DNA Repair 130 (2023) 103552

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

G-quadruplex resolution: From molecular mechanisms to physiological relevance

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ARTICLE INFO

Keywords:
G-quadruplex
G4
G4 ligand
G4 resolution
DNA replication
Genome integrity
R-loop
Epigenetics
Transcription regulation
Telomeres

ABSTRACT

Guanine-rich DNA sequences can fold into stable four-stranded structures called G-quadruplexes or G4s. Research in the past decade demonstrated that G4 structures are widespread in the genome and prevalent in regulatory regions of actively transcribed genes. The formation of G4s has been tightly linked to important biological processes including regulation of gene expression and genome maintenance. However, they can also pose a serious threat to genome integrity especially by impeding DNA replication, and G4-associated somatic mutations have been found accumulated in the cancer genomes. Specialised DNA helicases and single stranded DNA binding proteins that can resolve G4 structures play a crucial role in preventing genome instability. The large variety of G4 unfolding proteins suggest the presence of multiple G4 resolution mechanisms in cells. Recently, there has been considerable progress in our detailed understanding of how G4s are resolved, especially during DNA replication. In this review, we first discuss the current knowledge of the genomic G4 landscapes and the impact of G4 structures on DNA replication and genome integrity. We then describe the recent progress on the mechanisms that resolve G4 structures and their physiological relevance. Finally, we discuss therapeutic opportunities to target G4 structures.

1. Introduction

Genomic DNA predominantly forms a right-handed double helical structure, referred to as the B-form conformation [1,2]. However, DNA can also adopt several types of stable non-canonical secondary structures. One such structure, a G-quadruplex or G4, specifically forms in guanine-rich sequences in the genome [3,4] (Fig. 1A). This four-stranded structure arises by the stacking of two or more guanine tetrads (G-quartets), in which four guanine residues interact through non-canonical Hoogsteen hydrogen bonds [5] (Fig. 1B). Stacking of G-quartets is facilitated by the presence of monovalent cations and π-π interactions [6] (Fig. 1A, B), thereby forming a thermodynamically stable structure. G4 structures can form in a unimolecular or intermolecular fashion in vitro, and can adopt diverse topologies arising from different strand polarities and the composition of interconnecting loops [7] (Fig. 1C). In some cases, one G4 sequence can fold into several G4 structures with different topologies [6,8–11] (Fig. 1D). Since the Watson-Crick base pairing in duplex DNA is energetically more favourable than the Hoogsteen base pairing, G4 structures preferentially form in single stranded DNA (ssDNA) or upon transient melting of double stranded DNA (dsDNA) [12]. G4s can also form in RNA, for more information we refer the reader to excellent reviews covering RNA G4s [13–15].

While the presence of DNA G4 structures has been debated, converging lines of evidence indicate that these structures do form in cells, and also have cellular functions. G4 structures have been detected in various organisms including humans by immunostaining with several G4-specific antibodies [16–21] and by live-cell imaging with fluorescent G4 ligands [22–24]. Moreover, numerous proteins have been identified to specifically recognise G4 structures with up to picomolar affinity [25,26]. G4 sequences and structures are found enriched in regulatory loci such as active promoters, enhancers and telomeres [27,28], and they have been implicated in several key biological processes, such as transcription [29–31], telomere homeostasis [32–34], and DNA replication [35–38] and repair [39,40]. Emerging evidence suggests that G4s can also act as an epigenetic structure that contributes to cell-type specific transcriptome [30,41] and higher order chromatin structures [42].

Paradoxically, accumulating evidence also indicates that G4

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https://doi.org/10.1016/j.dnarep.2023.103552
Received 24 May 2023; Received in revised form 29 July 2023; Accepted 1 August 2023
Available online 3 August 2023

structures can promote DNA breaks. In some cases, these are controlled by G4 binding proteins and connected to a physiological role for these structures, such as in ensuring antigenic variation in pathological bacteria [43,44], or in class switch recombination (CSR) in B cells [45–48]. For example, during CSR, regulated double-strand breaks (DSBs) are initiated by activation-induced deaminase (AID) [49] that directly binds to G4s [45]. In other cases, G4-induced breaks are unscheduled and linked to genome instability and aging [50,51]. Genetic and biochemical studies uncovered numerous helicases and DNA binding proteins that can unwind G4 structures, several of which, when defected, lead to human disorders with high predisposition to cancer and neurodegeneration [52–54], suggesting a potential link between G4 stabilisation and these diseases. Notably, in tumors, G4s are associated with chromosomal break points [55–60] and altered transcriptional programs that support tumor development [30,61]. When not properly resolved G4 structures stall the DNA replication machinery [62,63], which can cause DNA double-strand breaks (DSBs) [64–66]. While timely G4 resolution is therefore important to maintain genome integrity and prevent tumorigenesis, our understanding of the mechanisms that resolve these structures is still in infancy.

In this review, we provide an update on the mechanisms that regulate G4 structures with an emphasis on G4 resolution and its physiological relevance. We first highlight recent progress in the determination of G4 landscapes in genomes. Then we discuss the impact of G4s on genome integrity, and examine recent mechanistic insights into G4 resolution. Finally, we discuss the role of G4 resolution in disease and therapeutic opportunities to target G4 structures.

2. G-quadruplex distribution in the genome

2.1. G4 mapping

Early biophysical studies uncovered that G4 stability is influenced by the number of stacked G-quartets [67] and the length and composition of loops [68–71]. Based on these findings, an initial G4 consensus sequence ($G_{3-5}N_{1-7}G_{3-5}N_{1-7}G_{3-5}N_{1-7}G_{3-5}$) was proposed [71,72]. Computational algorithms using this consensus predicted that the human genome contains over 370,000 sequences with the potential to form a G4 structure [71]. More recently, computational tools that accommodated additional parameters for polymorphic G4 structures [73,74] or used different strategies such as sliding window-based scoring systems [75] and machine learning [76–78] predicted that the number of potential G4 structures is substantially higher.

To obtain a genome-wide map of potential G4-forming sequences (PQSs), an experimental approach was established that combines DNA polymerase stalling at G4 structures with high-throughput sequencing [79] (G4-seq, Fig. 2A). G4-seq detected more than 500,000 polymerase stalling sites that localised to 5' ends of G4 sequences in the genome [79], establishing a robust genome-wide PQS map. Notably, this number could be an underestimation, since recent human genome sequencing data revealed that substantial G-rich repeats had been excluded from the previous reference genome [80].

The utilisation of G4 structure-specific antibodies for chromatin immunoprecipitation followed by high-throughput sequencing (G4 ChIP-seq, Fig. 2B) was a first approach to directly map G4 structures in cells [27]. In human HaCaT keratinocytes, G4 ChIP-seq detected ~10,000 G4 structures [27]. However, careful consideration is required when interpreting the number of detected G4s, as it may be affected by experimental conditions such as formaldehyde fixation and sonication prior to G4 capture that could affect G4 abundance. In addition, G4

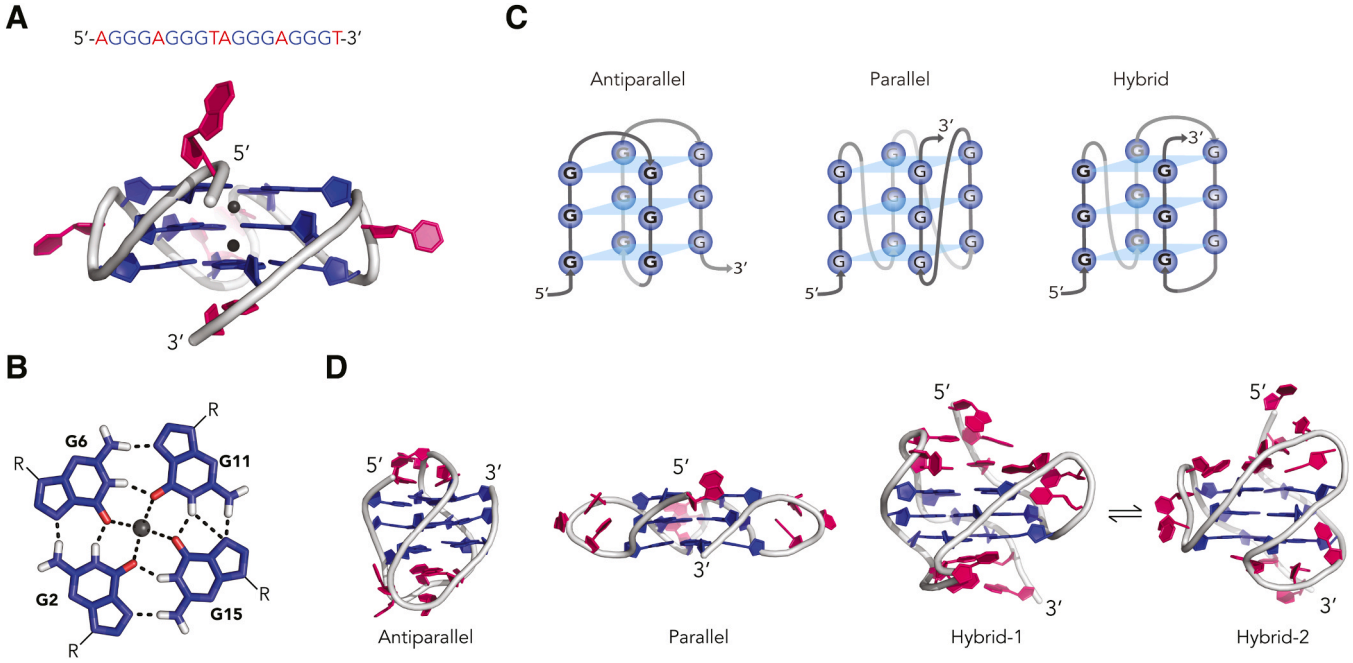


Fig. 1. G-quadruplex structures. A. The crystal structure of an intramolecular, parallel G4 from the human *c-MYC* promoter sequence (PDB ID: 6AU4 [281]; the sequence is indicated on top). Guanines are indicated in blue, bases in interconnecting loops in red. Spheres, monovalent cations. B. The structure of a G-quartet formed in the *c-MYC* G4 structure (A). Four coplanar guanines (G2, G6, G11, and G15) are stabilised by Hoogsteen hydrogen bonds and a central cation. Cations stabilise the structure with a strength in the following order: $K^+ > Na^+ > NH_4^+ > Li^+$. The H and O atoms are coloured white and red, respectively. Dashed lines, hydrogen bonds. C. Schematic of an antiparallel (left), parallel (middle), and a hybrid (right) G4 structure. Hoogsteen hydrogen bonds are shown in blue. Strand polarities are indicated with arrowheads. D. G4 structures with different topologies from a human telomere sequence. The telomere sequence is folded into an antiparallel G4 with the cation Na^+ (PDBID: 143D [8]). It can be also folded into parallel (PDBID: 1KF1 [6]) and hybrid G4 conformers with K^+ as a cation. In solution containing K^+, the telomere sequence is predominantly folded into two different hybrid conformations [9–11] with a dynamic equilibrium between hybrid-1 (PDBID: 2HY9 [282]) and hybrid-2 (PDBID: 2JPZ [283]). Bases are coloured as in (A).

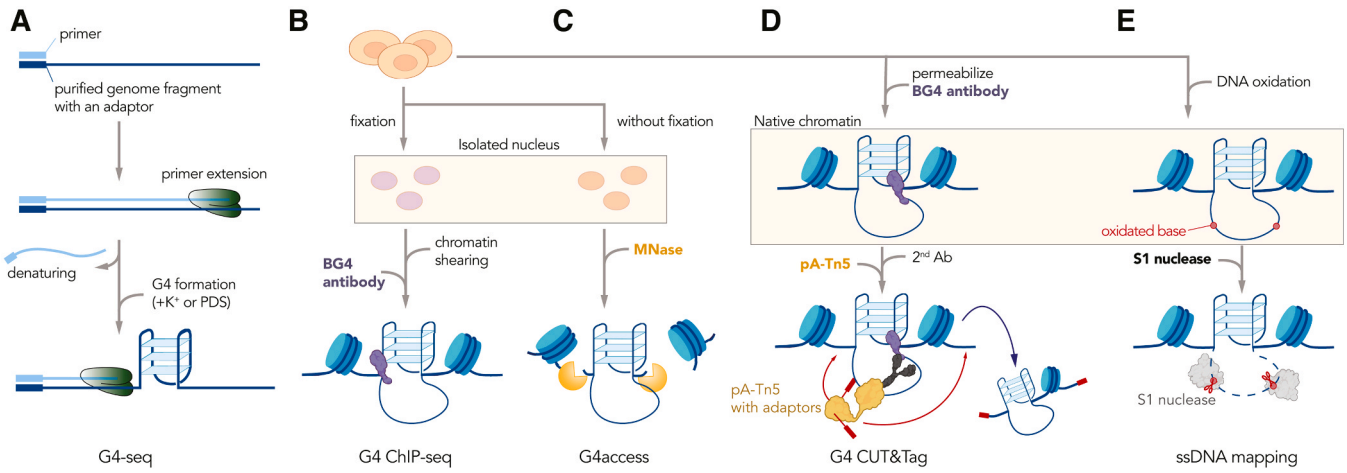


Fig. 2. Approaches for G-quadruplex mapping. A. Schematic of G4-seq [79]. Genomic DNA is isolated, digested, and sequencing adapters are ligated to the fragments (dark blue box). The DNA fragments are first sequenced under conditions that prevent G4 formation, and a second time in a G4-stabilizing condition (either in the presence of cations, or the G4 stabiliser pyridostatin). The two sequencing reads are subsequently compared to determine the stalling positions of DNA polymerase (green). B. Schematic of G4 ChIP-seq [27]. Chromatin is isolated from fixed cells and sheared by sonication. G4-containing chromatin fragments are subsequently precipitated with a G4-specific antibody BG4 (purple) and subjected to sequencing. This method was also performed with other G4-specific antibodies such as 1H6 and D1 (see also Table 1). C. Schematic of G4access [87]. Chromatin is isolated from non-fixed cells and treated with micrococcal nuclease (MNase). Subnucleosomal DNA fragments (<146 bp) are collected and subjected to sequencing. Based on the assumption that G4 structures are resistant against MNase, these fragments should be enriched for G4 structure forming sequences. Positions of G4 structures are subsequently determined through computational analyses of nuclease footprints using a PQS prediction tool. D. Schematic of G4 CUT&Tag [27]. Cells are gently permeabilised and treated with the FLAG-tagged BG4 antibody to capture G4s in chromatin. The anti-FLAG antibody (2nd Ab) is subsequently added to tether the pA-Tn5 transposase, which digests chromatin surrounding the BG4 antibody and simultaneously integrates adapters. The tagmented chromatin is extracted and sequenced. This approach does not involve cell fixation and detergent treatment that could induce epitope masking or affect G4 formation. E. Schematic of chemical G4 mapping [86]. Cells are treated with potassium permanganate ($KMnO_4$) that selectively oxidises single-stranded DNA. Chromatin is then isolated and digested by S1 nuclease. Positions of G4 structures are subsequently determined through sequencing and subsequent computational analyses of nuclease footprints using a PQS prediction tool.

ChIP-seq requires a large number of cells, which complicates G4 mapping particularly in noncancerous primary cells or at single-cell resolution.

Alternative approaches thus have been developed to capture G4 structures under more physiological conditions. Currently, the most sensitive method for in situ G4 mapping uses the cleavage under targets and tagmentation strategy [81] (G4 CUT&Tag, Fig. 2D). In this method, G4 structures in permeabilised but not fixed cells are bound by a G4-specific antibody, which then tethers a protein A-Tn5 transposase fusion protein (pA-Tn5) to the G4 sites [28,82]. Cleavage and direct ligation of adapters by Tn5 at antibody-bound chromatin loci then allow for robust and efficient high-throughput sequencing. G4 CUT&Tag approximately doubled the number of detected G4s in human cells with improved resolution compared to G4 ChIP-seq [82]. This method has recently been extended to single-nuclei (sn) G4 CUT&Tag to map G4 structures at single-cell resolution [83]. In human cancer cells, snG4 CUT&Tag successfully detected ~700 G4 peaks per cell on average and, when aggregated, this recapitulated G4 profiles obtained from bulk mapping [83]. Consistent with this, a similar number of G4 structures was detected by single-molecule imaging in single human cancer cells [23]. These G4 mapping data indicate that there is a large cell-to-cell variability in G4 position, and also that only a small fraction of all PQSs folds into a G4 structure. The latter suggests that formation of these structures is efficiently suppressed in cells but how G4 formation and resolution at specific positions is regulated largely remains to be elucidated.

It should be noted that G4-specific antibodies that have been used for G4 mapping exhibit a binding preference for specific G4 conformations (Table 1). Therefore, careful assessment is necessary when interpreting G4 abundance based on the antibodies used for mapping. The commonly used G4-specific antibody, BG4, has a higher affinity toward parallel G4s [84], which may result in underestimation of anti-parallel and hybrid G4 abundance. Similarly, the D1 antibody specifically binds to parallel G4s [18]. Furthermore, the 1H6 antibody shows some off-target specificity toward poly-thymidine ssDNA and exhibits a strong preference for G4s with poly-thymidine loops [85]. Thus, further development of G4 detection antibodies would be beneficial to better determine G4

Table 1
Representative G-quadruplex-specific antibodies used for G4 mapping.

Antibody	Substrate	Application	Features and Limitations
BG4 (scFv) [20]	DNA and RNA G4s [20]	IF [20], SMLM [63], G4 ChIP-seq [27], G4 CUT&Tag [28,82,83]	BG4 can detect a single DNA G4 in IF assays [63]. The antibody binds to parallell, anti-parallel, and hybrid G4s [20] but with lower affinities toward the latter two conformations [84]. It stablises a telomeric G4 [296].
1H6 (mouse monoclonal IgG) [17]	DNA G4s [17]	IF [17], G4 ChIP-seq [297]	1H6 exhibits off-target binding to PolyT DNA and does not bind to G4s without thymidines present in the loops. It is not clear if this antibody recognizes a single DNA G4 in IF.
D1 (scFv) [18]	DNA G4s [18]	IF [18], G4 ChIP-seq [18]	D1 specifically binds to parallel G4s [18]. It is not clear if this antibody recognizes a single DNA G4 in IF.
SG4 (nanobody) [21]	DNA G4s [21]	G4 ChIP-seq [21], Live-cell imaging [21]	SG4 binds to parallel, anti-parallel, and hybrid G4s [21]. Extensive biochemical characterisation of SG4 remains to be performed.

scFV, single-chain variable fragment; IF, immunofluorescence; SMLM, single-molecule localisation microscopy.

structure locations in cells.

Two recently developed G4 mapping methods circumvent both the use of G4 antibodies and formaldehyde fixation during the procedure. One of these methods exploits S1 nuclease to map ssDNA regions formed at PQSs [86] (Fig. 2E), while the other approach utilises micrococcal nuclease to map secondary structures at PQSs [87] (G4access, Fig. 2C). Both approaches detect the comparable number of G4s to G4 ChIP-seq methods in mammalian cells. However, nuclease treatment in these approaches is performed under non-physiological conditions which may have some influence on the results.

2.2. G4 distribution

Immunofluorescent staining with G4 structure-specific antibodies [16–21] or G4 probing with small molecules [22–24] displays signals throughout nuclei in human cells, raising a central question where G4 structures form in the genome. G4-seq demonstrated that PQSs are enriched in gene regulatory regions including promoters and 5' untranslated regions (UTRs) [79]. Although the G4 enrichment in 5' UTRs is limited to mammals and PQSs are rather depleted in bacterial coding regions [88–90], their overrepresentation in promoters has been observed in many organisms from humans to bacteria [88,91–96], suggesting their conserved functions in regulation of transcription. Consistent with this, G4 ChIP-seq detected G4 structures predominantly in promoters in human cells [27] (Fig. 3, i). Strikingly, over 98% of the detected G4 peaks resided at nucleosome depleted regions and they often colocalised with transcriptionally active markers such as trimethylation of histone H3 lysine 4 (H3K4me3) [27] (Fig. 3, i). Similar localisation was also observed by G4 CUT&Tag [28,82] and by antibody-independent S1 mapping [86] and G4access [87], strongly indicating G4 enrichment in open chromatin. Furthermore, G4 CUT&Tag unveiled G4 enrichment also in active enhancers in both mouse and human cells [28,82] (Fig. 3, ii). This characteristic distribution was observed in both non-cancerous and cancer cells. Consistent with their prevalence in open chromatin, G4 forming sites are mostly hypomethylated [97] (Fig. 3, i). Moreover, G4access revealed that, at imprinted genomic loci in mESCs, G4s are selectively formed in unmethylated active alleles [87]. The strong co-localisation of G4 structures with regulatory elements of transcriptionally active genes implicates G4s as epigenetic features for active transcription sites in mammalian cells.

G4 landscapes in different cell lines share only partial overlap, indicating cell-type specific G4 regulation [27,30,83,98]. G4 distribution does not seem to be regulated by cell-type specific transcription as inhibition of RNA Pol II activity does not affect G4 formation in chromatin [28,31]. Rather, accumulating evidence suggests that G4 structures actively enhance transcription to maintain cell identity. Interestingly, the positions of G4s seem to colocalise with topological associated domain (TAD) boundaries genome-wide, and G4s show insulation ability for promoter-enhancer (P-E) interaction [42] (Fig. 3, ii). Consistently, cohesin involved in DNA looping, preferentially localises surrounding G4s [99]. Furthermore, disruption of specific G4 motif leads to a reduction of P-E looping in the locus [99]. While these data suggest a potentially interesting role for G4 structures in loop extrusion in the regulatory elements, further investigation is required to confirm this model. In addition, over 99% of G4 structures overlap with transcription factor binding sites in human cells [42]. Consistently, several transcription factors (TFs) such as SP2, NRF1, FUS, and MYC exhibit G4 binding activity in vitro independently of the presence of their consensus motif, and colocalise with G4s in cells [29,30,42]. Based on this data, it has been proposed that G4s can act as structural 'hubs' that recruit TFs, thereby shaping cell-type specific transcriptomes. However, it is noteworthy that although over 90% G4s are detected in open chromatin, only ~10% of the open chromatin regions contain G4s, suggesting G4 regulatory role is limited to specific loci [30]. It is also currently unclear whether TFs are directly guided to G4s. Importantly, G4s also colocalise with histone marks for active transcription and R-loops, non-B DNA structures in which one strand consists of a DNA:RNA hybrid (reviewed elsewhere [100–102]) (Fig. 3, i), both implicated in TF recruitment [103,104]. Additional investigation is needed to

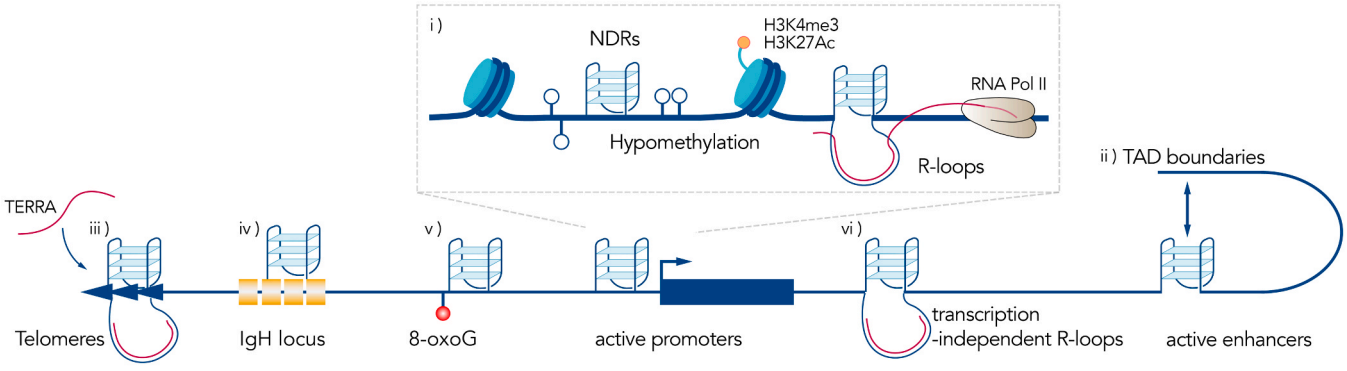


Fig. 3. G-quadruplex distribution. G4s are prevalent in regulatory regions of actively transcribed genes (i). G4 CUT&Tag detected about 60% of total G4 peaks in active promoters in both mESCs and human cancer cells [28,82]. These peaks often colocalise with RNA Pol II, R-loops, and open chromatin marks such as H3K4me3, H3K27Ac, nucleosome depleted regions (NDRs), and hypomethylated regions [28,82]. In both mouse and human ESCs, G4s are prevalent in bivalent promoters (not shown) [28,41]. G4 peaks were also detected in active enhancers in mouse and human ESCs (ii) [28,41]. Consistently, G4s are enriched in topological associated domain (TAD) boundaries especially for promoter-enhancer interaction [42]. Although YY1 plays an important role in DNA looping and colocalises with G4 structures [99], whether and how these structures play a direct role in DNA looping is currently unknown. About 25% of total G4 immunostaining signals overlap with telomeres in human cancer cells (iii) [20]. During telomeric DNA synthesis in S phase, G4s efficiently colocalise with telomerase [33] and enhance its processivity [32]. In telomeres, G4s are important for TERRA recruitment, which leads to *in trans* R-loop formation in the proximity of G4s [109,268]. While the TERRA R-loops are important for recruitment of telomere maintenance factors, some cancers that do not upregulate telomerase exploit the structures to induce break-induced replication-mediated telomere lengthening [109,152,153]. This colocalisation with transcription-independent R-loops is not likely limited to telomeres as G4 CUT&Tag detected R-loop peaks overlapping with G4 peaks genome-wide in the absence of ongoing transcription in mESCs (vi) [28]. Upon oxidative stress, G4 formation is robustly induced through the binding of APE1 to Apurinic/apyrimidinic sites [39] which is generated as an intermediate of base excision repair (v). APE1-induced G4s are associated with transcription activation in response to oxidative stress [284,285]. G4s appear to play an important role in class switch recombination in the IgH locus through direct interaction with a central enzyme activation-induced cytidine deaminase in CSR (iv) [45]. In pathological bacteria, a similar G4-dependent mechanism that creates antigenic variation has been identified [43,44]. This process requires R-loop formation on the non-G4 strand across from the structure, although the nuclease responsible for DNA incision during this process has yet to be identified (not shown). Importantly, detailed functions of G4s in both CSR and antigenic variation remain to be elucidated.

further characterise the mechanism of G4-mediated transcriptional regulation.

It remains a key question how G4 landscapes are established and whether and how they are inherited in daughter cells after mitosis. Since G4s localise to open chromatin, G4 formation might be controlled by mitotically inherited chromatin features. Consistently, G4s are observed in G1 phase in proliferating cells [20,22] when euchromatin is established [105]. In line with this possibility, an HDAC inhibitor [27,30,87] or loss of DNA methylase DMNT1 [97,106], both promoting open chromatin, enhances G4 formation, suggesting that euchromatin establishment and G4 formation is coupled. In contrast, at least during differentiation, G4 formation can precede transcriptionally active open chromatin establishment [41]. An important future challenge is to establish causal relationships between G4 formation and epigenetic modifications to better understand the mechanisms of G4 landscape establishment.

3. Impact of G-quadruplexes on genome integrity

G4 structures have been described to contribute to genome stability. Initially, in *S. lemnae* [107] and *S. cerevisiae* [108], it was shown that G4 structures function as telomeric end-capping structures to prevent their nucleolytic attrition. Subsequently in mammalian cells, they have been shown to enhance telomerase activity [32,33] and also to facilitate telomeric repeat–containing RNAs (TERRAs) association with telomeres [109], which recruits a number of telomere-binding factors [110–112] (Fig. 3, iii). Both mechanisms are crucial for mammalian telomere maintenance. Furthermore, in response to oxidative DNA damage and activation of base excision repair, G4 structure formation enhances the transcription of specific proteins associated with DNA repair and cell survival [113–115] (Fig. 3, v). Moreover, recent work shows that the yeast protein Zuo1 binds to and stabilises G4s, and promotes recruitment of repair factors to UV damage sites to maintain genome integrity [40]. Consistent with an early role in NER, G4 formation is stimulated in response to both oxidative and UV-mediated DNA damage [40,116]. These observations suggest a potential role for G4 structures as an early 'alarm signal' that flags DNA damage to facilitate DNA repair.

However, when not properly regulated, G4s are associated with genome instability. Early studies identified genome-wide deletions that specifically start around the 3′ end of PQSs in a mutant *C.elegans* strain defective in the G4 unwinding helicase DOG-1/FANCJ [64,117]. Subsequent studies revealed that exposure of human cells to G4 stabilizing ligands enhances G4 levels and induces DSBs, mitotic defects and chromosomal aberrations [66,118–122]. G4 structures, especially when persistent or stabilised, inhibit progression of both RNA and DNA polymerases [123–126]. Mounting evidence suggests that DNA replication stress induced by persistent G4 structures plays a prominent role in promoting genome instability, although G4-related transcriptional changes may also be indirectly involved [82,127–129]. DNA breakage induced by G4 ligands depends to a large extent on DNA replication in human cells [66,121,130], and is also frequently associated with telomere dysfunction [131,132] and consequential senescence [133]. Even though the eukaryotic replication machinery (replisome) includes a subcomplex called the Fork Protection Complex (FPC), consisting of TIMELESS-TIPIN, CLASPIN, and AND-1, that can detect and facilitate resolution of secondary DNA structures [134–136], G4 structures can still block DNA replication. This was first demonstrated in *S. cerevisiae* where replication slowdown was observed close to endogenous G4 sequences [137] or an inserted array of G4 sequences [138]. More recently, biochemical studies provided further proof that both the yeast (*S. cerevisiae)* [139–141] and *X. laevis* [62] replisomes stall at G4 structures. Moreover, recent single-molecule localisation microscopy (SMLM) imaging elegantly demonstrated replisome stalling at G4 structures in human cells [63]. Consistent with these observations, cells arrest in G2 phase [119] upon G4 stabilisation, indicative of incomplete DNA replication and accumulation of DNA damage. Furthermore, model organisms such as *C. elegans* [64,117,142] and *S. cerevisiae* [138,143,144], when compromised in their ability to unfold G4s, accumulate G4-specific deletion signatures that are consistent with DNA replication blockage causing these deletions. Therefore, G4-associated genome instability appears to largely result from the replication machinery colliding with the G4 structure. Interestingly, as a result of replication stalling persistent G4s also cause inheritable loss of epigenetic information adjacent to the structures, triggering irreversible transcriptional alteration in daughter cells [145–147]. Given the number of G4 structures detected in human single cells compared to other types of DNA lesions (Table 2), G4 structures pose a remarkable threat to genome integrity during genome duplication.

Genome instability induced by G4 structures can also in part be caused by their association with R-loops that can also stall replisomes and can cause replication-transcription conflicts and genome instability [148–150]. Over 70% of the G4 structures detected by CUT&Tag colocalise with R-loops in human and mouse cells [28,151] (Fig. 3, i and vi). Interestingly, G4 stabilisation enhances R-loop formation in proximity of G4 sites [118], and vice versa, R-loop induction also enhances G4 structure formation [109,152]. At least in telomeres, these colocalised G4s and R-loops likely form a strong roadblock for the replisome that can be a threat for genome integrity [152,153] (Fig. 3, iii).

The mechanisms of DSB formation upon replication fork stalling at G4s is unclear, but the endonucleases DNA2 [154], MUS81 [130,155] and MRE11 [156,157] have been implicated in this process. The repair of these breaks likely involves homologous recombination (HR) based on the observation that G4 stabilizing ligands cause synthetic lethality in HR-deficient cancer cells [121,158,159] (Fig. 4). Consistently, defects in proteins involved in HR, such as BRCA1, BRCA2, EXO1 and BLM lead to genome instability at PQSs [121,158–161]. In difficult-to-replicate loci such as common fragile sites, persistent G4 structures can prevent replication fork convergence [162,163]. This can induce fork collapse upon activation of CDK1-Cyclin B in G2 phase [164], leading to one-ended DSBs at G4 structures. These lesions can be repaired through a non-canonical HR-mediated process known as mitotic DNA synthesis (MiDAS) that requires the RAD52 recombinase [165] (Fig. 4). Consistently, cell-based reporter experiments showed that RAD52 promotes DSB repair in G4-forming regions [155]. While proteins involved in G4-related DSB repair are beginning to emerge, little is known about details and the fidelity of repair mechanisms of G4-containing DNA ends.

Together, the resolution of G4 structures before or during DNA replication prevents DSB induction and promotes genome integrity. The next section will focus on the recent advances in our understanding of these G4 resolution mechanisms.

4. Mechanisms of G-quadruplex resolution

4.1. G4 unfolding proteins

Continuous DNA replication past G4s requires unwinding of these structures which needs the action of additional proteins beyond the core replisome. Eukaryotes have evolved over a dozen helicases that can resolve G4 structures and in some cases can act redundantly (G4 helicases). The function and biochemical activity of all currently known G4 helicases have been extensively discussed in several excellent reviews [53,166–169]. In *S. cerevisiae*, PIF1 seems to be the predominant G4 helicase that facilitates replication past G4 structures [170,171]. Although PIF1 is evolutionarily conserved, its function appears to be mostly limited to the mitochondria in human cells [172]. In vertebrates, FANCJ plays an important role in G4 resolution and prevents deletions in the vicinity of G4s [65]. In addition, FANCJ is defective in hereditary breast cancers and the cancer-predisposition syndrome Fanconi anaemia (FA), and linked to the repair of DNA interstrand crosslinks by the FA pathway [173–176]. Consistent with redundancy in G4 helicase function, recent studies have shown that the DHX36 helicase can unfold

Table 2
Comparison of estimated frequencies of endogenous DNA lesions and G-quadruplexes.

	Endogenous DNA lesions										
DNA lesion	DSB	ICL	Cytosine deamination	Cyclopurine adducts	Depyrimidination	8-oxoG	Malondialdehyde adducts	Alkylation adducts	G-quadruplex	Depurination	SSB
Frequency per cell per day	10^1	10^1	10^2	10^2	10^2	10^3	10^3	10^3	10^{3*}	10^4	10^4

Adapted from Yousefzadeh et al., 2021 [298] and Lindahl et al., 2000 [299]. DSB, double-strand break; ICL, DNA interstrand crosslink; 8-oxoG, 8-oxoguanine; SSB, single-strand break. *per cell per cell cycle [83].

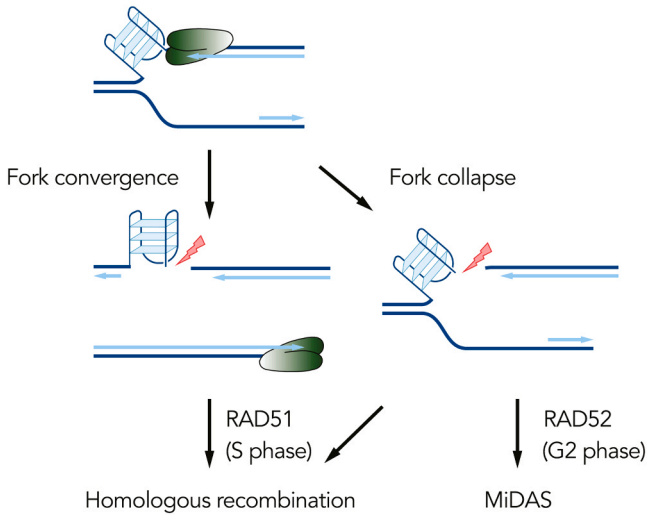


Fig. 4. Consequence of persistent G-quadruplexes. Schematic of DNA double-strand break (DSB) formation and its repair upon persistent DNA replication stalling at a G4 on the leading strand template. Fork breakage at G4s can induce two different DSBs. When the replication forks converge before DNA breakage or DNA polymerase stalls at a G4 on the lagging strand template (see also Fig. 5B), a two-ended DSB is induced. DNA breakage can also take place before fork convergence, resulting in a one-ended DSB. Both DSBs appear to be repaired through homologous recombination-directed mechanisms mediated by RAD51. When the replication fork persistently stalls until G2 entry without fork convergence, the fork is collapsed and a one-ended DSB is induced [164]. This can occur especially at common fragile sites where the interorigin distance is considerably larger [286]. This break can be repaired through a distinct mechanism referred to as mitotic DNA synthesis (MiDAS) mediated by RAD52 [163,165].

parallel G4s during DNA replication in the absence of FANCJ [62,177]. Genetic studies have identified other G4 helicases required for faithful telomere replication in mammals. The G4 helicases WRN and BLM redundantly prevent replication stress and genome instability in telomeres [178–180]. Both helicases also prevent dysregulated expression of genes harbouring PQSs in their promoter, suggesting genome-wide roles for BLM and WRN in G4 suppression [181–184]. Telomere replication is further facilitated by another G4 helicase RTEL1 [185,186].

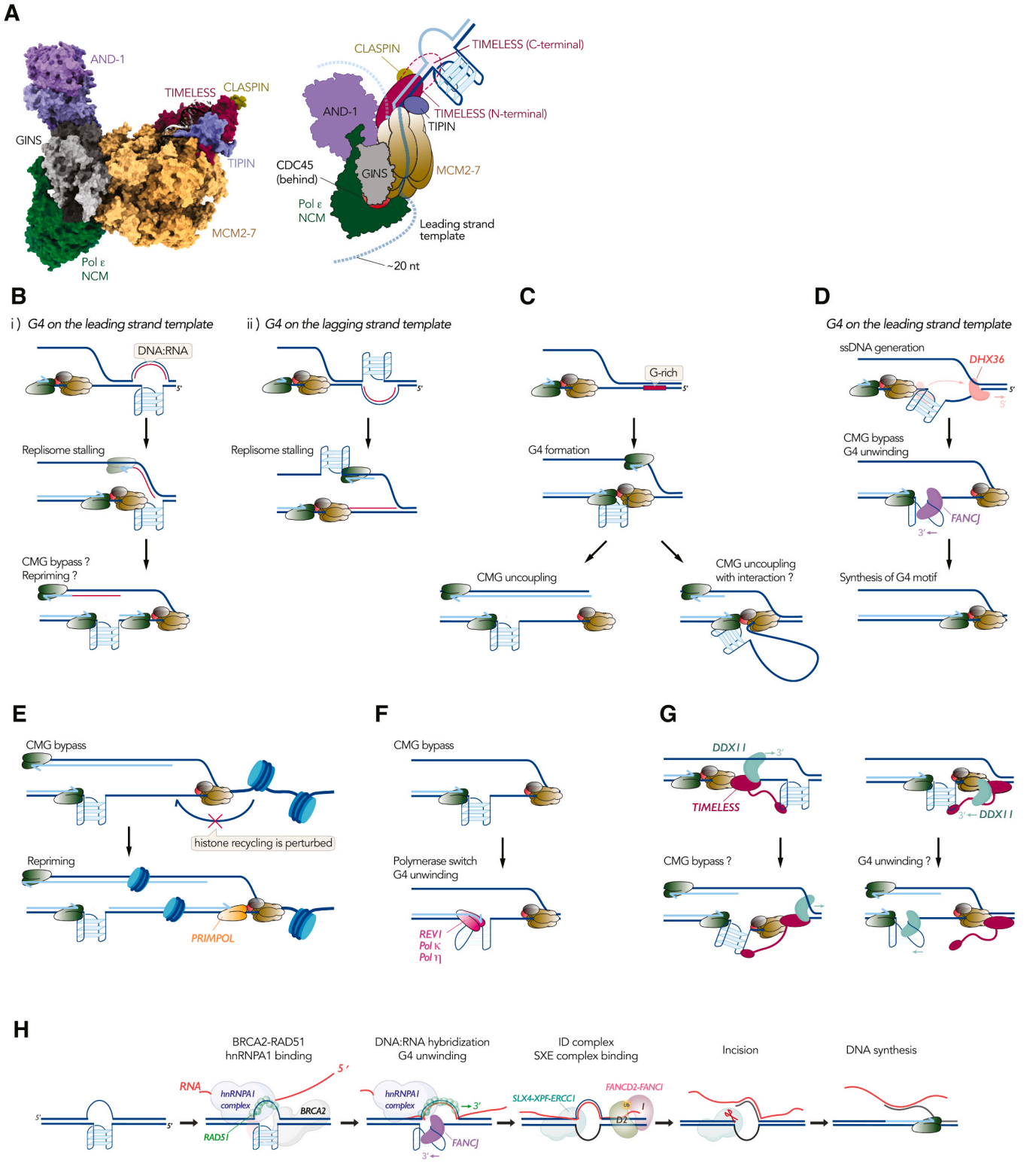
Most G4 helicases identified to date belong either to the SF1 or SF2 superfamily which require ssDNA adjacent to the G4 for unwinding [53,166–168], providing some clues about the mechanism of their regulation. Importantly, although the helicases share similar catalytic domains, they unfold G4s with a unique directionality [53,166–168], and likewise, exhibit certain selectivity for specific G4 topologies and inter- or intramolecular G4 structures [187,188]. In addition, their catalytic activity can be modulated by ssDNA binding proteins such as Replication Protein A (RPA) and POT1-TPP1. RPA, a heterotrimeric complex consisting of RPA1, RPA2 and RPA3, robustly stimulates the helicase activity of BLM [189], WRN [189], FANCJ [63,190], and DNA2 [191] through direct interaction in vitro. Similarly, POT1-TPP1, a dimeric complex that specifically binds to the telomeric G-rich strand, stimulates BLM and WRN [192]. Notably, both ssDNA binding proteins also have the propensity to melt telomeric G4s by themselves [193–196]. Single-molecule studies suggested that they destabilise G4s by binding to a transiently unfolded G-tract through repetitive interaction [197–199]. POT-TPP1 unfolds telomeric G4s [197], while RPA also unfolds non-telomeric G4s, specifically the less stable G4s [198]. Furthermore, analogous to these ssDNA binding proteins, most G4 helicases can melt G4s in an ATPase-independent manner in vitro [187,200–204]. However, the physiological significance of this activity has been debated as ATPase-dead G4 helicases cannot restore G4-related defects in the respective knockout cells [137,163,205–209] but rather exhibit dominant-negative behaviour in vivo [180,210–213] (reviewed in [214]). Based on these observations it can be inferred that there must be a variety of mechanisms that resolve G4s with different topologies, or at a different time or location. Despite extensive efforts in identification and characterisation of G4 helicases over years, only recently comprehensive biochemical mechanisms of how G4 helicases act in physiological circumstances are beginning to be elucidated.

4.2. *Consequences of G4 encounter by the yeast replisome*

Several G4 helicases, including PIF1, FANCJ, and RTEL1, directly interact with the replisome [186,215–217], suggesting that G4 resolution, at least in part, is coupled to DNA replication. Two recent studies [139,140] use the reconstituted DNA replication system based on purified *budding yeast (S. cerevisiae)* proteins to investigate replisome-G4 encounter. The eukaryotic core replisome consists of DNA polymerase Pol δ and Pol ε, the fork protection complex (FPC), and the replicative helicase CDC45-MCM2–7-GINS (CMG) that translocates on the leading strand template ahead of the polymerases (Fig. 5A). Firstly, Kumar et al. [139] demonstrated that a G4 structure on the leading strand template can directly cause replisome stalling. In this study, G4 structure formation was facilitated on a substrate plasmid by the induction of a DNA:RNA hybrid on the opposite DNA strand prior to replication initiation. The DNA:RNA hybrid present on the lagging strand template induces potent replisome stalling on the G-rich leading strand template, indicating that a G4 structure efficiently blocks CMG (Fig. 5B, i). Interestingly, on some substrate molecules, CMG uncoupling occurred and fork restart was observed downstream the intact G4. CMG thus has an intrinsic capacity to bypass a G4 structure albeit inefficiently, while the polymerase remains stalled at the G4 (Fig. 5B, i). Similarly, G4s on the lagging strand template, facilitated by DNA:RNA hybrid formation on the leading strand, inhibit lagging strand synthesis (Fig. 5B, ii). While the replisome also arrests at the DNA:RNA hybrid on the leading strand template, CMG can efficiently bypass this structure, by translocation past the DNA:RNA hybrid. Subsequently, the 3' RNA end can be used to restart the leading strand synthesis. This requires RNA extension by Pol α, which triggers strand elongation by either Pol ε or Pol δ. This is consistent with observations that DNA:RNA hybrids on the leading strand template cause only mild replication stress and little DNA damages in bacteria and in human cells [149,150].

The second study by Casas-Delucchi et al. [140] used different DNA templates containing a repeat sequence capable of forming G4s but these

are not preformed. Interestingly, in some substrate molecules, the leading strand DNA synthesis was blocked and the CMG was uncoupled from the polymerase at these sequences. Furthermore, the same group recently showed that, in this system, G4 formation is induced as a result of DNA replication and a single parallel G4 motif is sufficient to induce CMG-polymerase uncoupling [141]. This suggests that a G4 structure can form on the leading strand template behind CMG, and impede DNA replication (Fig. 5C). Consistent with its G4 unwinding function in *S. cerevisiae*, addition of the PIF1 helicase largely alleviated polymerase stalling at G4s in this system [139–141]. However, whether PIF1 performs this function in similar circumstances in vivo remains to be determined.



(caption on next page)

Fig. 5. Mechanisms of G-quadruplex resolution. A. The cryo-EM structure of the core human replisome (PDBID: 7PFO [135], left) and a schematic illustration of the replisome approaching a G4 structure (right). MCM2–7 unwinds the template duplex DNA in the front of the fork by threading the leading strand template through its central channel. The leading strand template exiting from the MCM2–7 channel appears to span ~20 nt at maximum to the catalytic centre of the replicative polymerase based on CMG footprints observed in *Xenopus* egg extracts [219]. Consistent with this, a docking model of a cryo-EM structure of the *S. cerevisiae* Pol ε holoenzyme [287] to the human core replisome positions the active site of Pol ε 110–140 Å from the MCM2–7 pore exit [135]. This ssDNA is sufficient for formation of a minimal G4 structure on the leading strand template. The C-terminal region of TIMELESS oriented to the front of MCM2–7, that is not present in the structure, is represented as a dashed oval. TRAIP has been identified to constitutively interact with replisome and ubiquitinate protein obstacles crosslinked to DNA at the leading edge, which facilitates protein degradation and bypass of CMG [288] (not shown). NCM, non-catalytic module. B. Models for replication of a DNA:RNA hybrid and a G4 structure in the reconstituted yeast replication system [139]. When a DNA:RNA hybrid is formed on the lagging strand template, G4 formation is induced on the leading strand template (i). The replisome efficiently stalls at the G4. This is probably due to steric hindrance of the G4 with the MCM2–7 central channel, because the pore entrance (~20 Å in diameter [289]) does not fit even a minimal G4 (~24 Å in diameter [290]). A fraction of stalled replisomes appear to bypass the G4 and restart leading strand synthesis by repriming. Similar leading strand repriming was observed at a cyclobutane pyrimidine dimer (CPD) on the leading strand template in the yeast replication system [220]. When a DNA:RNA hybrid is formed on the leading strand template, G4 formation is induced on the lagging strand template (ii). The replisome also stalls at the hybrid, but efficiently restarts. MCM2–7 can thread the DNA:RNA hybrid with the central pore, allowing CMG to bypass the hybrid region. Once CMG is bypassed, the 3' end of RNA molecule is extended by Pol α, and further extended predominantly by DNA polymerase ε (not shown). In contrast, the G4 on the lagging strand potently blocks DNA polymerase, inhibiting Okazaki fragment maturation. C. Models for replication of a G-rich sequence [140,141]. When a PQS is present on the leading strand template, a G4 can be formed likely behind CMG but ahead of DNA polymerase, resulting in uncoupling of CMG and DNA polymerase (left). Similar replication intermediates (middle cartoon) have been found in human cells [63]. In this study, CMG is situated downstream a G4 but still in close vicinity to the stalled DNA polymerase. This leads to an alternative model of replication intermediate where functional uncoupling of the CMG and polymerase results in the leading strand template looping out between the exit of MCM2–7 central channel and the catalytic domain of polymerase ε (right). D. Model for replication of a G4 structure in *Xenopus* egg extracts [62]. A parallel G4 on the leading strand template potently blocks the *X. laevis* replisome. This collision triggers ssDNA generation downstream the G4 through the duplex unwinding by DHX36 which bound to the G4 with picomolar affinity [291] prior to replisome collision. This induces CMG bypass past the G4. This may involve transient opening of a CMG gate as has also been suggested for other replisome blocking lesions [222]. Although purified MCM10, an accessory factor of replisome, promotes the ring opening in vitro [292], a role for MCM10 in CMG bypass has not been clarified. Once CMG is bypassed, FANCJ is recruited to the G4 and unfolds the structure. This reaction strictly requires DNA polymerase collision with the G4 structure. Given the recent finding that FANCJ also unfolds proteins crosslinked to DNA [293], trapped proteins on genomic DNA might be a preferable substrate for FANCJ. When FANCJ is absent, DHX36 unwinds the G4, conferring robust G4 unwinding. Since G4 unwinding by DHX36 requires a 3' ssDNA tail, stalled DNA polymerase appears to be efficiently unloaded from the leading strand template. Once the structure has been resolved, DNA synthesis past the G4 sequence occurs without stalling. It is unclear what DNA polymerase acts on this G4 motif synthesis. E. Model for replication of G4 structures involving PRIMPOL [223]. Replication of a G4 structure on the leading strand template induces CMG uncoupling. Prolonged CMG uncoupling perturbs histone recycling from ahead of CMG onto nascent daughter strands, leading to epigenetic instability. PRIMPOL is recruited downstream the G4 and restarts leading strand synthesis by repriming. The repriming intermediates might act as a landing pad for G4 helicases as PRIMPOL is also important for G4 resolution at least in human cells [224]. F. Model for replication of G4 structures involving translesion synthesis (TLS) polymerases [145,147]. After CMG uncoupling at a G4 structure, TLS polymerase REV1 plays a role in G4 resolution as observed in the *Bu-1* locus of DT40 cells. Importantly, the C-terminal region of REV1 that interacts with other TLS polymerases such as Polκ and Polη is important for G4 resolution, while the catalytic activity of REV1 is dispensable [146]. This may suggest that REV1 acts as a structural hub for other TLS polyemerases. Although as Pol κ and Pol η have been shown to destabilise G4s in vitro [228,230] and important to prevent genome instability upon G4 stabilisation in vivo [226], it is currently unclear whether these polymerases collaborate with REV1 to resolve G4s. G. Models for replication of G4 structures involving TIMELESS and DDX11 [136]. TIMELESS constitutively binds to the leading edge of CMG, regulating fork speed by contacting MCM2–7 (see also Fig. 5A). The C-terminal region of TIMELESS exhibits a specific sub-micromolar G4 binding activity, and is important for G4 resolution in the *Bu-1* locus of DT40 cells. Since the TIMELESS configuration in the replisome positions this domain ahead of MCM2–7 [135], this domain appears to sense G4s that are pre-formed on the leading strand template (left) or on the lagging strand template (not shown). This mechanism also requires recruitment of DDX11 through direct interaction with TIMELESS. Since DDX11 is a 5'– 3' helicase, it might translocate on the lagging strand to generate ssDNA for CMG bypass (bottom, left). Alternatively, it might unwind the G4s on the leading strand after CMG bypass (bottom, right) or on the lagging strand (not shown). In the former case, loss of DDX11 may lead to CMG uncoupling upon DNA polymerase stalling at a G4 on the leading strand template, which is consistent with the loss of epigenetic information observed in the *Bu-1* locus of the *DDX11*-knockout DT40 cells. The TIMELESS configuration is also stabilised through direct binding to the dsDNA template [135], which can be affected in the presence of a G4 on the template. In addition, it has been shown to dissociate from the replisome in response to oxidative stress [294]. It is thus conceivable that it recruits DDX11 after detaching from MCM2–7. Loss of DDX11 causes only minor sensitivity to G4 ligands [188], suggesting that other G4 helicases act redundantly in G4 resolution. Consistent with this, WRN and BLM have been shown to promote processive replication in the *Bu-1* locus [146], preventing loss of epigenetic information. H. Models for replication-independent G4 resolution in *Xenopus* egg extracts. Once G4 is formed, the RAD51 nucleoprotein filament is quickly assembled on the G4-opposing strand by BRCA2. Consistently, a recent report showed that BRCA2 directly binds to G4s with lower micromolar affinity [34]. RAD51 promotes hybridisation of homologous RNA transcripts to form a G-loop. This also requires the hnRNPA1 complex that shuttles RNA transcripts. Consistent with this, G4 stabilisation rapidly induces R-loop formation in the proximity of G4s in cells [118]. The processive assembly of RAD51 most likely induces branch migration. This creates ssDNA downstream the G4 and triggers G4 unfolding by FANCJ, inducing G-loop-to-R-loop conversion. The resulting R-loop recruits the FANCI-FANCD2 (ID) complex, which induces monoubiquitination of FANCD2, a crucial process for subsequent recruitment of the SLX4-XPF-ERCC1 nuclease complex (SXE). This complex selectively incises the RNA-containing strand, and DNA polymerase re-synthesises the strand from the DNA end. This synthesis is localised mostly within ~200 bp from the G4 motif, which is consistent with recently observed repair synthesis near PQSs in post-mitotic neurons [295].

4.3. Replication-dependent G4 resolution in Xenopus egg extract

A stepwise mechanism for G4 resolution that is activated by G4-replisome collision was recently outlined by replicating a plasmid containing a parallel and site-specific G4 structure in *Xenopus* egg extract [62]. In this system, CMG collision with the G4 on the leading strand template induces translocation of the DHX36 helicase, that binds the G4 prior to fork approach, on the leading strand template to generate ssDNA downstream of the G4 structure (Fig. 5D). This allows CMG bypass past the intact G4 structure (Fig. 5D), leading to CMG-polymerase uncoupling. In the absence of DHX36, FANCJ generates this downstream ssDNA by translocating along the lagging strand template. Subsequent polymerase stalling at the G4 structure after CMG bypass promotes G4 unwinding (Fig. 5D). Similarly, a G4 structure on the lagging strand template does not stall CMG but still requires DNA replication stalling for unwinding (Fig. 5D). Unwinding of both leading and lagging strand G4s primarily depends on FANCJ (Fig. 5D). In the absence of FANCJ, DHX36 acts redundantly.

While this is a robust mechanism for replication-coupled G4 unwinding by DHX36 and FANCJ, it has only been shown on a parallel G4 substrate. Direct evidence that G4 structures with different topologies block the eukaryotic replisome is currently missing, and mechanisms of their resolution remain to be elucidated. Given the other G4 helicases being involved in genome maintenance at G4 structures, other mechanisms that unwind G4 structures during DNA replication are likely to exist (discussed further below).

4.4. Replication-coupled G4 formation and resolution in cells

Recent SMLM imaging in human cells elegantly demonstrated the location of G4 structures in the context of the replisome [63]. This high-resolution imaging technique detected stalled replisomes with a folded G4 structure between CMG and the 3' nascent strand end in ~2% of total ongoing forks (Fig. 5C). Importantly, this intermediate structure is similar to what is proposed to be the substrate for FANCJ in the *Xenopus* egg extract system (Fig. 5D) [62], indicating that the mechanism of G4 resolution could be essentially the same. Consistent with this possibility, the stalled intermediate is enhanced when FANCJ is depleted from cells, and stalling is further exacerbated by treatment with a G4 ligand that inhibits G4 unfolding by other helicases including DHX36 [218]. In contrast, the G4 ligand treatment does not influence the intermediate accumulation in the FANCJ-proficient cells, corroborating the primary role of FANCJ in G4 unwinding at replication forks. Furthermore, this study showed that FANCJ-mediated G4 unwinding promotes RPA binding to the replisome, indicating that unwound G4 motifs are immediately bound to RPA to prevent their refolding.

One central question that arises from this study is whether the stalling intermediate results from CMG bypass past a pre-existing G4, or by G4 formation behind CMG on the leading strand template. Although these possibilities are not mutually exclusive, the studies using the SMLM imaging and the yeast replication system [63,140,141] both favour the latter model, which would be consistent with elevated numbers of G4s in S phase [20]. CMG footprints observed in vertebrate replisome indicate that at maximum ~20 nt ssDNA can be exposed between the exit channel of the CMG and the catalytic centre of the replicative polymerase [219], which would only be enough for minimal G4 structures to form. However, transient uncoupling of the polymerase and CMG helicase could occur on a regular basis in response to variations in DNA synthesis rates, DNA damages, and nucleotide supplies, which can facilitate G4 formation behind CMG. Importantly, SMLM imaging showed that CMG downstream the G4 remains in the vicinity of the DNA polymerase situated upstream of the G4 even after induction of their uncoupling. This raises an interesting possibility that CMG retains interaction with the DNA polymerase, looping out the leading strand template during unwinding (Fig. 5C). Importantly, the processivity of CMG is considerably reduced upon uncoupling in both yeast replication systems and *Xenopus* egg extracts [220–222]. In line with these observations, SMLM analysis showed limited RPA binding at G4-containing replisomes. On the other hand, given the abundant G4 structures in chromatin during the G1-S transition when replication initiation is prepared [23], preformed G4 structures will very likely also be encountered by the replisome. Therefore, it is most conceivable that both situations can occur.

G4-induced uncoupling of CMG helicase and polymerase activity can disrupt histone recycling from the parental template to the replicated daughter strands, resulting in local loss of epigenetic histone marks as observed in cells [145–147]. A recent cell-based study revealed that a DNA primase-polymerase PRIMPOL plays an important role to prevent the epigenetic instability in a well-establish locus, the *Bu-1A* gene in DT40 cells [223], whose expression is dependent on continuous replication of a G4 forming sequence on the leading strand template [145–147]. Although PRIMPOL does not directly unfold G4 structures, it can reprime ~6 nt ahead of a G4 structure in vitro [223]. Mutations in its catalytic core severely impaired expression of the *BU*-1A in DT40 cells, suggesting that PRIMPOL-mediated repriming is required for timely replication of G4-containing regions (Fig. 5E). In addition, loss of PRIMPOL elevates cellular G4 formation and sensitises human cells to G4 ligands [224], suggesting that PRIMPOL also promotes G4 resolution. It should be noted that PRIMPOL also forms nuclear foci throughout cell cycle upon G4 stabilisation [224]. It is thus conceivable that PRIMPOL prevents epigenetic instability by facilitating G4 resolution prior to replication.

4.5. G4 resolution by TLS polymerases

An early study in *C.elegans* revealed that, when FANCJ is absent, the translesion synthesis (TLS) polymerases Pol κ and Pol η can prevent deletions at G4 motifs [225]. Consistent with a role for these TLS polymerases in G4 resolution, knockdown of Pol κ or Pol η moderately sensitises human cancer cells to a G4 ligand and elevates the γH2AX level in a PQS-containing oncogenic promoter [226]. Accumulating evidence also suggests that another TLS polymerase REV1 plays an important role in G4 resolution. Loss of REV1 leads to mutations and deletions near a G4 motif [227] in human cells and induces epigenetic instability near G4s on the leading strand template in DT40 cells [145, 147]. Pol κ, Pol η, and REV1 bind to G4s with a ~10 nM affinity, destabilise the structures, and can extend DNA synthesis at a G4 structure in vitro [228–230] albeit with varying efficiency. Importantly, these activities of the TLS polymerases are detected specifically for unstable G4s, suggesting that G4 resolution by these TLS polymerases may be limited to specific loci (Fig. 5F). Importantly, REV1 can unfold G4s independently of DNA synthesis [229] and efficiently accumulates on DNA damage sites outside S phase [231]. REV1 might thus play a role in G4 resolution also outside DNA replication.

4.6. G4 resolution at the replication fork

A recent cell-based study [136] described an additional G4 unwinding mechanism that functions independently of FANCJ. This mechanism requires TIMELESS, a component of FPC that travels with the replisome at the leading edge of the CMG helicase (Fig. 5A) [135]. Interestingly, a G4 binding domain was identified in the C-terminal region of TIMELESS. Deletion of this domain strongly reduced expression of the *BU*-1 locus in DT40 cells. This indicates that the TIMELESS-G4 interaction is important for processive replication, and therefor likely for resolution of the G4 structure during replication. Consistent with the observation in the yeast replication system that FPC alone cannot resolve G4 structures in vitro [139–141], this function of TIMELESS requires direct interaction with the G4 helicase DDX11. Since DDX11 accumulates on chromatin upon G4 stabilisation, it appears to be recruited by TIMELSS when replisome encounters a G4 structure. Importantly, based on the structure of the human replisome, the C-terminal G4 binding domain of TIMELESS, although not fully visible in the structure, appears to be positioned in the front of CMG [135] (Fig. 5A). It thus most likely senses a pre-formed G4 in the DNA ahead of the fork, or a G4 in the ssDNA on the lagging strand template (Fig. 5G). It will be of great interest to determine the conformation of TIMELESS and DDX11 in the replisome stalled at a G4 for better understanding of the mechanistic details of this G4 resolution pathway.

4.7. Replication-independent G4 resolution

To minimise the CMG uncoupling, and associated ssDNA exposure, it would be more beneficial for cells to resolve G4 structures prior to DNA replication. Recent studies uncovered rapid G4 unfolding dynamics in asynchronous cells [23], in which most G4s are unfolded in 30 minutes even after G4 stabilisation [118]. This suggests that G4s are resolved throughout the cell cycle and not only during DNA-replication. This is assumed to occur through a mechanism involving helicases-mediated G4 unwinding followed by reannealing of the two strands. However, such a simple snapback mechanism might be an oversimplification. Recent cryo-EM work demonstrated that G4 structure formation in duplex DNA does not cause drastic unwinding of the DNA around the G4 [232], therefore, the ssDNA required for G4 helicase binding may not be available. In addition, the displaced strand upon G4 formation is immediately bound by RPA that strongly inhibits reannealing. Reannealing is further inhibited when DNA:RNA hybrids are generated near G4s [109,118]. Moreover, given the important roles for G4 structures in transcriptional regulation, their resolution outside DNA replication must

be tightly controlled. These facts suggest that more complex mechanisms may regulate G4s independent of DNA replication. Such mechanisms would be also crucial for non-dividing cells.

We recently described one such replication-independent G4 resolution mechanism using a plasmid containing a parallel G4 structure *Xenopus* egg extract (Fig. 5H) [177]. In this mechanism, G4 resolution is initiated by the formation of DNA:RNA hybrid in the displaced strand across from the G4 structure, forming a G-loop (Fig. 5H). Importantly, G-loop formation is independent of active transcription, instead, RNA transcripts homologous to the non-G4 strand are recruited *in trans* through homologous pairing by the RAD51 recombinase, which is loaded on the G4-opposing strand by BRCA2. G-loop formation triggers expansion of the bubble region, creating ssDNA required for FANCJ binding (Fig. 5H). Furthermore, G-loop formation induces mono-ubiquitination of DNA repair complex FANCI-FANCD2, as seen also in cells [233,234], and promotes site-specific incision of the hybrid strand by recruiting the scaffolding protein SLX4 and the XPF-ERCC1 nuclease (Fig. 5H). This incision requires prior G4 resolution by FANCJ and once the DNA:RNA hybrid strand is incised, the non-G4 strand is resynthesised from the DNA end, displacing the hybrid-containing strand (Fig. 5H).

The renewal of the non-G4 strand in this mechanism could be advantageous for genome integrity because this strand is often vulnerable to APOBEC-induced mutagenesis due to its C-richness [235–238]. In line with this idea, APOBEC-induces mutations are remarkably excluded in PQSs in cells [239]. It is noteworthy that, although this mechanism is not necessarily coupled to active transcription, it is tightly regulated by RNA transcript levels (Fig. 5H). This RNA-dependent regulation could thus enable G4 suppression at selective loci where the RNA transcript level is low while retaining G4s at actively transcribed loci, leading to cell-type specific G4 regulation.

5. G-quadruplexes in pathology and treatment of disease

5.1. Pathological consequences of G4s

Copy number variations, as well as structural variants were found substantially enriched at PQSs in multiple cancer genomes [56–61], indicating that G4 structures represent vulnerable genomic regions that contribute to malignant transformation and cancer development. In line with these observations, germline mutations in G4 unwinding proteins have been found in diseases characterised by high cancer predisposition [52,53]. In addition, DHX36 has been classified as an oncogene [240], and found upregulated in multiple cancers [241,242]. Furthermore, several G4 unwinding proteins have been associated with diseases that display premature aging of specific tissues ascribed to telomere instability, congenital microcephaly and neurodegeneration [54]. While loss of G4 helicases often results in increased levels of G4 structures [17,63,163,179,208], direct evidence that these diseases are caused by a defect in G4 resolution is currently missing. This has proven difficult to obtain, especially since these helicases often have multiple cellular functions. However, it suggests that G4 suppression could, at least in part, play a role in differentiation and maintenance of the neural lineage. Alternatively, it is also conceivable that combined defects of multiple cellular processes contribute to onset of the diseases. For example, defects in FANCJ lead to FA [173–175], a recessive disorder caused by defective repair of endogenous DNA interstrand crosslinks [243], but whether the defect in G4 resolution plays an additional role in the pathogenesis is not clear. Interestingly, FANCJ knockout mice exhibit an FA phenotype [244], which is not obvious in most other FA gene knockout mice [245,246]. This could result from the additional problems caused by impaired G4 resolution such as replication stress and transcriptional dysregulation. In line with this notion, FA patients bearing FANCJ mutations suffer from more severe symptoms with a shorter survival period among the FA subtypes [247]. Clearly, further clinical studies are needed for better understanding of the pathological consequences resulting from G4 resolution deficiency, specifically in uncharacterised diseases associated with premature aging, congenital microcephaly, and neurodegeneration.

G4s are highly enriched in oncogenic promotors in the human genome [71]. Moreover, G4s have been shown to regulate the transcription of a number of highly relevant oncogenes, such as *VEGF* [248], *MYC* [127], *KRAS* [128], and *KIT* [249]. Consistently, in breast cancer, G4s were shown to regulate transcription in promoters of highly amplified genes related to cancer development, shaping transcriptomes that are tightly correlated with intratumor heterogeneity [61]. These findings further highlight the potential relevance of transcriptional regulation by G4s in cancers.

5.2. G4-targeting therapeutics

Accumulating evidence indicates that G4s are promising therapeutic targets in cancer treatment [250,251]. The antitumor activity of G4 ligands is achieved by at least three mechanisms. The first mechanism is induction of replication stress and subsequent DSB formation. Consistent with this, and similar to inhibition of PARP1/2 [252,253], stabilisation of G4 structures results in synthetic lethality in HR-deficient cancer cells [121,158,159]. Notably, G4 ligands exert the selective anti-tumor activity also upon acquired resistance to PARP inhibitors [121,159]. A phase I clinical trial of the most promising G4 ligand CX-5461 has been recently completed and provided the clinical proof-of-concept for the G4 stabilizing strategy in HR-deficient cancers [254]. The second potential therapeutic mechanism is inhibition of telomerase [119,255] or the telomere protection complex [256], which causes telomeric instability and consequential senescence and cell death. The third mechanism is downregulation of oncogene expression, leading to severe inhibition of cell growth and subsequent cell death [127,128,248,249]. Although G4 formation at promoters is important for active transcription, their stabilisation has been shown to inhibit the transcription of vital oncogenes including *MYC* [66,127], the most frequently amplified oncogene, and correlates with tumor aggression and poor clinical outcome [257].

Since early studies showed a strong anti-tumor effect of G4 ligands [127,133], over a thousand G4 ligands have been developed [258]. Their clinical use is, however, not approved yet. This is primarily due to their poor selectivity for specific promotors, which represents a central barrier for therapeutic application as it induces potential side effects on normal tissues. While recent work has led to the promising discovery of several G4 ligands with a higher selectivity to specific G4 structures [259–261], careful consideration is required when interpreting clinical efficacy of the ligands due to their potential off-targets. For example, multiple lines of evidence have demonstrated that CX-5461 and another G4 ligand pyridostatin also trap topoisomerase 2 (TOP2) at stabilised G4 structures and induce DSBs during transcription [262–265]. Importantly, TOP2 is widely expressed in normal tissues and off-target TOP2 poisoning have been implicated in late-emerging fatal adverse events including acute leukemias [266] and cardiotoxicity [267]. Ongoing phase II clinical trials of CX-5461 thus may need additional examination on the long-term safety concern.

A novel, potentially promising, strategy to target G4 structures is RNA-based therapeutics. Recent studies have suggested that G4 structures can be stabilised through the invasion of RNA transcripts into the non-G4 strand *in trans* [48,177,268,269]. Consistent with this concept, siRNAs directed to gene promoters have been shown to severely inhibit their downstream transcription [270,271]. Because stable G4 structure formation has been found in multiple oncogenic promoters in cancers [61], development of RNA cocktails that simultaneously targets such G4s might offer a reliable and specific therapeutic strategy against various cancer types.

Importantly, PQSs have been also found in virtually all viruses. Accumulating evidence indicates that G4 structures are involved in multiple key steps of viral life cycles (reviewed elsewhere [272–274]). Notably, most primate lentiviruses, including human immunodeficiency

virus (HIV), harbor conserved G4 motifs in their long terminal repeat regions [92,275], and exploit the G4 structures for transcription activation after integration to the host genome [276,277]. Consistent with this, G4 stabilizing ligands exert a remarkable antiproliferative effect to HIV by both suppressing provirus transcription and blocking reverse transcription [278,279]. Therefore, the strategies for specific G4 stabilisation could be highly applicable also for antiviral therapy. Since the current antiviral therapy is mostly reliant on a single strategy that inhibits viral proteins, this new approach would be especially beneficial for treatment of drug-resistant viruses as frequently seen for HIV [280].

6. Conclusion

Our understanding of the regulation and impact of G4 structures has tremendously increased in the past years owing to the development of new methods to map G4s throughout the genome, to visualise individual G4s in cells, and to monitor G4 resolution in various biological contexts. Although numerous G4 helicases have been identified, we lack knowledge on which specific cellular G4 structures they act on, what molecular mechanisms act for G4 resolution, and how G4 resolution is regulated in cellular contexts. Further insights are important for a fundamental understanding of how dysregulation of G4 structures contribute to pathogenesis, and to develop new strategies to alleviate or exploit G4-associated defects in therapy.

Author statement

All authors have read and approved the content of the submitted manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

Data availability

No data was used for the research described in the article.

Acknowledgements

We thank Francesca Mattiroli for feedback on the manuscript. P.K. is supported by the European Research Council (ERC) through an ERC consolidator grant (ERCCOG 101003210-XlinkRepair) and by the Oncode Institute, which is partly financed by the Dutch Cancer Society (KWF). K.S. was supported by the Japanese Biochemical Society through Osamu Hayaishi Memorial Scholarship for Study Abroad.

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