37]
BLM	Human Chicken	3′-to-5′ DNA helicase	Prevent genetic instability at G-quadruplex sites (not G-quadruplex specific).	[26,39]
DOG-1	*C. elegans*	5′-to-3′ DNA helicase (FancJ homologue)	Prevent genetic instability at G-quadruplex sites.	[28,29]
FANCJ	Human Chicken	5′-to-3′ DNA helicase	Prevent genetic and epigenetic instability at G-quadruplex sites (acting in concert with REV1 and/or WRN/BLM).	[30,31,39]
PIF1	*S. cerevisiae* Human	5′-to-3′ DNA helicase	Prevent genetic instability at G-quadruplex sites and inhibits telomere lengthening	[25,32–34]
RecQ	Gonococci *E. coli*	3′-to-5′ DNA helicase	Control G-quadruplex induced recombination events in pathogens	[35]
REV1	Chicken	Y family translesion polymerase	Prevent epigenetic instability at G-quadruplex sites	[38,39]
WRN	Human Chicken	3′-to-5′ DNA helicase	Prevent genetic instability at G-quadruplex sites (not G-quadruplex specific)	[27,39]

potent unwinder of G-quadruplex structures *in vitro*, were mapped by ChIP-Seq [25•] to G-quadruplex motifs in a significant subset of the high-confidence Pif1-binding sites. Again consistent with an association in replication, Pif1 was more strongly associated with G-quadruplex motifs in late S phase and DNA Pol2 levels are higher at G-quadruplex sites in the absence of Pif1, suggestive of pausing. This approach experimentally identified 138 (of the 558 predicted) quadruplex motifs in the genome of *S. cerevisiae*. These observations are complemented by studies that employed super-resolution microscopy with fluorescent tagging of a PDS derivative that showed significant co-localization with Pif1 foci in human U2OS cells [24••].

Both visualization and mapping experiments [17,20••,24••,25•] suggest that G-quadruplex DNA formation is associated with replication. It is worth noting that the creation of single-strand gaps on the lagging strand at replication forks may create a context particularly prone to G-quadruplex formation. Thus any biological events that need DNA in single stranded form may be affected by the formation of metastable secondary structures such as G-quadruplex DNA.

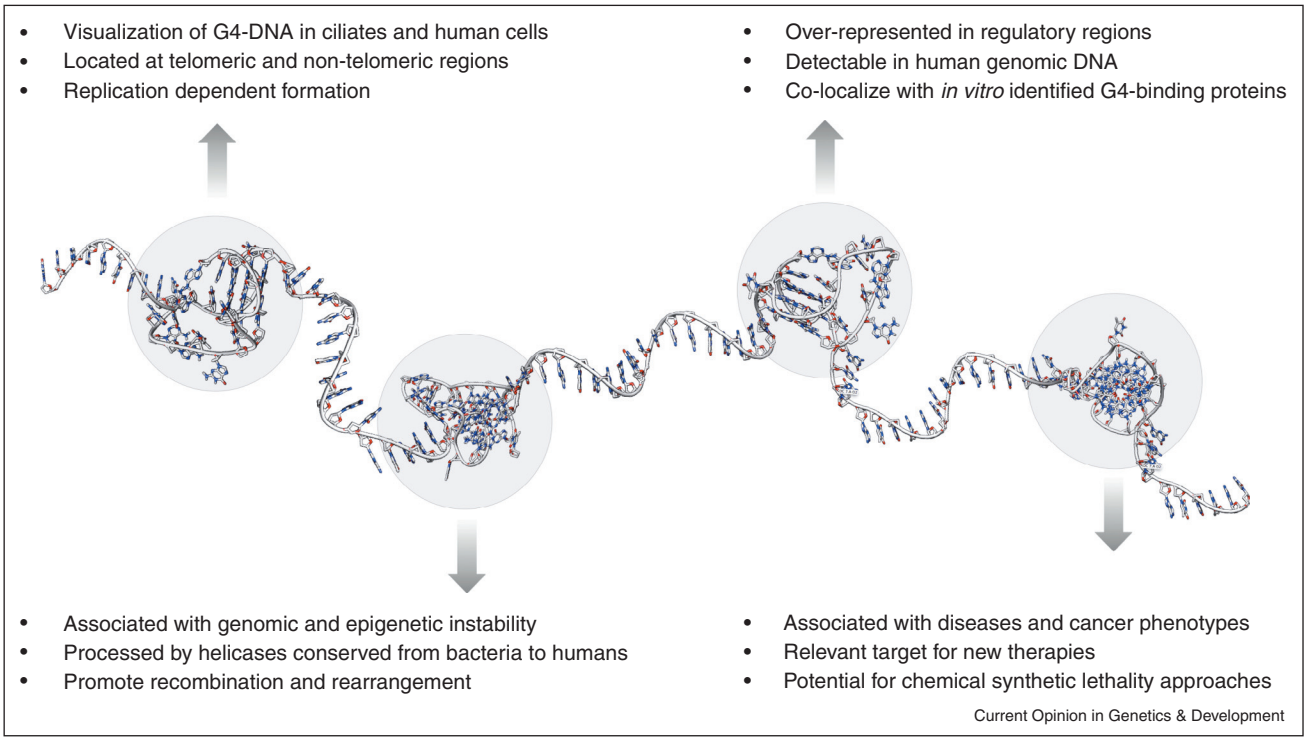
Relevance of G-quadruplex DNA

We now focus on recent articles that describe biological consequences that are linked to quadruplex DNA. Many natural proteins have been identified that interact with quadruplex-DNA and Table 1 illustrates a range of protein activities that support the relevance of G-quadruplex DNA to replication and transcription.

Genome integrity is essential to maintain normal cell function, and malfunctioning in DNA replication or repair can lead to genetic instability and disease. Biochemical studies have shown that G-quadruplex DNA can be resolved, in particular, by the RecQ family of helicases that include BLM [26] and WRN [27]. In addition, Lansdorp *et al*. showed that disruption of DEAH helicase named *dog-1* (deletion of guanine rich DNA) in *Caenorhabditis elegans* triggers deletions of upstream guanine-rich DNA [28], especially in regions with at least 22 consecutive guanines. It would thus appear that G-quadruplex DNA could promote genetic rearrangements *in vivo* [29]. The human homologue of DOG-1 is FANCJ, which is mutated in Fanconi anemia patients, and is also able to unwind G-quadruplex DNA *in vitro*. FANCJ-deficient cells display elevated levels of DNA damage when treated with the G-quadruplex ligand telomestatin [30], and genome analysis of DNA deletions in a patient-derived FANCJ loss-of-function cell line indicates a bias in breakpoint locations proximal to predicted G-quadruplex sites [31]. Furthermore, absence of Pif1, a distant homologue to the RecD bacterial helicase, also promotes genetic instability at alleles of the G-rich human minisatellite CEB1 inserted in the *S. cerevisiae* genome, but not of other tandem repeats [32]. Inactivation of other DNA helicases, including Sgs1 (*S. cerevisiae* RecQ homologue), had no effect on CEB1 stability. Still in *S. cerevisiae*, replication fork progression is slowed particularly at G-quadruplex motifs, in the presence of the replication inhibitor hydroxyurea, in Pif1 deficient cells [25•]. As, the G-quadruplex unwinding properties of Pif1 helicases are conserved from bacteria to humans, this suggests the possibility of evolutionary selection of proteins that maintain genomic stability at quadruplex sites [33••].

DNA damage can lead to chromosomal rearrangements at mitosis following creation of strand breaks and it is

Figure 3



Summary of the main concept discussed.

evident that G-quadruplexes can induce such strand breaks, although the mechanistic details have not yet been elucidated. In Pif1-deficient yeast gross chromosomal rearrangements (GCR) are stimulated by the introduction of sequence motifs shown to form G-quadruplex structure [33••] or G-quadruplex-containing minisatellites as CEB1 [32,34•]. Furthermore, the treatment of WT (Pif1-positive) cells with the quadruplex ligand PhenDC3 leads to a similar induction of chromosomal rearrangements [34•]. The structural details of the GCR depended on the orientation of the G-quadruplex motifs, and the nature of the rearrangements are more complex than broken chromosomes healing by *de novo* telomere addition, but include a combination of deletions, mutations and insertions [33••,34•]. Further support for a G-quadruplex-dependent recombination mechanism comes from Cahoon *et al.* who identified a cis-acting quadruplex motif near the variable pilin genes of the human pathogen *Neisseria gonorrhoeae* that controls recombination of the antigenically locus and avoid immune detection [35]. Disruption of the element either by mutagenesis or by targeting with a G-quadruplex ligand prevented recombination and pilin antigenic variation. The *Neisseria g.* RecQ helicase is required to process nicks produced by the quadruplex forming sequence.

An example of another apparently G-quadruplex related genetic disease has emerged from studies on *ATRX*, an X-linked gene of the SWI/SNF family, in which mutations lead to a rare form of syndromal mental retardation α-thalassaemia, caused by a down-regulation of α-globin expression [36]. Gibbons *et al.* showed that ATRX binds to G-rich tandem repeat sequences in both telomeres and euchromatin. Chip-Seq experiments on human and mouse confirmed the preference for ATRX to bind to G-quadruplex encoding DNA [37•] since 50% of ATRX binding sites overlapped with putative quadruplex sequences. Consistent with the binding of quadruplex structures, recombinant ATRX was shown to bind G-quadruplex DNA *in vitro* with high affinity. The genes associated with the ATRX-binding repeats show deregulated expression when *ATRX* is mutated. Specific attention was given to the variable tandem repeat within the cluster of alpha-like globin genes, and the authors demonstrated that a larger repeat led to a greater degree of down-regulation. Due to its important role in incorporating the histone variant H3.3 into telomeric, ribosomal and pericentromeric DNA, the authors propose that ATRX might act by modifying the epigenetic state of the guanine-rich repeats containing genes.

A recent hypothesis suggests that G-quadruplex DNA is involved in the regulation and the maintenance of epigenetic regulation of gene expression. Studies on the Y family translesion polymerase REV1 in DT40 chicken cells by Sale *et al.* showed that the presence of G-quadruplex DNA influences the preservation of histone marks in daughter chromosomes when the replication machinery is compromised [38]. The authors propose a model in which the failure to maintain processive DNA replication at G-quadruplex DNA in REV1-defficient cells leads to an uncoupling of DNA synthesis from histone recycling, resulting in localized loss of chromatin marks. Insertion of a G-quadruplex sequence in a silent locus, ρ-Globin, leads to expression derepression in REV1-defficient cells. A similar process caused deactivation of transcriptionally active genes and a microarray analysis of REV-1 deficient DT40 cells showed genome-wide reprogramming of gene transcription [39•]. The epigenetic link between G-quadruplexes and the replication machinery appears to be controlled by coordinating FANCJ with REV1 and the WRN/BLM helicases, each of which has been associated with resolving G-quadruplex DNA.

The genome-wide analysis of DNA breakpoints associated with somatic copy-number alterations (SCNAs) identified G-quadruplex DNA as a hallmark of fragile sites [40]. Abnormal hypomethylation in the vicinity of putative quadruplex sequences is also a common feature of many breakpoint hotspots. The authors propose that abnormal hypomethylation in genomic regions enriched in G-quadruplex DNA drives tissue-specific mutational landscapes in cancer.

The involvement of G-quadruplex DNA in genomic instability and site-specific DNA damage, has led to a suggestion that a combination of G-quadruplex ligands either with inhibitors of DNA repair or associated pathways could be an efficacious strategy for consideration in the future treatment of tumors. For example the G-quadruplex ligand RHPS4 potentiates the antitumor activity of Camptothecins in models of solid tumor [41]. The treatment of mice with irinotecan followed by RHSP4 shows a synergistic effect in reducing the growth of xenographs. Likewise, the WRN helicase inhibitor, NSC 19630, sensitizes cancer cells to the G-quadruplex ligand telomestatin [42]. NSC 19630 induces apoptosis in a WRN-dependent manner and induces a greater number of γH2AX foci, which is a phenotype associated with G-quadruplex DNA and G-quadruplex ligands [24••]. Similarly, the G-quadruplex ligand PDS acts synergistically with NU7441, an inhibitor of the DNA-PK kinase crucial for non-homologous end joining repair of DNA double strand breaks [24••,43]. These experiments, together with the explicit evidence that ligands such as PDS trap G-quadruplexes in the nucleus [20••], implicate G-quadruplex DNA as a relevant molecular target to potentially exploit genomic instability as a vulnerability in cancers.

Conclusions

Taken together the results of recent studies on visualization of G-quadruplex DNA in cells, experimental mapping of G-quadruplex structures in genomic DNA and biological studies on proteins that maintain genome integrity at G-quadruplex sites have provided compelling new data on this DNA secondary structure (Figure 3). Important future challenges include the need to elucidate the mechanism(s) by which G-quadruplex DNA can modulate transcription, replication and also genome integrity. Furthermore, several cellular functions have been recently linked with G-quadruplex DNA such as the selection of replication origins [44] and the modulation of transcriptional termination via the formation of DNA:RNA hybrid quadruplex in R-loops [45], which warrant detailed investigation. Such investigations are now enabled by the recent advancements in tools and methods to probe such questions in a biologically relevant context.

Acknowledgements

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- •• of outstanding interest

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