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How to untie G-quadruplex knots and why?

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Summary.

For more than two decades now, the prime objective of the chemical biology community studying G-quadruplexes (G4s) has been to use chemicals to interact with and stabilize G4s (*i.e.*, G4-ligands) in cells to obtain mechanistic interpretations. This strategy has been undoubtedly successful, as demonstrated by the spectacular recent advances. However, these insights have also led to a fundamental rethinking of G4-targeting strategies: in light of the prevalence of G4s in the human genome, transcriptome and ncRNAome (collectively referred to as the G4ome), and particularly their involvement in human diseases (cancers and neuropathologies), should we continue to search for and develop G4-stabilizing ligands or would it not be wise to invest efforts in designing and exploiting molecular tools able to unfold G4s? Perhaps provocative, this question invites us to take a new and fresh look at the wealth of data accumulated on the functional relevance of G4s. In this review, we first focus on how, when and where G4s fold in cells; then, we describe the enzymatic systems the cells have evolved to counteract G4 folding (*i.e.*, G4-helicases) and how they have been used as biomolecular tools to manipulate G4s in cells; finally, we present the strategies currently being implemented by chemical biologists on G4-ligands and G4-helicase to devise new molecular G4-unwinding agents.

1. Introduction

The central dogma of biology, heralded by Francis Crick in 1957,(1-3) has been instrumental in the emergence of modern biology. In science, a dogma is “*usually based on some recognized authority (here, one of the discoverers of the DNA double helix structure)(4-6) whose pronouncements are unconditionally accepted*”.(7) A dogma is therefore useful as it provides a basis of accepted knowledge that does not need to be re-demonstrated in each new study. Somewhat paradoxically, a dogma also fuels studies aiming at challenging its ideas, through permanent verification or reconsideration.(8) A dogma is therefore bound to evolve, most often at a pace dictated by the technical advances in the field.

Crick’s dogma describes the flow of genetic information from DNA to proteins. In his view, DNA is a barcode waiting to be scanned to meet cellular needs. This point has recently been challenged: while the role of DNA as the repository of genetic information is unquestionable, researchers have uncovered that DNA is not structurally placid and that it can fold into a variety of structures that deviate from the Watson-Crick duplex. The exact roles of this structural plasticity are only now unfolding.

The first-in-class example of alternative DNA structures is undoubtedly the G-quadruplex (G4), a four-stranded structure that folds from guanine (G)-rich sequences.(9) This structure is highly thermodynamically stable and folds spontaneously when a G-rich DNA strand escapes its duplex structure, *i.e.*, when the double helix melts to allow for DNA transactions (replication, transcription). Initially suspected to form at telomeres,(10) bioinformatics(11-13) and then sequencing techniques(14-17) have progressively raised G4s to the status of key genetic levers, highly prevalent in the human genome, governing gene expression and genomic stability.(18-21) After three decades of research, the time is now ripe to look at the wealth of knowledge accumulated with hindsight: are G4s the promising genetic switches able to defeat Crick’s dogma? Or are they one-off, *ad hoc* events that threaten the genetic integrity of human cells?

As discussed hereafter, there is no easy way to address these questions and every answer calls for some nuance. Nature has evolved an efficient enzymatic arsenal (helicases) to regulate G4 formation *in vivo*, as if to comply with Crick’s dogma. Scientists are also now conceiving molecular tools to exploit G4 unwinding systems to uncover the roles of G4s in genetic diseases, in an approach that fits the general concept of chemical biology defined by Linus Pauling in 1954 (see section 4a).(22)

In the following sections, we will attempt to portray the G4 landscape in its true colors: what is a G4? G4-DNA *versus* G4-RNA? Where, when and how do G4s fold within cells? At what cost and for which mechanistic uses? We go on to describe the cell's innate strategy to regulate G4 formation using helicases. Next, we detail the approaches currently investigated by chemical biologists to harness nature's own techniques to untie G4 knots and exploit them in oncology, ageing and age-related diseases. One thing is sure: the visionary ideas of Francis Crick, expressed more than 60 years ago, continue to spark debates among scientists and provide a fertile ground on which ever more innovative science is flourishing.

2. A nucleic acid structure with a strong identity

2a. G-quadruplex-forming sequences are pervasive in the human genome and transcriptome. G4 structure(23) hinges on the self-association of four guanines to form a supramolecular macrocycle referred to as the G-quartet (**figure 1A**).⁽²⁴⁾ The stability of the G-quartet originates in the 8 hydrogen (H) bonds on both Watson-Crick⁽⁴⁾ and Hoogsteen⁽²⁵⁾ faces, (4 H-bonds per G, whereas canonical GC association involves only 3 Watson-Crick interactions per base) reinforced by either π -⁽²⁶⁾ or σ -bonds,⁽²⁷⁾ or both. In physiological conditions, the presence of cations such as K^+ or Na^+ is instrumental within the G-quartet, being coordinated to the four inwardly pointing carbonyl groups of the Gs, thereby decreasing the high electronic density at the heart of the macrocycle and overcoming the enthalpic penalty of G4 formation.⁽²⁸⁾

A nucleic acid sequence, both DNA and RNA, is termed a putative G-quadruplex forming sequence (PQFS) if it comprises four or more runs of consecutive Gs (at least two, but more often three).^(11-13,17) Upon strand folding, consecutive Gs will form consecutive G-quartets that will stack on each other, further stabilizing the resulting edifice through π -stacking interactions and cation chelation between G-quartet layers (**figure 1A**).

Bioinformatics studies identified >300,000 DNA PQFS in the human genome, corresponding to the sequence $G_{\geq 3}N_xG_{\geq 3}N_xG_{\geq 3}N_xG_{\geq 3}$ (where G is guanine, and N any intervening nucleobase, and x ranges from 1 to 7).⁽¹¹⁾ Later, the definition of PQFS was extended to higher x values (*i.e.* longer loops with $x \leq 15$,⁽²⁹⁾ then ≤ 21)⁽¹⁴⁾ and, coupled with sequencing-based techniques,^(17,30) the number of PQFS was raised to > 500,000 sequences corresponding to $G_{\geq 3}N_xG_{\geq 3}N_yG_{\geq 3}N_xG_{\geq 3}$ ($x \leq 7$, $y \leq 21$, in K^+ -rich conditions). The prevalence of G4-DNA in our genome is thus firmly established; that of RNA PQFS has also been scrutinized, bearing in mind

that structures and functions are more intimately correlated in RNA than in DNA.(31-33) G4-RNA are pervasive as well, with > 3,000 PQFS corresponding to $G_{23}N_xG_{23}N_yG_{23}N_xG_{23}$ ($x \leq 12$, $y \leq 21$, in K^+ -rich conditions) in a pool of purified polyadenylated (polyA) mRNAs,(34) and > 6,000 PQFS from rough cell extracts (including coding and non-coding RNAs).(35)

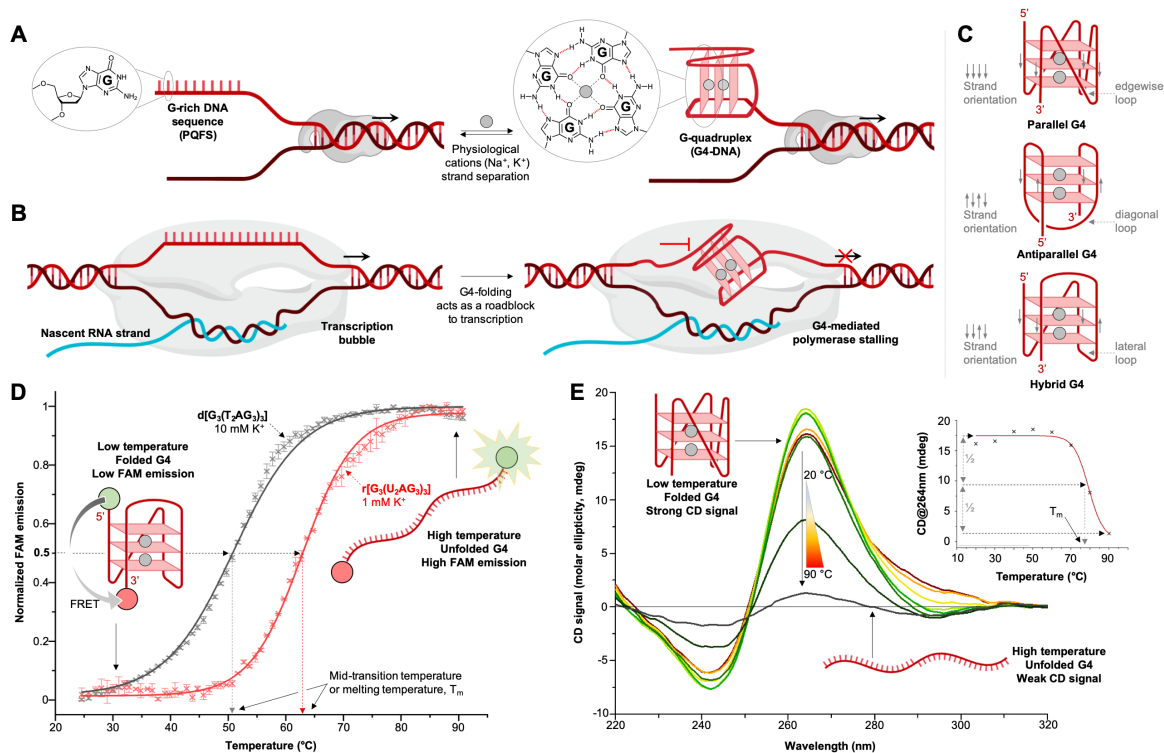


Figure 1. Schematic representation of (A) the transient formation of a G-quadruplex-DNA (G4-DNA) when a putative quadruplex forming sequence (PQFS) is freed from the duplex structure (inserts show detailed chemical structures of guanine (G) and G-quartet); (B) the G4-mediated stalling of the RNA polymerase during transcription; and (C) the possible G4 topologies: parallel, antiparallel and hybrid G4. [Created with BioRender] Example of the thermal denaturation of (D) a G4 (G4-DNA, black line; G4-RNA, red line) labeled with a fluorescein (FAM, green circle) at its 5'-end and a tetramethylrhodamine (red circle) at its 3'-end, monitored by the modification of the FAM fluorescence (*FRET-melting assay*); and (E) an unlabeled G4-RNA, monitored by the modification of its circular dichroism (CD) signal (*CD-melting assay*).

2b. The formation of G-quadruplexes is intimately linked to genetic transactions. The strong bias in the genomic/transcriptomic distribution of PQFS towards key functional regions lends credence to widespread G4 regulatory roles (telomeres, replication origins and gene promoters for G4-DNA; 3'- and 5'-untranslated regions (UTR) for G4-RNA).(19,21,36) For instance, the notable enrichment of DNA PQFS in gene promoters (with > 8,000 PQFS localised < 1kb upstream of gene transcription start sites (TSS))(37) is suggestive of a G4-mediated control of gene expression at the transcriptional level. Similarly, the strong enrichment of RNA PQFS in UTRs (with > 2,000 and > 500 PQFS in 3'- and 5'-UTR, respectively, in polyA mRNA)(34)

is suggestive of a G4-mediated control of gene expression at the translational level. However, whether G4s contribute to or perturb cellular functions faces systematic analysis. For instance, G4-DNA can, on one hand, act as a roadblock to polymerases (*i.e.* inhibiting gene expression) and on the other hand help recruit transcription factors (*i.e.* favoring gene expression).(38) Similarly, G4-RNA in 5'-UTRs can, on one hand, act as a physical obstacle to ribosome entry (cap-dependent translation; *i.e.* inhibiting gene expression) and, on the other hand, facilitate ribosomes' entry (cap-independent translation; *i.e.* favoring gene expression).(39,40) To address these conundrums further research is required.

The formation of G4s in cells, whose prevalence has been demonstrated through a wealth of optical methods,(41,42) is thus intimately linked to genetic transactions (**figure 1B**) that are replication and transcription for G4-DNA, and translation for G4-RNA. ChIP-seq investigations performed with the BG4 antibody confirmed an enrichment of G4s at transcriptionally active sites, linked to nucleosome depletion and open chromatin state.(14) Recently, a G4P-ChIP technique, performed with a dimeric, truncated DHX36 helicase (see section 3c), confirmed both the prevalence of QFS in living human cells (> 120,000 G4P peaks, 60% of which in promoter regions) and the transcription-dependence of G4 formation.(43) Similarly, while the formation of G4-RNA seems to be globally repressed *in vivo*,(44) functional ncRNAs involved in gene expression (among many other biological functions)(45) exert their cellular effects *via* the transient formation of G4-RNA.(35) G4 folding *in vivo* could thus represent another layer of non-genetic, non-epigenetic but structural regulation of gene expression.

2c. G-quadruplexes are structurally defined and thermodynamically stable. The three-dimensional structure of G4s is dictated by the sequence they fold from. The G-runs provide the structural basis of the G4 core, allowing G-quartets to stack on top of each other. The number and nature of the intervening nucleobases N (*vide supra*) offer structural diversity, forming loops of different length (≤ 21), orientation (lateral, diagonal or edgewise loops, **figure 1C**) and nature (single-stranded or forming bulges). G4-DNA has high structural plasticity (adopting conformations most often referred to as parallel, antiparallel and hybrid-type G4 structure, or types I-III)(46), while G4-RNA is mostly restricted to a parallel structure, owing to the influence of the additional 2'-OH of the RNA backbone believed to be involved in an additional extended H-bond network. This network might also explain why G4-RNA are generally more stable than their G4-DNA counterparts (*vide infra*).

Subtle changes in either the nucleic acid sequences (*i.e.* the nature of N_x) or the experimental conditions in which the PQFS are studied (*i.e.* the cationic nature of the buffer, the stabilizing Na^+ or K^+ *versus* the non-stabilizing Li^+) have profound effects on the way G4s fold upon themselves and on the stability of the resulting edifices. A wide array of biophysical and spectroscopic methods have been developed to understand the thermodynamic and kinetic parameters that govern G4 folding.(47,48) Experiments based on an increase of temperature (so-called melting) have been particularly popular:(49-51) the temperature-promoted transition from the folded (low temperature) to the unfolded conformation (high temperature) of a PQFS can easily be monitored by fluorescence (*e.g.* FRET, for fluorescence resonance energy transfer, **figure 1D**),(50,52) absorbance (UV-vis)(53) or circular dichroism (CD, **figure 1E**)(54,55) spectroscopy and has been used to conveniently characterize G4 stability *via* the measurement of the mid-transition temperature (or melting temperature, T_m) at which 50% of the G4 is unfolded. For instance, melting experiments have been instrumental to demonstrate that both the length and nature of nucleobases constituting the G4 loops (N_x) are critical for G4 stability. As an example, the A-to-T substitution in the 3 loops of the G4-DNA $d[{}^5(G_3N_3)_3G_3{}^3]$ triggers a dramatic drop in G4 stability as shown by a decrease of the T_m from 74 (N = T) to 50 °C (N = A) assessed by FRET-melting, in a 1mM K^+ -containing buffer.(56) Similarly, increasing the length of the central loop of the G4-DNA $d[{}^5G_3TG_3T_xG_3TG_3{}^3]$ destabilizes the resulting G4 as demonstrated by a decrease of the T_m from 84 ($x = 2$) to 62 °C ($x = 15$) assessed by UV-melting in a 100 mM K^+ -containing buffer.(57) The difference of stability between G4-DNA and G4-RNA was also conveniently demonstrated by both CD-melting,(58) with a $T_m = 65$ °C for $d[{}^5G_3(T_2AG_3)_3{}^3]$ (the human telomeric sequence) *versus* 73 °C for $r[{}^5G_3(U_2AG_3)_3{}^3]$ (the telomeric transcript) in 100 mM K^+ and by FRET-melting (**figure 1D**), with a T_m of 51 °C for $d[{}^5G_3(T_2AG_3)_3{}^3]$ (in 10 mM K^+) *versus* 63 °C for $r[{}^5G_3(U_2AG_3)_3{}^3]$ (in 1 mM K^+).

The stability of G4s determined *in vitro* corresponds to their ability to trigger genetic instability *in vivo*. For instance, a study in *S. Cerevisiae* has demonstrated that shortening the central loop (underlined) of the CEB25 G4 $d[{}^5A_2G_3TG_3\underline{IGTA_2GTGT}G_3TG_3T^3]$ from 9- to 1-nt loop $d[{}^5A_2G_3TG_3\underline{I}G_3TG_3T^3]$ increases the stability of the resulting G4s (with $T_m = 55$ and 74 °C, respectively), which correlates with a spectacular increase of the genomic instability, from 0.5 to 67% of minisatellites rearrangements in helicase-deficient colonies.(59) These results indicate the extent to which G4s threaten the genetic stability and the importance of the G4-

helicases in safeguarding this stability (further discussed below). Initially postulated to be 'melting proteins' by Sen & Gilbert in 1988,(9) the pivotal roles of helicases in managing the threat of G4s was uncovered only nine years later, with the description of the SV40 large T-ag helicase.(60)

3. Enzymatic unwinding of DNA and RNA(61,62)

3a. Helicases & genetic diseases. Helicases were discovered in 1976, initially referred to as 'DNA unwinding proteins'(61-63) and the term helicase was officially coined in 1979.(64) Helicases are enzymes with the eponymous ability to unwind DNA helices. A total of 95 different helicases, 31 for DNA and 64 for RNA, have now been reported.(65) The two most studied families are the RecQ family, including Bloom (BLM), Werner (WRN), and RecQ-like helicase 1, 4 and 5 (RECQL1, RECQL4 and RECQL5, respectively); and the iron-sulfur (Fe-S) family, including xeroderma pigmentosum group D (XPD), Fanconi anaemia complementation group J (FANCI, also known as BRCA1 interacting protein C-terminal Helicase 1 (BRIP1)), regulator of telomere length helicase 1 (RTEL1) and DEAD/H-Box helicase 11 (DDX11).(66) Accumulating evidence points to the critical role of helicases in genome maintenance,(67) being involved in DNA damage response and repair.(65) They exert their safeguarding activity *via* the resolution of higher-order DNA structures that are transiently formed either during DNA repair (*e.g.* the Holliday junction(68,69) intermediate of homologous recombination) or during genetic transactions (*e.g.* the 5' flap intermediates during replication,(70) or the R-loops during transcription).(71) Helicases also participate in genome maintenance, resolving higher-order structures that fold from hard-to-replicate sequences,(72,73) chiefly G4s (*vide infra*),(74-76) that can act as physical barriers to polymerase advancement along their DNA substrates.

Most of the information collected about helicases stems from the diseases originating in their dysregulation. For instance, overexpression of RECQL1(77) and RECQL5(78) is a signature of several cancers(79) and FANCI is frequently mutated in breast cancers.(80) Mutations in helicase genes are also responsible for severe inheritable genetic disorders: Cockayne syndrome (dwarfism, mental retardation, skin and skeletal abnormalities) is due to the mutation of XPD;(81,82) Bloom syndrome (growth retardation and immunodeficiency) to that of BLM;(83) Werner syndrome (or 'adult progeria', early ageing) to that of WRN;(84) Rothmund-Thomson syndrome (or 'poikiloderma congenitale', abnormalities in skin and

skeleton, premature ageing) to that of RecQL4;(85) Fanconi anemia (developmental abnormalities, bone marrow failure) to that of FANCI;(86-88) etc.

Collectively, these examples show that the lack of helicase activity triggers the accumulation of unresolved forked DNA that is responsible for severe genetic disorders and cancer predisposition. They also confirm that structure-prone DNA sequences can jeopardize overall genetic stability. A way to tackle this threat is to use helicases as molecular tools to unfold these higher-order structures. As further detailed hereafter, this has been particularly studied with the subfamily of helicases known as the G4-helicases.(74-76)

Table 1. Main mammalian G4-helicases.

Name	Superfamily, (89,90) family	Composition	Dir.	Targets	Binding partner	Associated disease
BLM (91,92)	SF2 RecQ-like helicase	1417 aa 159 kDa	3' → 5'	G4-DNA; both inter- and intramolecular G4; forked DNA structures; both ATP-dependent and independent	RPA POT1	Bloom syndrome(83)
WRN (92,93)	SF2 RecQ-like helicase	1432 aa 162 kDa	3' → 5'	G4-DNA; both inter- and intramolecular G4; forked and triplex DNA; both ATP-dependent and independent	RPA POT1	Werner syndrome(84)
FANCI (94,95) (BACH1) (BRIP1)	SF2 Fe-S helicase	1249 aa 140 kDa	5' → 3'	G4-DNA; both inter- and intramolecular G4; forked DNA; D-loop	RPA REV1	Fanconi anemia(86-88)
RTEL1 (96,97)	SF2 DEAH-box helicase	1219 aa 133 kDa	5' → 3'	G4-DNA; intramolecular G4; telomeric G4; T-loops	PCNA	Dyskeratosis congenita & Hoyeraal-Hreidarsson syndrome(98-101)
Pif1 (102-104)	SF1 Pif-like helicase	641 aa 69 kDa	5' → 3'	G4-DNA; telomeric G4; mitochondrial G4	RPA PCNA	Cancer predisposition(105)
DHX36 (106) (RHAU) (G4R1)	SF2 DEAH-box helicase	1008 aa 114 kDa	3' → 5'	G4-DNA & G4-RNA; preference for parallel G4; both ATP-dependent and independent	-	-

3b. An enzymatic machinery to unfold G-quadruplexes. Beyond RecQ and Fe-S helicases, a novel overlapping family is now being classified, the G4-helicases.(65,74-76,107) These helicases unwind G4s *in vitro* and evidence is now accumulating to support a similar activity *in vivo*. The most studied mammalian G4-helicases are undoubtedly BLM, WRN, FANCI and RTEL1, along with Pif1 (Petite integration frequency 1)(108,109) and DHX36 (DEAH (Asp-Glu-Ala-His)-box Helicase 36)(110) (**table 1**).

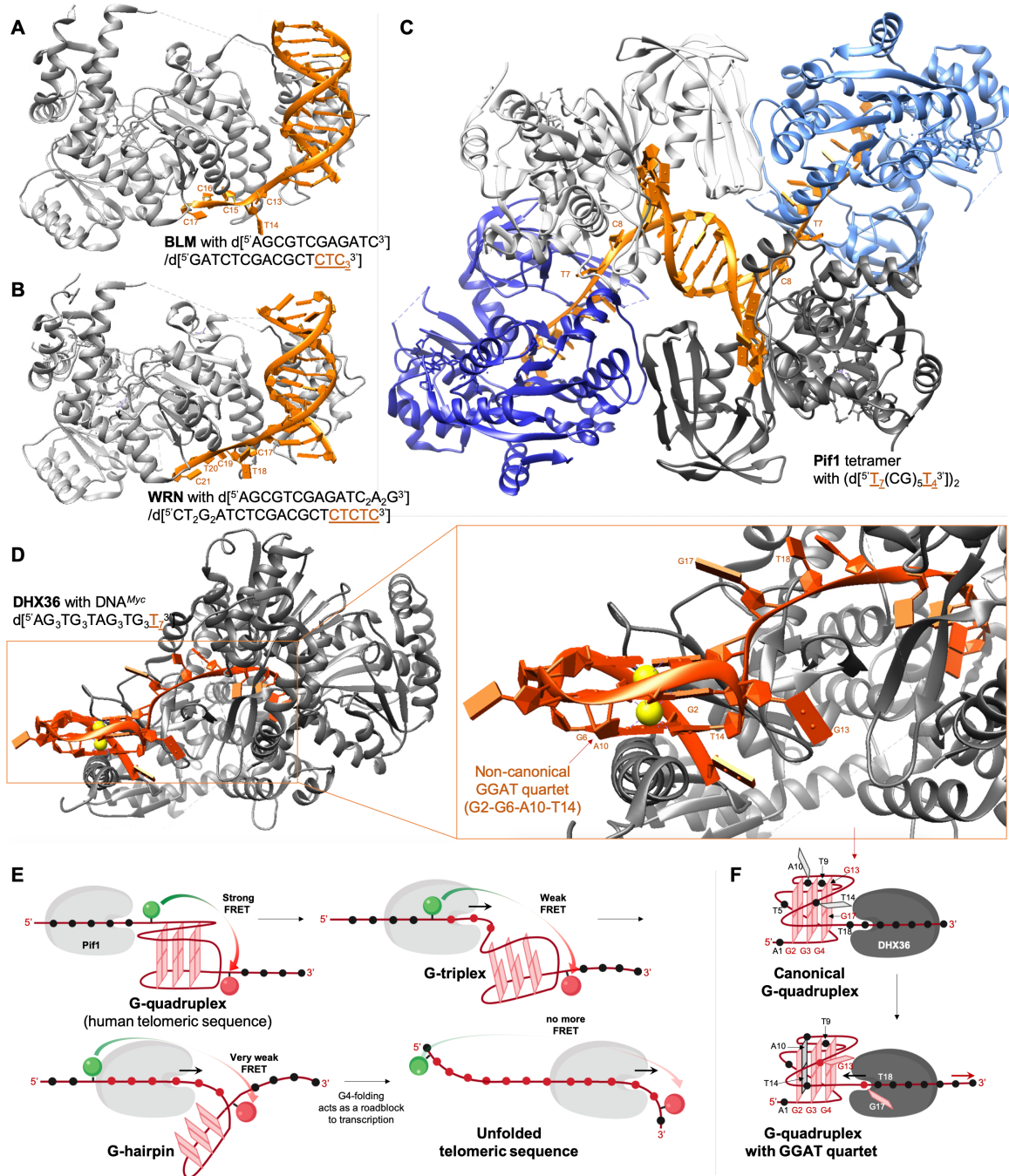


Figure 2. X-ray structures of both BLM (A, PDB ID: 4CGZ)(111) and WRN (B, PDB ID: 4O3M)(112) in complex with a 3'-overhang of a dsDNA, of Pif1 with both 3'- and 5'-overhang of a dsDNA (or forked DNA) (C, PDB ID: 6L3G)(113) and of DHX36 in complex with a 3'-overhang of a G4 (D, PDB ID: 5VHE)(114). The DNA sequences are indicated; nucleotides in orange belong to the overhang. [Created with Chimera] Zakian's and Ferré-d'Amaré's models for G4-helicase-mediated G4-unfolding by Pif1 (E)(115) and DHX36 (F)(114) respectively, based on single-molecule FRET experiments and the X-ray crystallography structure seen in panel D. [Created with BioRender]

* **BLM**: this nuclear helicase(116) comprises 4 main domains: the oligomerizable N-terminus domain, the core helicase domain, the RecQ C-terminal (RQC) domain and the helicase and RNaseD C-terminal (HRDC) domain. The N-terminus domain enables, in the

absence of DNA, the oligomerization of BLM in a hexameric ring structure,(117,118) which regulates and protects the protein before disassembly to provide the active, monomeric BLM helicase.(119,120) BLM binds to 3'-tailed duplex DNA(121) through its core helicase subdomains (PDB ID: 4CGZ, **figure 2A**),(111) which are also responsible for the ATP-dependent 3'->5' helicase activity of BLM on duplex DNA.(122) The HRDC domain governs interaction with ssDNA by specific amino acids (Lys1227, His1236, Tyr1237, Asn1239, Thr1243, Val1244, Leu1246, Asp1264, Gly1265)(123). It is involved in resolving double Holliday junctions (DHJ),(124) particularly, the BLM-topoisomerase III complex(125,126) is known to unwind DHJs implicated in cross-over events during HR.(127) Sgs1, the BLM helicase ortholog in *S. cerevisiae*, was among the first reported as G4-helicases in 1999(128) and has been minutely studied since.(129) A pioneering study by L. H. Hurley showed that the G4-ligand PIPER(130,131) prevents the action of Sgs1 on G4s,(132) demonstrating the direct G4/Sgs1 interaction, as well as the potential use of helicases for screening G4-ligands, which was further developed notably by single-molecule FRET (smFRET) against pyridostatin (PDS) and PhenDC3 for instance.(133) Soon after, the G4-resolving activity of BLM was reported,(91) and shown to be similarly impaired by a G4-ligand BRACO19.(134) The structure specificity of BLM was established by polyacrylamide gel electrophoresis (PAGE) experiments,(92,135) showing its preference for G4s ($K_d = 4$ nM) over DHJ ($K_d = 76$ nM).(136) In this study, the porphyrinic ligand NMM (*N*-methylmesoporphyrin IX) was shown to inhibit the action of BLM, trapping the helicase on its G4 substrate where it consumes ATP without unfolding its substrate. BLM is involved in key DNA damage repair processes (such as DSB resection,(137) HR,(138) DNA repair(139) and the alternative lengthening of telomere (ALT) mechanism)(140) and its deficiency triggers chromosomal fragility, notably *via* elevated sister chromatid exchange (SCE) at G4 sites.(141) Its ability to resolve DHJs (helicase core and HRDC domain)(124) or unwind duplex-DNA (RQC domains) is ATP-dependent,(122) while its G4-resolving activity (helicase core, RQC and HRDC domains) can be performed without ATP hydrolysis.(142) It was suggested that this occurs through the cooperation between the HRDC (which binds the ssDNA) and the RQC domains (which acts as a helicase domain), enabling this unusual ATP-independent G4-unwinding mechanism,(143) although multiple mechanisms have been described.(144)

* WRN: this nuclear helicase was the second member of the super family 2 (SF2) to be discovered in humans.(84) This 3'->5' helicase contains the central seven-motif domain

common to the RecQ family, which catalyzes G4 unwinding, with an exonuclease domain present at the N-terminus.(145) The ability of WRN to unwind G4s *in vitro* was initially described with a peculiar bimolecular G4 that folds from the trinucleotide repeat d(CGG)_n-containing sequence responsible for Fragile X syndrome.(93) The functional difference between BLM and WRN was difficult to disentangle(146) as neither of them unwind blunt-ended DNA and both of them unwind intra-, bi-molecular and multimeric G4s with similar efficiency; however, it was found that WRN unfolds a 12-bp bulged DNA with a 25-fold higher efficiency than BLM.(92,147) A rationale was provided to explain the similarity in G4 binding of these two helicases: crystallographic studies highlighted a common RQC motif, which contains the winged helix motif, found to be critical for duplex DNA-binding. Of particular importance is a conserved serine (Ser1121 for BLM, Ser989 for WRN) that forms a H-bond with the phosphate backbone of the duplex DNA (PDB ID: 3AAF(148) and 4O3M(112) for WRN and BLM, respectively, **figure 2B**) and the quadruplex DNA.(149) Another key motif is the β-wing, which mediated DNA strand separation via two residues at the end of the loop (Asn1164 and Asp1165 for BLM, Phe1037 and Met1038 for WRN) that act as a ‘hairpin scalpel’ to separate the 3' terminal base-pair.(150) This mechanism can be extrapolated to the unwinding of G4, with the opening of the accessible G-quartet by the hairpin scalpel. Studies in which Phe1037 was mutated to Ala confirmed its central role with both duplex (double-stranded DNA, dsDNA) and G4-DNA.(151) For all RecQ helicases, ATP hydrolysis is the driving force required for the processivity of the enzyme along the nucleic acid structure and the RQC domains are responsible for DNA-binding and unwinding activity.

* FANCI: this helicase was discovered for its interaction with the tumor suppressor breast cancer 1 (BRCA1), explaining why it is also referred to as BRCA1 interacting protein C-terminal Helicase 1 (BRIP1) or BRCA1 associated C-terminal helicase (BACH1). A few years later the mutation of the corresponding gene was identified as being responsible for a rare genetic condition known as FA (*vide supra*). This FANCI gene encodes for a 1,249 amino acid-protein comprising eight conserved motifs involved in ATP hydrolysis. *In vitro*, FANCI efficiently unwinds dsDNA and different types of G4s,(95) while the other members of Fe-S family such as XPD and DDX11 are not able to resolve intramolecular G4s.(152) FANCI unwinds G4s in a 5'→3' directionality and can cooperate with the RecQ helicases WRN and BLM to do so *in vitro*.(153,154) Accumulating evidence indicates a G4-resolving activity in cells, since the deficiency of FANCI in FA cell lines(152) and of FANCI homolog Dog1 in *C. elegans*(155,156)

results in deletions at genomic G4 sites. However, the precise mechanism by which FANCD1 unwinds G4s remains to be fully understood: the role of the Fe-S domain (with its four conserved cysteines) is evoked, since mutations of the Fe-S cluster in XPD (on Cys88S and Cys105S residues) results in a loss of helicase activity (on dsDNA) while the ATP hydrolysis activity is maintained.(157) This hypothesis is currently debated as a series of biochemical studies performed with different Fe-S helicases did not support the Fe-S cluster role in G4 resolution and clearly distinguishes FANCD1 from other the Fe-S helicases DDX11 and XPD, which is further substantiated by the observation that treatments with the G4 ligand telomestatin (TMS) triggers DNA damage in FANCD1- but not in DDX11- or XPD-deficient cells.(152)

* RTEL1: RTEL1, which belongs to the DEAH subfamily of SF2,(158) is a 5'→3' helicase(159) with a structural affinity and unwinding mechanism similar to XPD and FANCD1.(160) The N-terminal domain of the helicase comprises a region with four cysteine residues hosting the Fe-S clusters, which has been demonstrated to be dispensable for DNA binding (chemical alteration studies).(160) RTEL1 was originally discovered as the dominant mediator of telomere length regulation in mice,(161) selectively disrupting the telomeric loop (T-loop) formed by the invasion of the G-overhang in double stranded telomeres.(97) The absence of RTEL1 activity in murine embryonic stem cells triggers telomere length dysregulations and chromosome fusions,(161) thus demonstrating that RTEL1 acts on telomeres in a manner reminiscent to WRN(162) or BLM.(163) However, the roles of these helicases at telomeres is not redundant as the regulation of telomere length by RTEL1 is telomerase dependent,(164) while WRN acts through a mechanism referred to as alternative lengthening of telomere (ALT).(162) RTEL1 dysregulation induces limited telomeric damage, decreased the recruitment of protection of telomere 1 (POT1, see section 3b) to telomeres,(165) and an increased rate of sister chromatid exchanges, suggesting a resolving role of the recombination intermediates.(166) In a recent study, RTEL1-depleted cells were associated to an increased R-loop and G4 accumulation.(167) The unwinding ability of RTEL1 is now extended to the whole genome, as a maintenance factor in hard-to-replicate *loci*. The combination of G4 ligand RHPS4(168) with RTEL1 depletion results an increased lethality of RHPS4. Additionally, ATPase-impaired RTEL1 showed an accumulation of R-loop foci, thus further indicating its ATPase/helicase function. As aforementioned, mutation in RTEL1 gene is associated with HHS, a severe form of dyskeratosis congenita. Two mutations were identified,

one in the catalytic core at the ATP-binding site, and another in the C-terminal region believed to affect the interaction of RTEL1 with its putative cellular partners.(98) Polymorphism in the introns of RTEL1 is also associated with brain tumors,(169) and either its upregulation or downregulation have been linked with breast and gastrointestinal cancers.(170-172)

* Pif1: belonging to the SF1 family,(90) Pif1 has a conserved G4 unwinding activity from yeast to humans. Its structure comprises two highly conserved motifs: on one side the helicase domains (seven motifs), which catalyze ATP-dependent G4 unwinding *per se* in the 5'→3' direction; and on the other side a 21 amino acid region which is also involved in activities that require ATP (173) and which is the signature of its family.(174) The first Pif1 to be identified was in *S. cerevisiae* (ScPif1) by genomic investigations in mitochondria.(175) Amongst the large family of Pif1 helicases, ScPif1 and SpPif1 (from *S. pombe*) are the most commonly used models for human Pif1 (hPif1) to investigate their role in managing G4s, owing to the difficulties in expressing and purifying full-length hPif1. One of the first *in vitro* studies performed with purified Pif1 showed its ability to unwind intermolecular G4s by PAGE experiments. Since then, several high-throughput screening methods, based on fluorescence measurements, have been developed and applied with both inter- and intramolecular G4s. By smFRET assays, Pif1 has been shown to unwind G4 more efficiently than dsDNA, however the presence of a G4 at the 5'-end of a G4/dsDNA construct seems to stimulate dsDNA opening.(176) Pif1 does not show preference for a specific G4 conformation, processing parallel c-MYC G4 and antiparallel telomeric G4 at the same rate, however a single-stranded DNA (ssDNA) extension on their 5'-end is required for helicase loading.(177) Pif1 activity was also shown to be impeded by G4 ligands (including PDS, PhenDC3 and BRACO19).(177) ScPif1 and five prokaryotic Pif1 helicases are potent G4 unwinders *in vitro*(178) and prevent genomic instability in yeast.(59) Despite dozens of studies reported, the exact nature of the Pif1/G4 interaction is not yet fully understood. It was proposed that scPif1 is monomeric in solution but dimerizes upon DNA binding.(179) Structural data collected with *Bacteroides sp* Pif1 (BaPif1) in the presence of ssDNA or ATP analogs combined with mutagenesis studies highlight the functional role of a 21 amino acid motif called Pif1's signature domain, and in particular, two amino acids Gly249 and Ala355. These two residues are located in the hinge region between the 2A and 2B domains and their mutation to Pro triggers defective ss-, ds- and G4-DNA-unwinding activities.(180) Recently, the structure of two BaPif1 molecules bound to a forked dsDNA was elucidated with high resolution (3.3Å, PDB ID: 6L3G, **figure 2C**), providing

new insights into the unwinding mechanism. Herein, the fork unfolding occurs *via* the opening of the first base-pair on the 5'-side, induced by geometrical strain, while the other helicases bind to the 3'-side to stabilize the single strand and prevent the displaced strand from refolding.(113) Regarding the G4 unfolding, a model was proposed by Zakian *et al.* on the basis of smFRET results, which comprises step-by-step G4 unfolding involving G-triplex and G-hairpin intermediates (**figure 2E**),(115) whose relevance was previously established by both computational(181) and experimental methods (*e.g.* atomic force microscopy,(182) smFRET(183) and microscale thermophoresis).(184)

* DHX36: in contrast with the previously described helicases, DHX36 unwinds both DNA and RNA. This helicase, also referred to as RNA Helicase associated with AU-rich element (RHAU) or G4-resolvase 1 (G4R1), has a very high affinity for both G4-DNA and G4-RNA, with a strong preference for the parallel G4 topology.(166) DHX36 is a member of the DEAH/RNA helicase A (RHA) subfamily, exhibiting two RecA-like domains.(185) Its N-terminus is characterized by a glycine rich element and a DHX36-specific motif (DSM).(114,186) The crystal structure of drosophila homolog dmDHX36 obtained with different nucleic acid sequences including G-rich single strands shows a preferential binding of potential G4 structure.(187) More recently, the co-crystallization of human DHX36/c-Myc G4-DNA showed that the non-polar planar surface of the DSM binds atop the G4 structure in a manner similar to G4-ligands (PDB ID: 5VHE, **figure 2D**).(188) The previously reported c-Myc crystal structure(189) showed that it folds into a parallel G4 with three G-quartets. However, in the presence of DHX36, its C-terminus forms an extensive H-bond network with the sugar-phosphate DNA backbone, forming a complex containing a G4-DNA with two G-quartets and a non-canonical A-T-G-G quartet at the 5'-end (**figure 2F**).(114) A subsequent rotation of the G4/helicase complex (the C-terminal and RecA domains rotate by 28° and 14°, respectively) is supposed to trigger the G4 unfolding in an ATP-independent manner, a mechanical rotation that is dynamically counteracted by spontaneous G4 refolding. This partial opening is disruptive enough to allow for the hybridization of the complementary strand via Watson-Crick base pairing. G4-RNA unwinding is thought to occur via a different mechanism in which ATP hydrolysis is required.(38,190) The depletion of DHX36 results in the impairment of a wide range of mRNA, which accumulate in stress granules.(191) The activity of DHX36 was also shown to be impeded by G4 ligands (including the PDS and PhenDC3).(192)

G4-helicases are thus key players for controlling G4 dynamics in cells and their catalytic efficiency can be modulated by a series of DNA-binding protein partners including the replication protein A (RPA), POT1, the proliferating cell nuclear antigen (PCNA) and the DNA repair protein REV1 (Table 1). RPA is a ssDNA binding protein (ssBP) described to greatly enhance the DNA unwinding properties of both Fe-S and RecQ helicases, although RPA was also shown to unfold telomeric G4 *in vitro* alone.(193,194) This heterotrimeric protein complex, which comprises 3 subunits commonly referred to as RPA70, RPA32 and RPA14 (70, 32 and 14 kDa, respectively), is a key player in DNA replication, recombination and repair. RPA rapidly binds ssDNA in a non-sequence specific manner *via* the N-terminus part of RPA70, which is also implicated in the interaction with helicases.(195) RPA physically interacts with WRN,(196) BLM,(197) Pif1(198,199) and FANCI(94) and stimulates their helicase activity mostly by recruiting them where needed (*e.g.*, replication forks, DNA damage), coating the unwound ssDNA (thus preventing its refolding) and tethering the helicase during the unwinding process.(195) WRN and BLM also interact with another ssBP, POT1, that does not unfold G4 *per se* but traps the unfolded telomeric sequence(200) although a more active G4 disrupting ability is discussed,(201) notably through a conformational selection.(202) POT1 drives helicases to telomeres to remove telomeric G4s,(203) subsequently ensuring the protection of the unwound 3' ssDNA tail and preventing telomeric losses.(204) Comparatively, POT1 has a higher affinity and a better specificity for telomeres than RPA, and it is still unclear whether POT1 alone or a POT1/RPA association is responsible for facilitating the WRN-mediated telomere replication.(205) PCNA, involved in DNA replication and repair, is a trimeric protein that acts as a “molecular tool belt” wrapping itself around DNA and recruiting other proteins at replication sites. Pif1(199,206) and RTEL1(207) are known to interact efficiently with PCNA through their PCNA interaction motif (PIP Box). This association is crucial to allow for the replication to occur properly through G4-forming sequences, avoiding replication fork stalling or collapse mostly *via* an accurate coordination of the helicase activity with the replisome progression. FANCI also possesses a PIP box involved in both PCNA and REV1 recruitment, the latter being either PCNA-mediated or direct, since this box might also act as a REV1 interacting region (RIR).(208) REV1 is a DNA repair polymerase with a high affinity for G4s,(208,209) which might contribute to G4 melting in a non-enzymatic manner similar to RPA,(210) or allow for DNA replication through the unwound G4 sequence.(211,212)

3c. G4-helicases as biomolecular tools. Beyond their roles in cells, helicases can also be interesting biotechnological tools: the ease with which Pif1 can be fused with tags and expressed in cells has made it a privileged tool for deciphering mechanisms at the cellular level. For instance, labeled Pif1 has found many different applications, among which variants of Pif1 fused with green fluorescent protein (GFP) rank highly. For instance, Zakian *et al.* expressed Pfh1p-GFP (the GFP-labeled ScPif1 homolog) in *S. Pombe* strains for immunoblotting purposes to study the different isoforms of Pfh1p and localize Pfh1p in subcellular compartments by optical imaging (**figure 3a**).⁽²¹³⁾ Pfh1p-GFP was found to accumulate in both nuclei (particularly nucleoli) and mitochondria. Optical imaging was also used to demonstrate the recruitment of Pfh1p at DNA damage through its colocalization with the red fluorescent protein-labeled DNA repair and recombination protein, RFP-Rad22p. Balasubramanian, Jackson *et al.* expressed GFP-hPif1 α (the GFP-labeled nuclear isoform of hPif1) to study the distribution of G4s within the cell nucleus *via* colocalization with the G4-specific small molecule PDS- α , which was tagged *in situ* (once the ligand is at G4 *loci* in human U2OS osteosarcoma cells) with AlexaFluor594 by *in situ click* reaction (**figure 3b**).^(214,215) Jimeno, Huertas *et al.* used the same GFP-hPif1 α to demonstrate the recruitment of hPif α at DNA damage sites (triggered by either the DSB-inducing agents neocarzinostatin and doxycycline, or ionizing radiation), in colocalization studies using an antibody raised against the DNA damage marker γ H2AX (**figure 3c**).⁽²¹⁶⁾ GFP-hPif1 α was also used to demonstrate the association of Pif1 with BRCA1 in cells, *via* co-immunoprecipitation (coIP) with HIS6x-BRCA1 (the recombinant histidine (HIS)-tagged BRCA1) in a binding assay (pull down). In this study, Pif1 was overexpressed to rescue the genetic instability triggered by PDS treatment.⁽²¹⁶⁾ A similar approach was followed by Tsvetkov *et al.* who expressed GFP-Pif1 in neurons⁽²¹⁷⁾ to assess the evolution of the G4 landscape with ageing, showing an increase of G4s with age. In this study, G4 ligand treatment (PDS) was shown to affect neuronal autophagy, and Pif1 was overexpressed to rescue autophagic deficits in cultured neurons exposed to PDS. These results were in line with a previous report in which PDS was shown to accelerate aging of primary rat cortical neurons, triggering G4-mediated transcriptional pausing which impairs mitochondrial functions and disrupts neuronal energetics.⁽²¹⁸⁾ The use of other fluorophore-tagged Pif1 has been reported, according to a classical chemical protein modification strategy: for instance, Dou, Xi *et al.* labeled Pif1p with Cy3-maleimide and the

resulting Cy3-Pif1 was used for *in vitro* studies (smFRET) to gain further insights into the G4 unwinding mechanism of Pif1.(219)

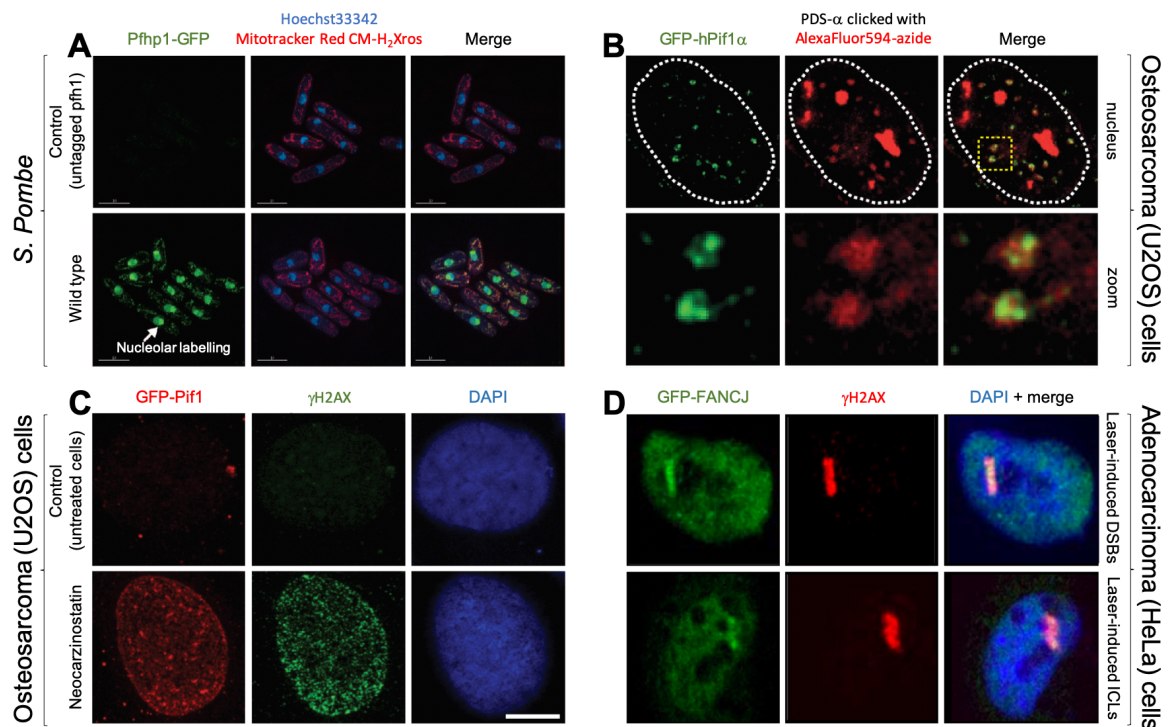


Figure 3. Optical imaging performed with GFP-labeled Pif1 (A-C) and FANCI (D) in yeast (*S. Pombe* strain, A) and human cancer cells (U2OS (B,C) and HeLa cells (D)) showed the subcellular localization of Pif1 (A),(213) the presence of G4s in human cells through the colocalization with both G4-specific small-molecule (PDS- α) and protein (hPif1 α) (B),(214) and demonstrated the recruitment of Pif1 (C)(216) and FANCI (D)(220) to DNA-damage foci.

Various other tags have been fused with Pif1, mostly for purification purposes. For instance, to demonstrate the helicase activity of *S. Pombe* Pfh1p, Zakian *et al.* fused its gene with GST (glutathione S-transferase) and isolated the resulting GST-Pfh1p-h in *S. cerevisiae* on glutathione sepharose.(221) The GST tag, which precludes the helicase activity, could then be removed by thrombin digestion for a final purification by fractionation on heparin sepharose. The same group also fused ScPif1 with Myc protein in order to precipitate the resulting Pif1-Myc construct with anti-Myc antibodies, then used the construct in chromatin immunoprecipitation (ChIP) followed by qPCR(104) or sequencing analyses.(222,223) These sequencing studies demonstrated that Pif1 interacts with G4-forming motifs in relevant cellular contexts and with other cellular components such as the ubiquitin ligase complex member MMS1 and the helicase RRM3. The same construct was recently used by Géli, Corda *et al.* to demonstrate by coIP that Pif1 interacts with RPA to resolve G4s at human

minisatellites CEB1 *loci* in yeasts.(224) Furthermore, Raney *et al.* fused ScPif1 with the TAP tag (for tandem affinity purification, comprising two protein A domains and the calmodulin binding peptide) for affinity purification purposes. Pif1-TAP served as molecular bait to pull down proteins associated with Pif1 *in vivo*, revealing a strong association with the mitochondrial ssBP Rim1. This led to the hypothesis that the Pif1-Rim1 complex could be a key factor in mitochondrial DNA (mtDNA) maintenance, where Rim1 recruits Pif1 into the active replication complex to resolve G4 structures.(225) In a similar approach, Raney, Kim *et al.* fused ScPif1 with hemagglutinin (HA) to perform a coIP (using anti-HA coated beads) with Pif1-HA and the transcriptional activator and G4 binder, Sub1. Again, it was hypothesized that Sub1 could recruit G4-resolving helicases at G4 sites during transcription, thereby suppressing G4-associated genome instability and offering protection against toxicity triggered by a G4 ligand, in this case the porphyrin TMPyP4 (further discussed below, see section 4b).(226)

Pif1 has received a great deal of attention, however other helicases have been genetically engineered and the resulting constructs used to further study their cellular activities. For example, Brosh *et al.* expressed wild type and mutated FANC-J fused with GFP in chicken DT40 cells for coIP investigations, thus identifying BRCA1 as a FANC-J interaction partner, and visualizing the recruitment of FANC-J to DNA damage sites upon exposure to the DNA cross-linking agent mitomycin C or the G4 ligand TMS.(227) This approach was further developed later, in both DT40 and HeLa cells, expanding the panel of FANC-J mutations studied (defective in dimerization, processivity and helicase activity), testing other DNA damaging agents (TMS, DNA crosslinking agent cisplatin, DNA polymerase inhibitor aphidicholin and DSB-inducer bleomycin), and identifying other interaction partners (*e.g.* DNA repair protein MRE11 that recruits FANC-J to DSB sites). The investigators used thorough colocalization investigations (with DNA damage marker γ H2AX, HR marker RAD51 and interstrand cross-link repair marker XPB) (**figure 3d**).(220,228) Overall, genetically engineered helicases have proven to be highly efficient and versatile molecular tools to investigate helicase dynamics and function, as well as G4 landscapes in cells. More recently, a series of G4 probes (G4P) were designed on the basis of the dimerization of the G4-binding domain of DHX36 (a 23-amino acid segment referred to as RHAU23) conjugated to either His/Flag tags (for purification purposes) or NLS sequences (for controlling the subcellular localization of the G4P).(43) As discussed in section 2b, G4Ps enabled the detection of > 120,000 QFS in living cells (70% of which being associated with genes, 60% within 2kb around the TSS) *via* the G4P-

ChIP method, that is, substantially more QFS detected than by G4-ChIP performed with BG4, ascribed to its smaller size (6.7 kDa *versus* >30 kDa for BG4) and the technical difference in protocols implemented (G4P is incubated live while BG4 is added after cell fixation).

4. Chemical biology with G-quadruplexes

4a. How to stabilize G-quadruplexes and why? The notion of chemical biology dates back to a correspondence in 1975 between Linus Pauling and George W. Beadle, although they officially published the term in 1954.(22) Since then, the notion has progressively evolved towards a more general concept in which chemicals are used as “*reversible modulators*” of biological systems “*to gain a deep understanding of cell circuitry and disease biology*”.(229) The latter definition perfectly suits G4s and G4-ligands. Cell-permeable G4-specific small molecules have been used for decades to interact with G4s in cells in the hope of providing mechanistic interpretations. This approach has enabled significant progress beginning in the late 1990s.(230-232) However, most of the reported G4 ligands were intended to be used as drugs,(233-237) rather than chemical biology probes,(20,41,42) bypassing the stage of molecular mechanistic study in order to directly develop novel therapeutics, often in cancer.

From a chemical design point of view, the conformational polymorphism of G4-DNA (see section 2c) might explain why its targeting is challenging. This difficulty is further compounded by the fact that G4 conformation can commonly be ligand-induced. Targeting G4-RNA could be considered simpler for its relative topological simplicity (only forming parallel structures), however this topology lacks well-defined binding sites. Hundreds of G4-ligands have been designed and studied over the past decades,(233-237) with some successful examples (*e.g.* BRACO19,(238-240) PhenDC3,(241-244) PDS(214,244-246) and CX-5461),(244,247) which mostly interact through π -stacking atop the external accessible G-quartet (**Figure 4**). Notable advances have been made in terms of selectivity for the G4 structure over dsDNA, but this field still faces tremendous challenges: next-generation G4-ligands will have to meet expectations in terms of G4-DNA vs G4-RNA specificity,(248-251) and of their ability to discriminate different G4 topologies. This is particularly important given that an overwhelming majority G4 ligands have been designed against a particular subclass of G4s (*e.g.* G4s in promoters or telomeres); however, given the very high number of possible PQFS targets in our genome and transcriptome (see section 2a), the chance of success is limited. Yet, the anticancer properties of G4 ligands are undoubtedly established. The mechanisms by which

they exert this activity remain to be deciphered. The link between a global antiproliferative effect observed on ligand treatment and the cellular processes involving the G4s for which an *in vitro* affinity has been determined (*e.g.* G4-forming sequences from promoters or telomeres in solution) is often hastily established. As G4s are intimately linked to genetic transactions (see section 2b), G4 ligands can stabilize physically barriers to polymerases in general, triggering DNA damage (**Figure 4**).^(252,253) It may be more elegant to consider G4s as genetic switches with specific spatiotemporal control over key cellular processes, compared to considering G4s as genome-wide triggers for creating DNA damage, however this latter, simpler rationale offers a mechanistic model that accounts for most of the cell-based studies collected so far. Therefore, in light of the propensity of G4-stabilising ligands to trigger genetic instability, and the association with severe genetic dysregulations (see section 2c), it is surprising that, to date, most if not all efforts by chemical biologists have been focused on G4 stabilization rather than destabilization. This may stem from the difficulty in designing small molecules able to mimic G4 helicases.

4b. How to destabilize G-quadruplexes and why? The quest for molecules that could unwind G4s and rescue helicase deficiency is a yet emerging but highly promising topic.⁽²⁵⁴⁾ This research is technically impeded by the lack of standard assays and protocols to reliably assess G4 destabilization, in sharp contrast to the stabilization of G4s (see section 2c).

To date, only a handful of compounds have been described with such a property (**figure 4**), some of them being a subject of debate (*vide infra*). However, there is no consensus on the cellular relevance of these compounds as their unwinding capabilities were assessed *in vitro* with assays tailored for a single study only. Beyond established techniques of nucleic acid denaturation applied to G4s, including heating (see above) and urea,⁽²⁵⁵⁾ chemicals such as metallic ions (*e.g.*, copper)⁽²⁵⁶⁾ and complexes^(257,258) as well as natural compounds (*e.g.*, polyamines such as spermine) have been used in large excess as a way to study the dynamics of G4 folding and unfolding. The systematic analysis of the G4-unwinding agents really started with TMPyP4 (**figure 4B**), a ligand that has been thoroughly used for stabilizing G4s,^(259,260) owing to its large aromatic structure that overlaps efficiently the external G-quartet of a G4 while its positively charged pyridinium arms interact with the negatively charged G4 grooves.⁽²⁶¹⁾ For instance, TMPyP4 was used in the seminal study on the genetic expression regulation of the *c-myc* oncogene *via* stabilization of the G4 found in its promoter region.⁽²⁶²⁾

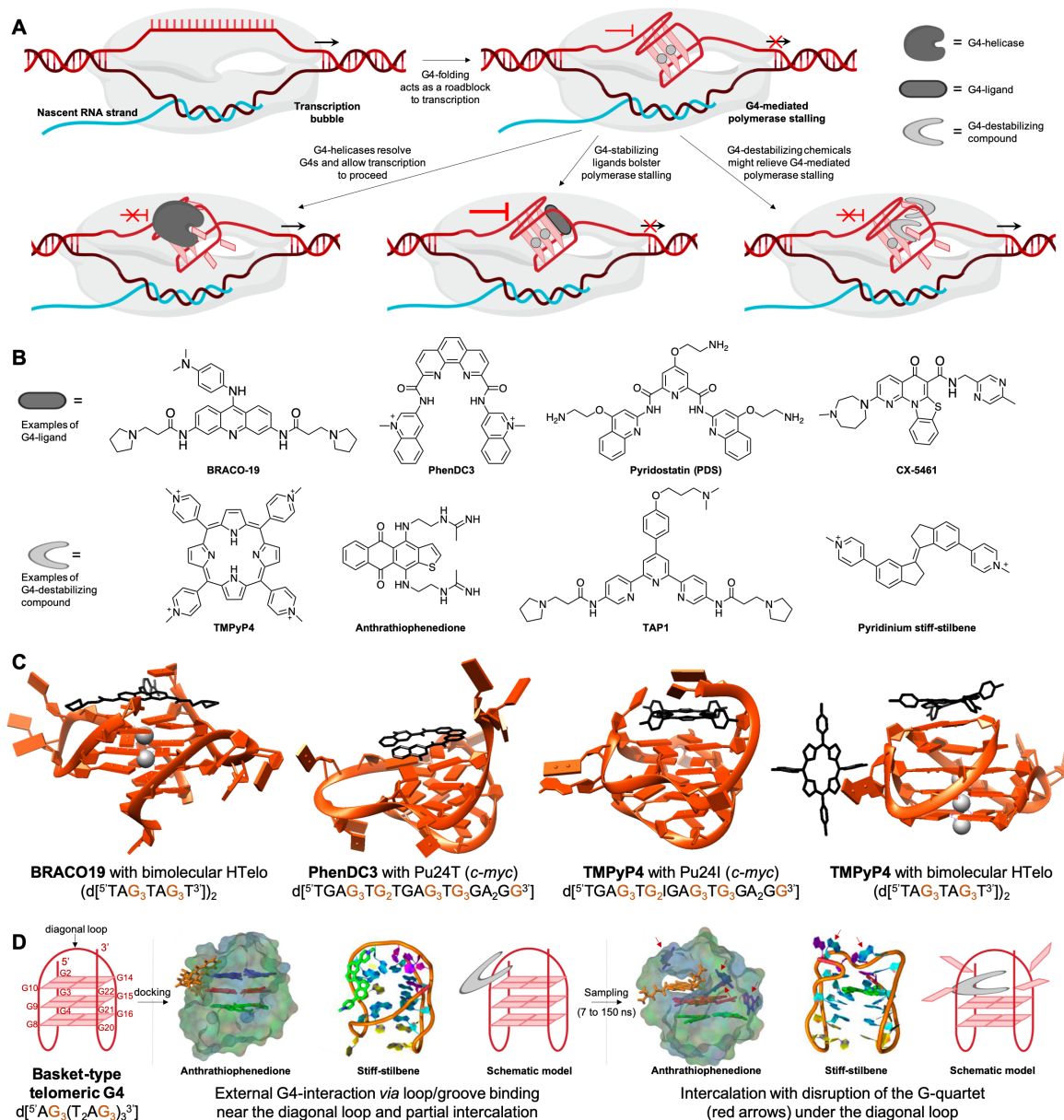


Figure 4. Schematic representation (A) of the G4-mediated stalling of the RNA polymerase during transcription along with the rescue pathway by the action of G4-helicases (left), a possible rescue pathway by the action of G4-destabilizing compounds (right), and, conversely, reinforcement of the crisis situation by G4-ligands (center). [Created with BioRender] Chemical structures (B) of representative G4-ligands (upper row, *i.e.* BRACO-19,(238) PhenDC3,(241) PDS(245) and CX-5461)(247) and of G4-destabilizing compounds (lower row, *i.e.* TMPyP4,(263) anthrathiophenedione,(264) TAP1(265) and stiff-stilbene).(266) Structures (C) of G4-ligand/G4 complexes obtained with BRACO19 in interaction with a bimolecular human telomeric (HTelo) G4 (PDB ID: 3CE5),(267) PhenDC3 in interaction with Pu24T (from *c-myc* promoter, PDB ID: 2MGN),(268) and TMPyP4 in interaction with both Pu24I (from *c-myc* promoter, PDB ID: 2A5R)(261) and the bimolecular HTelo (PDB ID: 2HRI),(269) from either X-ray (HTelo) or NMR investigations (*c-myc*). [Created with Chimera.] (D) Kaluzhny's and Galan's molecular dynamics models for small molecule-mediated G4-unfolding performed with the telomeric G4 (PDB ID: 143D)(270) and either the anthrathiophenedione(264) or stiff-stilbene.(266) [Created with BioRender]

The use of TMPyP4 as a G4-stabilizer has grown rapidly, despite a substantial body of research describing its ability to interact with other nucleic acid structures(271-274) including

duplex-DNA,(275,276) branched DNA,(277) G4s,(259) and even G4 loops (**figure 4C**).(269) This situation was complicated by a series of reports describing the G4-destabilizing properties of TMPyP4. Fry *et al.* reported on the destabilization of both DNA and RNA G4s that fold from (CGG)_n trinucleotide repeats involved in the Fragile X syndrome (localized in the first exon of the FMR1 gene and the 5'-UTR of the FMR1 mRNA, respectively).(278,279) These studies relied on a combination of *in vitro* (*e.g.* PAGE, melting assays)(278) and cell-based investigations (*e.g.* dual luciferase assay).(279) They concurred in demonstrating that TMPyP4 unfolds (CGG)_n G4s, but not telomeric G4s, in a manner reminiscent of the heterogeneous nuclear ribonucleoprotein CArG-binding factor A (CBF-A) known to unfold G4s, and enhance the translation efficiency by removing the G4 roadblock. Importantly, it was shown that only TMPyP4 was able to unfold G4s, and not its positional isomers TMPyP2 and TMPyP3. These results were contradicted by Kaluzhny *et al.* who reported the ability of TMPyP3 to unfold telomeric G4,(280) *via* G4 melting ($\Delta T_m = -8$ °C) and CD profiles (-30% of molar ellipticity at 296 nm) at low G4:ligand ratio (between 1:2 and 1:3). The former conclusion was however concurred by three independent studies: Hartig *et al.* showed that TMPyP4 efficiently unfolds the thrombin binding aptamer G4 *via* UV-melting and CD studies (almost complete disappearance of the CD signal at 295 nm at 1:4 G4:ligand ratio), thus hampering the anticoagulant protein/DNA interaction, but not TMPyP2;(281) Basu *et al.* demonstrated that TMPyP4 unwinds G4-RNA that folds from the 5'-UTR of MT3-MMP mRNA (r[⁵GAG₃AG₃AG₃AGAG₃A³], by CD (complete disappearance of the CD signal at 263 nm at 1:25 G4:ligand ratio), PAGE (performed at 1:200 G4:ligand ratio) and NMR analyses (at low G4:ligand ratio (1:1.4) but elevated concentrations (430 and 600 μ M of RNA and TMPyP4, respectively));(282) Pearson *et al.* showed that TMPyP4 distorts G4-RNA that folds from (GGGGCC)_n hexanucleotide repeats found in the chromosome 9 (C9ORF72 locus) and involved in both amyotrophic lateral sclerosis and frontotemporal dementia (ALS/FTD).(263) To do so, the authors performed PAGE (at >1:200 G4:ligand ratio) and CD analyses (at 1:10 G4:ligand ratio), later completing their studies with isothermal titration calorimetry (ITC, up to 1:40 G4:ligand ratio).(283) The authors provide initial mechanistic propositions that TMPyP4 can bind either atop the external G-quartet (strong, entropically-driven binding event) or intercalated between two G-quartets (weak, enthalpically-driven binding event), which results in G4 distortion. While the intercalation is debated, several structural analyses show that the porphyrin binds to external G-quartets along with nucleobases in the loops and grooves in a

diverse manner.(261,269) In light of the unspecific high affinity of TMPyP4 for DNA, it can be postulated that it unfolds G4 by interacting with and stabilizing the resulting ssDNA, intercalating in between adjacent nucleobases, since most of these results were collected at high to very high G4:ligand ratio (up to 1:200). However, no accurate structural information is available yet to support this model. Finally, to add an additional layer of complexity, it should be noted that *i-* the ability of TMPyP4 to stabilize or disrupt G4 structure can be modulated by the presence and nature of a metal within the porphyrin core,(284) and *ii-* the protocols and techniques used strongly influence the outcomes of the porphyrin/G4 interaction studies.(285)

Kaluzhny *et al.* also reported on an anthrathiophedione derivative (**figure 4B**) that similarly displays a higher affinity for randomly coiled DNA than for G4,(264) presumably *via* partial intercalation between adjacent nucleobases. This results in the unfolding of telomeric G4 notably monitored by the decrease of CD signal at 295 nm (at 1:7 G4:DNA ratio, in Na⁺-rich but not in K⁺-rich conditions). A working hypothesis, supported by molecular dynamics (DOCK 6.4 and Amber 8) performed with the NMR structure of the basket-type telomeric G4 (PDB ID: 143D, **figure 4D**),(270) is that the G4-disruption occurs in two steps: first, an external interaction of the anthrathiophedione with the loop; then, the G4-disruption *via* G-quartet interaction. The partial intercalation at the external G-quartet, and thus its partial distortion, mechanically lengthens the loops, which in turn destabilizes the resulting long-looped, two-quartet G4 and provides a greater access to ssDNA that the ligand can bind to, therefore accelerating the global G4 unfolding.

Another interesting example of G4 unfolders is a triaryl pyridine 1 (or TAP1, **figure 4B**) reported by Balasubramanian *et al.* as being able to disrupt the G4-DNA found in the c-kit promoter, which consequently results in an enhancement of the expression of this oncogene in human gastric cancer HGC-27 cells.(265) The TAP1 activity was investigated *in vitro via* CD (almost complete disappearance of the CD signal at 260 nm at 1:10 G4:ligand ratio) and NMR titrations (at low G4:ligand ratio (1:2) and elevated concentrations (190 and 380 μM of DNA and TMPyP4, respectively)). The properties of this compound, which belongs to a family of 20 TAP analogs initially investigated for their G4-stabilizing properties,(286) were further studied by atomic force microscopy (AFM).(287) It was demonstrated that a high concentration of TAP1 (500 μM) prevents G4 formation in the linearized pPH600 plasmid (-75% of G4 occurrence, with a half-maximal inhibition concentration (IC₅₀) of 100 μM TAP1). Once again

however, no structural rationale was provided to explain the G4-destabilizing properties of TAP1.

More recently, Galan *et al.* reported on the design and use of a stiff-stilbene ligand (**figure 4B**) able to regulate the G4 stabilization/unfolding in a photoresponsive manner.(266) This bispyridinium compound efficiently stabilizes telomeric G4s in K⁺-rich conditions (assessed by FRET-melting assay, with $\Delta T_m = 21^\circ\text{C}$), and inversely triggers the unfolding of the same G4 in Na⁺-rich conditions, presumably *via* a combination of groove binding and intercalation, as modelled by molecular dynamics simulations.(266) This compound can be photodegraded upon irradiation (20 min at 400 nm), thus enabling reversible folding and unfolding of the telomeric G4 upon repetitive cycles of addition of ligand (unfolding, at 1:10 G4:ligand ratio) and photoirradiation (refolding), assessed by CD (at 273 and 340 nm) and NMR signatures (in the 10 - 12 ppm region). Similar to the anthrathiophedione above, molecular dynamics (AutoDock, molecular dynamics (MD) and metadynamics (WTMetaD) simulations)(288,289) were performed with the NMR structure of the basket-type telomeric G4 (PDB ID: 143D, **figure 4D**);(270) the resulting model suggests a complex G4-disruption pathway involving multiple intermediates and binding/unbinding events that lead to G4 unfolding presumably through groove binding (kinetic event) followed by the intercalation of the ligand within the G4 structure, in which it acts as a lever that opens the G4 backbone.

Several derivatives were later developed,(290) and studied for their G4-stabilising properties, along with their antiproliferative activity against *T. brucei* parasite and HeLa cancer cells. NMR titrations confirmed the ability of bispyridinium stiff-stilbenes to disrupt the external G-quartet upon binding (1:2 G4:ligand ratio, in K⁺-conditions), which was also reported with a bispyridinium dithienylethene (DTE) derivative (at 1:3.6 G4:ligand ratio in both K⁺ and Na⁺). DTE can be reversibly photoswitched ($\lambda_{\text{ex}} = 450/635 \text{ nm}$) between a 'closed' and an 'open' conformation, the former behaving as a classical G4 external stacker, while the latter disrupts the external G-quartet through partial intercalation.(291)

Summary & outlook

Over twenty years of research have enabled us to attain our current molecular understanding of the roles that G4s play in human cells and diseases. The functional relevance of G4s has progressed at a pace dictated by the advances in chemical biology methodology. As the field currently stands, scientists are able to foster G4 cellular effects using G4-stabilizing

compounds (such as through inflicting severe damage on the cancer cell genome, thus inducing lethal genetic aberrations in anticancer strategies), as well as alleviate G4-mediated cellular processes using G4-resolving molecular tools, one of the key challenges on the way to providing those eagerly awaited therapeutics to fight neurological disorders and age-related diseases. The genetic diseases listed in Table 1 are currently suffering from severe drug attrition: most of the treatments applied so far are either protective (*e.g.*, protection from sunlight, against aggressive infections for Bloom's syndrome patients)(292) or prospective (*e.g.*, the targeting of p38 MAPK signaling pathway in Werner Syndrome-derived cells),(293) but none of them tackle the molecular origins of the disease. We do not propose to predict which direction should be followed within G4 research. However, we summarize here the knowledge and tools now available to enable further investigations into G4-unwinding. We described the two types of G4-unwinders that are currently being investigated: the G4-helicases on one hand, which are increasingly used as biomolecular tools mostly for optical imaging but also to reverse detrimental G4-mediated cellular effects; and small molecule G4-destabilizers on the other hand, with a particular focus on the current issues that must be addressed to make small molecules reliable candidates for manipulating G4s in cells. To date, these are mostly technical issues (*e.g.* the lack of standard assays to assess G4-destabilization *in vitro*)(294) but the recent results described here leave no doubts as to the forthcoming development of such promising molecular tools, which will find application ranging from fundamental research tools to potential new genetic disease therapy.

Significance

Small molecule-mediated G-quadruplex (G4)-stabilization has been the privileged chemical biology approach to gain insights into G4 cellular biology. An alternative approach is now emerging, relying on the use of molecular tools able to unfold G4s, which shed new light on G4 regulatory roles. In this review, we describe in detail the different strategies implemented to unwind G4s (using either unfolding proteins, known as G4-helicases, or G4-disrupting small-molecules), with all their strengths and weaknesses, and what could be the therapeutic consequences of manipulating G4 in cells to treat genetic diseases.

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Author contributions

P.L., J.M., F.R.S. and D.M. wrote the manuscript.

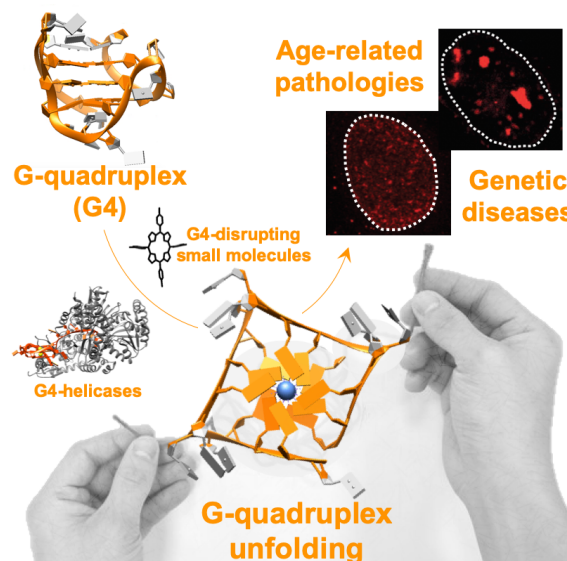
Declaration of interests.

The authors declare no competing interests.

Highlights

- G-quadruplexes (G4s) are pervasive in human genome and transcriptome
- Enzymes referred to as G4-helicases are known to unfold G4s in cells
- G4-helicases can be used as molecular tools to manipulate G4s in cells
- G4-stabilizing small molecules (G4-ligands) are currently studied as anticancer agents
- G4-disrupting molecules are new molecular tools with high therapeutic potential

Graphical abstract



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