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Non-B DNA structures as a booster of genome instability

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ABSTRACT

Non-canonical secondary structures (NCSs) are alternative nucleic acid structures that differ from the canonical B-DNA conformation. NCSs often occur in repetitive DNA sequences and can adopt different conformations depending on the sequence. The majority of these structures form in the context of physiological processes, such as transcription-associated R-loops, G4s, as well as hairpins and slipped-strand DNA, whose formation can be dependent on DNA replication. It is therefore not surprising that NCSs play important roles in the regulation of key biological processes. In the last years, increasing published data have supported their biological role thanks to genome-wide studies and the development of bioinformatic prediction tools. Data have also highlighted the pathological role of these secondary structures. Indeed, the alteration or stabilization of NCSs can cause the impairment of transcription and DNA replication, modification in chromatin structure and DNA damage. These events lead to a wide range of recombination events, deletions, mutations and chromosomal aberrations, well-known hallmarks of genome instability which are strongly associated with human diseases. In this review, we summarize molecular processes through which NCSs trigger genome instability, with a focus on G-quadruplex, i-motif, R-loop, Z-DNA, hairpin, cruciform and multi-stranded structures known as triplexes.

Contents

1. Introduction

Non-canonical secondary structures (NCSs) include a variety of

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Abbreviations

NCSs	Non-canonical secondary structures
G4s	G-quadruplexes
PrimPol	Primase-Polymerase
HRR	Homologous recombination repair
DSBs	DNA double strand breaks
PDS	Pyridostatin
CPT	Camptothecin
TLS	Translesion synthesis
UFBs	Ultra-fine bridges
NER	Nucleotide excision repair
RNAPII	RNA Polymerase II
RTCs	Replication-transcription conflicts
HO	Head-on
CD	Co-directional
SWNTs	single-walled carbon nanotube
R	Polypurine strand
Y	Polypyrimidine strand
TFO	Triplex forming oligonucleotide

nucleic acid structures that do not rely simply on the canonical Watson-Crick base pairing and strand complementarity. Nucleic acids can therefore adopt alternative conformations other than the classical B-DNA double helix structure. NCSs commonly form at repeated DNA regions and their stability and structural diversity can be influenced by various factors, including the nucleotide sequence. For example, inverted repeats can form hairpin or cruciform structures while mirror repeats tend to form triplexes. In addition, guanine-rich sequences can promote the formation of G-quadruplexes (G4s) and/or R-loops (Fig. 1A and B), whereas cytosine-rich sequences may form four-stranded structures, known as i-motifs. Despite these differences, the high similarity of NCSs forming sequences may allow the folding of two or more structures in the same DNA region, which may coexist or compete with each other. This is the case of G4s and R-loops, which, when located on the opposite strands of the same DNA region, form the so-called G-loop structures (Fig. 1C) [1]. Given the complementarity of their forming sequences, also G4s and i-motifs can also fold simultaneously on opposite strands or be mutually exclusive [2,3]. Experimental and computational studies have shown that NCSs can form in gene regulatory DNA regions, such as promoters, untranslated regions, telomeres, and replication initiation zones, proving their involvement in transcription, replication, recombination and other biological mechanisms [4,5]. The participation of non-B DNA structures in biological processes requires a finely tuning of their formation, removal and localization. Indeed, the failure of these control mechanisms leads to the dysregulation of cellular functions, resulting in genome instability. NCS-mediated genome instability is mainly associated with the impairment of DNA replication as all non-canonical structures can interfere with the ongoing replication fork and even when cells engage specialized polymerases to resume replication, the process is error-prone [6,7]. The outcomes of such interference are diverse and range from fork collapse and DNA damage to expansions and deletions of DNA portions as well as under-replication of DNA regions [7]. However, NCSs can also interfere with transcription by blocking ongoing RNA polymerase II (RNAPII) or by altering gene expression and can be targeted by endogenous nucleases or error-prone repair enzymes causing further instability [8,9]. Therefore, the dynamic equilibrium between canonical B-DNA and non-B DNA structures plays an important role in the regulation of cellular processes. Disruption of this equilibrium can have severe consequences leading to genome instability and, consequently, to several human diseases such as cancer [9–11].

In this regard, G4s have been found to accumulate at the L1 retrotransposon element in Alzheimer's disease [12], while mutations in RNaseH2 and ADAR1 (R-loops and Z-DNA regulatory enzymes, respectively) cause Aicardi-Goutieres syndrome [13,14]. Hairpin structures, as well as R-loops and triplexes, have been also linked to trinucleotide repeat expansions, largely associated with neurological diseases [9] and all of these structures have been found to increase mutational burden and to dysregulate cancer-related genes thus contributing to cancer onset [15]. A deeper insight into the biology of non-canonical structures and their impact on genome instability is, therefore, extremely important to develop therapeutic strategies to face such diseases. In recent years, research in this field has focused on the development of small molecules and ligands that can selectively recognize these non-canonical structures, making them potential therapeutic targets for several pathologies, including cancer. In addition, several studies have attempted to understand how these structures can be induced/stabilized as a way of further triggering genome instability. Indeed, the success of anti-cancer strategies usually relay on their ability to induce DNA damage, replication fork stalling or mitotic mis-segregation which in turn activate various responses, such as inhibition of cell proliferation and induction of cell death. Interestingly, recent discoveries have also established the existence of a complex crosstalk between genome instability and the innate immune system [16] and have pointed out how genome instability induced by some NCSs stimulates an anti-cancer immune response with interesting perspectives in the field of immunotherapy and combinational therapy. For example, G4 binders and Top1 poisons, which stabilize G4s and modulate R-loops amounts respectively, induce the formation of micronuclei, well-known markers of genome instability, which activate innate immune gene pathways in several cancer cells [17–19]. In this review, we describe the structural features of NCSs focusing on mutations, deletions, and other gross chromosomal rearrangements that occur as a consequence of their altered homeostasis during the main biological processes. In particular, we discuss the current state of knowledge

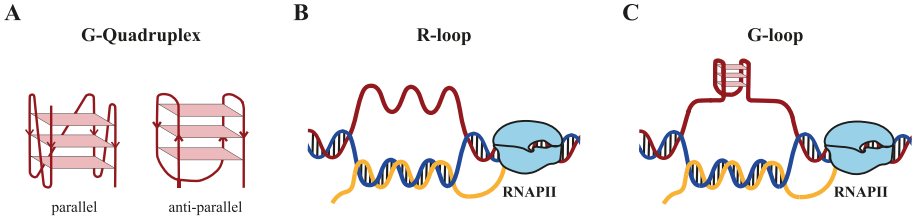

Fig. 1. Schematic representation of G4, R-loop and G-loop structures. **(A)** Two different conformations that G4s can adopt according to strand direction. **(B)** R-loop structure consists in an RNA strand annealed to its DNA template forming a hybrid duplex and a displaced non-template DNA strand. **(C)** G4 folding on the displaced DNA strand of R-loop results in a secondary structure known as G-loop. Created with Biorender.com.

on the molecular mechanisms of genetic instability induced by these structures and how this instability causes human diseases.

2. Non-canonical secondary structures and related genome instability

2.1. G-quadruplexes

G4s are currently one of the most abundant and studied non-B DNA structures involved in the regulation of various cellular processes. G4s are four-stranded nucleic acid structures that can originate from either DNA or RNA G-rich regions and are constituted by stacked guanine tetrads held together by Hoogsteen hydrogen bonds further stabilized by monovalent cations (K^+ or Na^+) coordinated in or between the G-tetrads. G4s can adopt different conformations depending on various factors such as strand stoichiometry, direction, and intervening nucleotide length [20,21] (Fig. 1A). Experimental and bioinformatic data have shown that the number of the canonical consensus of potential G4-forming sequences (PQSs) in the human genome is between 300,000 and 1.5 million [22,23]. According to computational and genome-wide studies, G4s are preferentially located at gene regulatory regions, such as promoters, enhancers, untranslated exon regions, telomeric DNA, immunoglobulin sites and recombination hot-spots [24]. Interestingly, G4s have been found to be particularly enriched in the promoter region of oncogenes such as c-*Myc*, *c-KIT*, *BCL2*, *KRAS*, *VEGF* and *SRC* [25], indicating a strong association of these structures with cancer. Given their abundance and their specific localization across the human genome, G4s play a critical role in the regulation of many biological processes such as replication initiation, telomere maintenance, DNA damage repair, high order chromatin organization and gene expression regulation [26–28]. As other non-B DNA structures, G4s can form in nucleosome-free DNA regions influencing the occupancy and positioning of nucleosomes in chromatin [4,26,29], further indicating that G4s play a key role in gene regulation and transcriptional control. In some cases, G4s can act as a platform for the binding of specific transcription factors and chromatin regulatory proteins, helping to maintain chromatin in an open state and facilitating gene expression [30,31]. Conversely, changes in the chromatin landscape can affect the folding and distribution of G4s across the genome. For example, methylation of the guanine residues in G4-forming sequences can modulate G4 formation and subsequently affect the binding of G4 interacting proteins [32]. In addition, chromatin remodeling can modulate pre-existing and de novo G4s formation. Interestingly, induced chromatin relaxation in HaCaT cells modulates G4s particularly in regulatory regions associated with high levels of transcription, indicating a positive correlation between G4s folding and transcriptional activity and pointing out that G4 structures may play a role in epigenetically mark the genome [26]. Given their high relevance in regulating key biological processes, G4 status is a critical point that can affect both physiological and pathological conditions. Indeed, during transcription and replication processes, negative supercoiling can facilitate the formation of stable G4s [33] which, if not promptly resolved by dedicated helicases or stabilized by G4-ligands, can represent a steric obstacle to RNA and DNA polymerase progression. The persistent blockage of these processes causes a general stress that can result in the generation of double strand breaks (DSBs), mutations, increased recombination events, chromosome aberrations and micronuclei formation. Accordingly, the loss of G4-related helicases, such as BLM and FANCJ, which are usually involved in the control of G4 homeostasis, leads to the development of pathologies characterized by G4-mediated high recombination events and DNA damage [34,35].

2.1.1. G-quadruplex related genome instability

Transcription-dependent instability induced by G4s has been shown to occur in yeast due to the high transcriptional rate at PQSs in the immunoglobulin Sμ region [36]. Furthermore, *in vivo* G4 stabilization by using different small molecules has been shown to trigger the G4-mediated transcriptional reprogramming of the BU-1 locus in DT-40 cells in a replication-dependent manner [37]. More in detail, delayed G4 processing mediated by specific helicases in the BU-1 locus can arrest DNA polymerase progression at the leading strand causing replisome uncoupling and an impairment of histone recycling/nucleosome reassembly. This modified pattern of histone modifications is epigenetically inherited across cellular divisions and results in a permanent alteration of *BU-1* gene expression [37]. Stabilization of G4s by using small ligands is known to induce deleterious effects on genome stability, including DNA damage. In this context, by generating the first genome-wide G4 map, Rodriguez et al. revealed that treatment with the G4 ligand Pyridostatin (PDS) caused DNA damage in chromosomal regions containing a high number of G4-forming sites in a manner dependent on transcription and replication [38]. Interestingly, DNA damage elicited by PDS at G4 sites occurred in some of the G4-associated oncogenes such as *c-Myc* and *SRC* resulting in the downregulation of these genes [38]. This is consistent with other published data showing that TMPyP4-mediated G4 stabilization inhibits transcription initiation by impairing transcription factors loadings at promoters and decreasing RNA polymerase II occupancy [39]. Hence, these findings provide evidence that the dysregulation of G4 structures can interfere with the normal functioning of the transcriptional machinery affecting genome stability and the expression levels of various genes, including disease-related genes [40]. Indeed, G4s has been associated with several pathologies, including cancer and neurodegenerative diseases [26,41] and published data converge on G4s as potentially effective pharmacological targets for DNA-targeted therapies, particularly in anticancer drug design. In particular, it has been shown that G4s are specifically enriched in the promoter region of oncogenes and are associated with somatic copy number amplification and structural variants in several cancer cells highlighting the role of these structures in cancer progression and in cancer-mediated genome instability [42,43]. It has been also demonstrated that actively formed G4s, rather than the G4 motif sequence, contribute to the generation of somatic structural variants (SVs) in cancers [42]. Moreover, SVs breakpoints related to G4s are associated with active chromatin markers and are enriched in regulatory regions of the genome, such as early replication origins and TAD boundaries [42], suggesting that G4-mediated impairment of chromatin organization, transcription, or DNA replication, may result in somatic breakpoints contributing to genome instability of cancer cells. The fact that the relationship between G4s and somatic structural variants is cancer type specific and can be modified by multiple factors, including genomic and epigenomic signatures and chromatin structures [26,42,43], suggests that G4s can be used as genomic markers for the prediction of somatic breakpoints in cancer.

The impairment of DNA replication due to G4s alteration/stabilization has been associated with improperly replicated DNA and DNA damage, which can promote mutagenesis (e.g., recombination, mutations and deletions), loss of genetic information and micronuclei formation strongly affecting the genome integrity. During replication, G4 folding negatively interferes with DNA synthesis of both lagging and leading strands [44]. This has been largely demonstrated with *in vitro* experiments showing that various DNA polymerases, involved in both DNA replication and repair, fail to bypass the G4 barrier [45]. For this reason, several helicases or nucleases are involved in the *in vivo* resolution of G4s during DNA replication, removing structural barriers and avoiding the stop of

DNA synthesis [18]. In *Saccharomyces cerevisiae* the PIF1 helicase is fundamental to maintain genome stability by preventing G4-mediated replication fork stalling and DNA breakage [46,47]. The BLM helicase has a role both in preventing G4-mediated replication stress, especially at telomere region [48,49] and in ensuring faithful chromosome segregation in human cells. A model of BLM helicase activity in decatenating sister chromatid bridges during anaphase has been shown to prevent micronuclei formation, chromosome aberrations or other mitotic-related defects that lead to genome instability [50,51]. Furthermore, Lee and co-workers have shown that the FANCJ helicase, in combination with RPA protein, processes G4-associated replication forks ensuring a proper replication stress response to promote ATR-mediated replication fork restart, thereby guaranteeing genome stability [52]. In *Caenorhabditis elegans,* cells lacking *dog1*, FANCJ helicase ortholog, accumulate site-specific genome deletions at G-C rich regions, including in predicted G4 motifs [53]. In addition, G4-associated helicases can recruit alternative DNA polymerases, such as REV1 and Pol ζ [54], to replicate G4-containing templates through a translesion synthesis (TLS) mechanism (Fig. 2A). TLS is a mechanism of DNA damage tolerance pathway that allows replication forks to overcome template obstacles, ensuring DNA replication progression. Among TLS polymerases, Polymerase η has been largely associated with G4s [55,56] and its activity, especially at common fragile sites, prevents replication perturbations at G4-forming sites. These perturbations have been associated with incompletely replicated DNA in G2/M, that can result in Ultra-Fine DNA bridges (UFBs) formation. If left unresolved, these structures can give rise to micronuclei containing fragile-site sequences [57]. DNA synthesis repriming is another mechanism that cells employ to counteract the G4 barrier through an obstacle-bypass mechanism. Schiavone et al. demonstrated that the DNA primase and DNA polymerase PrimPol is involved in this mechanism as it catalyses de novo synthesis of RNA primer, promoting the restart of DNA synthesis downstream of G4 replication impediment on the leading strand [58] (Fig. 2B). Consequently, it leaves un-replicated single-stranded DNA (ssDNA) gaps which must be filled by post-replicative mechanisms. Loss of PrimPol in human cells causes defects in replication fork progression and restart, increase in sister chromatid exchanges, mutagenesis and micronuclei formation [59,60]. By contrast, PrimPol repriming likely leads to increased genomic instability and DNA damage when PrimPol related ssDNA gaps cannot be properly repaired. Indeed, when left unrepaired, ssDNA gaps can collapse into DSBs in subsequent cell divisions [61]. Members of TLS and homologous recombination (HR) repair mechanisms mediate ssDNA gap filling depending on the cell phase [62], and loss of these factors significantly affects cell survival and increases genome instability and DNA damage [63]. In particular, BRCA1/2-deficient cells accumulate ssDNA gaps and spontaneous mutations due to the repriming of PrimPol [62,64] (Fig. 2B). G4-mediated DSBs may be formed through several mechanisms, previously reviewed in Miglietta et al., 2020, and different repair pathways are involved in their resolution. G4-induced DSBs can be processed by Polymerase θ-mediated alternative end joining, an intrinsically mutagenic mechanism that causes insertions or deletions at DNA damaged regions related to G4s, thereby contributing to the induction of genome instability [61]. DSBs can also be repaired by a HR-mediated mechanism using an intact homologous DNA sequence. However, HR-mediated repair can generate unresolved DNA intermediates which can lead to anaphase bridges and UFBs formation, potentially causing genome instability [65]. HR factors, such as BRCA1 and BRCA2, have also a role in protecting stalled replication forks from nuclease degradation [66,67], thus promoting the restart of replication and driving HR repair (Fig. 2B). This is in line with the fact that BRCA1/2-deficient cancer cells are particularly sensitive to G4 binders [68]. Indeed, stabilization of G4 structures in BRCA2-deficient cancer cells induces high levels of DSBs [69] which can activate different molecular pathways leading to either cell killing or genome instability. This finding is consistent with more recent data showing that G4-mediated replication stress can trigger non-random DNA segregation, a mitotic event in which the sister chromatids inherited asymmetrically the damaged newly synthetized DNA leading to cell-cycle arrest and cell death of the only damaged daughter cell [70]. In addition, De Magis et al. demonstrate that, besides DSBs, after G4s stabilization by using PDS and FG ligands, BRCA2-deficient cells show high levels of micronuclei with a mechanism involving unscheduled R-loop/G4s (G-loop) formation [66] (Fig. 1C). This G4s and R-loops interplay seems to have a crucial function in driving DNA damage and genome instability in different types of cancer cells. Interestingly, recent findings have shown that the stabilization of G4 and R-loop structures, mediated by G4 binders and Top1 poisons, may have an immune-stimulatory effect, since they can activate an innate immune cascade in human cancer cells through micronuclei accumulation [18,68]. In particular, G4-and R-loop- induced micronuclei are recognized by the cytoplasmic DNA sensor cGAS leading to STING activation, which plays a pivotal role in stimulating Type I Interferon and other immune-related pathways such as lymphocyte and T-cell migration, which are fundamental to elicit an adaptive immune tumour surveillance [16,18]. Therefore, these findings elucidate the potential anticancer activity that G4 binders- and Top1 poisons-mediated genome instability can promote in tumour cells opening the way for the development of new cancer therapies.

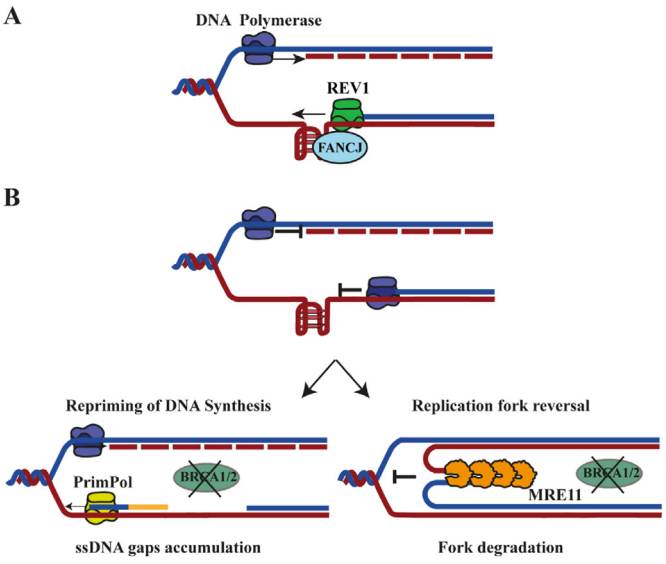

Fig. 2. Mechanisms through which G-quadruplexes can induce replication-dependent genome instability. The formation of G4 structures within the replication bubble represents an obstacle for the replication machinery limiting the progression of DNA polymerase and inducing replication fork stall. To avoid replication fork collapse, dedicated helicases, including FANCJ, are involved in the *in vivo* resolution of G4s. **(A)** FANCJ is also implicated in the recruitment of specialized DNA polymerases, as Rev1, able to replicate G4-containing templates through a TLS mechanism. **(B)** Besides TLS, PrimPol-mediated repriming and fork reversal are two mechanisms that counteract G4-barrier allowing the restart of DNA synthesis. PrimPol-mediated repriming (RNA primer is indicated in yellow) can promote genomic instability since it leaves ssDNA gap that, if left unrepaired, can collapse in DSBs and contribute to mutations. The absence of BRCA1/2 proteins exacerbates ssDNA gap accumulation and mutation events related to PrimPol repriming activity. BRCA1/2 proteins play also a pivotal role in fork reversal mechanism as their absence can lead to the degradation of the newly synthetized DNA by nucleases (e.g., MRE11) driving fork degradation. Created with Biorender.com.

2.2. R-loops

R-loops are hybrid structures composed of an RNA strand annealed to its template and a displaced non-template strand (Fig. 1B). Their formation is favoured by various DNA features such as negative super-helicity, nicked DNA or presence of GC skew [19,71]. As the accumulation of negative supercoils associated with ongoing RNA Polymerase II (RNAPII) favours RNA hybridization (thread back model), they mostly form during transcription [72,73]. These RNAPII-dependent R-loops can be divided into "promoter-paused", when they form as a consequence of RNAPII pausing at the promoter, and "elongation-associated", when their formation occurs at the gene body in association with transcription elongation [74]. In addition to these co-transcriptional R-loops (*in-cis*), accumulating evidence suggests that transcripts can hybridize post-transcriptionally to complementary sequences at a different locus from where they were transcribed (*in-trans*) [75–78]. Genome-wide studies revealed that R-loops cover from 3% to 8% of eukaryotic genomes and that they accumulate not only at highly transcribed regions but also at retrotransposons, antisense or non-coding RNAs and at repeated regions of telomeres and centromeres [71]. R-loops can regulate transcription initiation and termination, chromatin organization, DNA methylation, telomere maintenance, immunoglobulin class-switch recombination, DNA replication and repair processes [79,80]. However, altered R-loop levels, stability or position can have harmful consequences such as DNA damage and genome instability, hence cells developed several mechanisms to control R-loop homeostasis [71,80,81]. Despite the existence of factors which favour or stabilize R-loops formation, like DDX1 and DHX9 helicases or mitochondrial ssDNA-binding proteins [71], most of the reported data deal with preventing or resolving factors which counteract the formation of unscheduled R-loops. For example, proteins involved in post-transcriptional RNA processing/export (e.g., THO-complex), in the control of DNA topology during transcription (e.g., TOP1; TOP3B) or in chromatin remodeling (e.g., HDAC) prevent the formation of R-loops [19,71,73]. On the other side, RNases H proteins (RNase H1 or H2), which specifically degrade the RNA strand of the hybrid, or helicases like SETX, BLM or DDX19, which have DNA-RNA unwinding activity, belong to a multitude of cellular resolving factors. Although the mechanism is still poorly understood, components of Fanconi anemia (FA) pathway (e.g., BRCA1, BRCA2, FANCA, FANCD2, FANCM) or other damage repair factors (e.g., ATR, ATM, CHK1, CHK2) have also been implicated in the regulation of R-loop levels [71].

To date, it is still unclear what distinguishes, if any, pathological from physiological R-loops; however, when one or more of R-loop regulatory enzymes are depleted from cells and, more generally, when their regulation is altered, R-loops can cause DNA fragmentation, hyperrecombination, hypermutation, gross chromosomal rearrangements as well as UFBs and micronuclei formation [82–85], which have been implicated in the pathogenesis of several diseases, including cancer, neurological and autoimmune diseases [86–93].

2.2.1. R-loop related genome instability

How R-loops cause genome instability is still under investigation, but the higher exposure of the displaced ssDNA to spontaneous mutagenicity [77] or to the action of nucleases, deaminases and genotoxic agents has been revealed to contribute to such instability (Fig. 3). For example, the exposed single strand of R-loops can be targeted by deaminases which convert cytidine to uracil. In particular, it has been shown in yeast that Activation-Induced cytidine Deaminase, which is involved in immunoglobulin class-switch recombination, increases break/mutation/translocation rates in transcribed genes when R-loops augment after

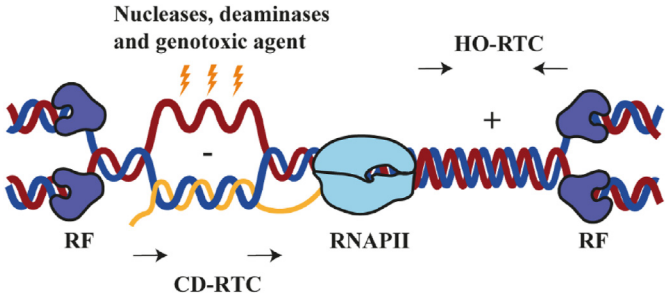

Fig. 3. Mechanisms through which R-loops can induce genome instability. R-loop-mediated genome instability is mainly related to the increase of replication-transcription conflicts (RTCs). These can be co-directional (CD-RTCs) or head-on (HO-RTCs) depending on weather replication fork (RF) and RNA Polymerase II (RNAPII) move in the same or opposite directions, respectively. Genome instability is also caused by nucleases, deaminases and genotoxic agents which target the single-stranded DNA of R-loops. As mentioned in the text (paragraph 2.2) the formation of a G4 structure in the single-strand displaced DNA (G-loop) further stabilizes R-loop, enhancing its detrimental effects. Created with Biorender.com.

mutating the THO complex [94,95]. Also in yeast, cytosine deamination by Fcy1 causes DNA breaks and contractions at CTG:CAG repeats [80], while McCann and colleagues proposed a model in which APOBEC3B deaminates displaced strands in R-loop structures [96]. Not only deaminases, but also nucleases target the ssDNA of R-loop structure likely to resolve it and leading to DSBs generation with potential recombinogenic effect. In this context, enzymes belonging to the nucleotide excision repair (NER) pathway, such as XPF and XPG, have been involved in R-loop processing [97–101]. The ssDNA of R-loop structures can also fold into other non-canonical structures such as G4s, forming the so-called G-loop, as mentioned before in section 2.1.1 (Fig. 1C) [1,19,69]. In a physiological context, the formation of G4s is useful, for example, to stabilize the R-loop structure favouring the deamination necessary to immunoglobulin class switching [102,103]; however, the presence of G-loop may be deleterious as it represents an obstacle for biological processes that promotes genome instability [19,69,104,105]. In this regard, De Magis and colleagues demonstrated that the stabilization of G4s by different G4-ligands induces the spreading of co-transcriptional R-loops to nearby regions containing G4 forming sequences. As already mentioned in section *2.1.1*, G-loop stabilization at these regions causes DNA damage increase, cell death and micronuclei formation depending on which ligand has been used [52].

Apart from R-loops exposure to cellular enzymes, R-loops (also in the form of G-loop) induce instability mainly by interfering with transcription and replication processes. Indeed, despite being by-products of transcription, R-loops can hinder the same process which gave them rise. In this context, mRNA annealed to its template may represent an obstacle for the other elongating RNAPIIs causing their pausing, stalling or backtracking. This situation may lead to DNA damage since transcription blocking R-loops are subsequently processed by XPF, XPG and the transcription-coupled nucleotide excision repair protein CSB [97,98]. DNA damage can subsequently be repaired thanks to BRCA1 and BRCA2 intervention with the consequent activation of FA pathway and the initiation of RAD51-dependent homology-directed repair [2,71]. BRCA1 and BRCA2 are also particularly relevant for hybrid homeostasis because they favour RNAPII elongation during transcription thus preventing R-loop formation [106–108]. Mutations in BRCA1/2, with consequent accumulation of R-loop-dependent DNA damage, have been found in breast, ovarian and other cancers. Conversely, there are cases, like the Ewing sarcoma cells, where the increased levels of R-loops cause BRCA1 sequestration thus impairing

homologous recombination repair and phenocopying BRCA1-deficient tumours [93,109]. BRCA1 also favours R-loops unwinding, especially during transcription termination, by recruiting SETX. The failure of SETX recruitment by BRCA1 causes R-loops increase at terminations sites [106]. On the other side, the fact that oculomotor apraxia 2 and amyotrophic lateral sclerosis type 4 are characterized by decreased levels of R-loops due to SETX gain of function [92,93,106] underlines how both reduced and increased levels of R-loops can be detrimental. Transcription-dependent R-loops can also alter the expression levels of genes involved in various disorders. For example, it has been reported that R-loops cause gene silencing at expanded trinucleotide repeats at *FXN* and *FMR1* genes consequently proving an R-loop role in Friedreich's ataxia and X fragile syndromes [110,111]. Anyway, transcription-mediated R-loop harmful effects are mostly related to the replication process.

Several studies in bacteria, yeast and human cells revealed that the major contribution of R-loops to genome instability is given by the impairment of replication processes, at least in cycling cells [71]. Indeed, R-loop structures, together with paused/blocked/backtracked RNAPII, represent a dangerous obstacle for replication fork progression, since they cause fork stall and collapse, thus contributing to DNA damage formation. Until now, the involvement of R-loops in increasing collisions between the replication and transcription machinery (RTCs) and consequent genome instability has been well established in *E.coli*, yeast and human cells [112–114]. Depending on the reciprocal direction of replication and transcription machinery, RTCs can be head-on (HO) or co-directional (CD) (Fig. 3). The effect of these conflicts on R-loop homeostasis is still controversial since some data demonstrate that a different RTCs orientation can promote a further increase of R-loops or their resolution [115,116], while others suggest that R-loop formation is orientation-independent [117]. However, all agree that HO collisions are much more injurious, causing replication stalling and DNA damage, while the CD collisions are less detrimental maybe because of replication fork ability to dissolve R-loops [118–120]. To this extent, Promonet and co-workers showed that the alteration of R-loop homeostasis resulting from Top1 depletion causes DNA damage mainly at regions where HO conflicts take place [121]. As previously observed by Manzo et al., DNA breaks are not directly caused by Top1 removal, as Top1 depleted cells block in G0/G1 phase with minimal DNA damage despite having altered levels of R-loops [104]. However, under experimental conditions that allow the G1-S transition, cells exhibit high levels of R-loop associated damage, which has been demonstrated to be due to HO-RTCs [121]. In this context, several proteins such as topoisomerases, helicases or proteins belonging to repair pathways are engaged to protect replication fork from R-loop persistence [92]. For example, Top1 plays an important role in preventing R-loop-related genome instability, mainly by reducing negative supercoiling associated with biological processes [19]. In this regard, Marinello and colleagues showed that the blockage of Top1 by CPT poisoning causes R-loop mediated DNA damage and micronuclei increase [17]. On the other side, BRCA2 recruits RNase H2 to DSBs in S/G2 cells to allow hybrid resolution [122]. Mutations in at least one of the three subunits of RNase H2 and consequent high levels of R-loops have been found in Aicardi-Goutieres syndrome, a neuroinflammatory disease characterized by the chronic activation of the IFN-α-mediated immune response [80,93,123]. It has been proposed that the activation of the immune system is due to an excessive accumulation of endogenous nucleic acids, but it has not been shown whether these are micronuclei or free cytoplasmic R-loops [123]. Indeed, while immune gene activation by micronuclei has been widely studied in the last years, only few papers recently shown how cytoplasmic RNA-DNA hybrid accumulation, occurring for example after the depletion of SETX or BRCA1, can stimulate the innate immune response by activating the cGAS-STING pathway [101,124].

Together with the mechanisms described above, it has also been proposed that chromatin modification may be involved in R-loop mediated replication instability. According to this model, R-loops may cause H3S10 phosphorylation leading to DNA condensation which, in turn, could result in a DNA replication barrier [125,126]. Such impediment could be particularly detrimental at fragile sites where a more closed chromatin, together with R-loop induced RTCs, favours replication failure [113,127]. An additional mechanism through which R-loops can promote mutagenesis is a non-canonical replication where the hybrid is used as a primer independently from the presence of a replication origin. Indeed, it has been proved that, in *E. coli* and in yeast, the accumulation of R-loops, given by the lack of R-loops regulating enzymes, can favour the priming of replication forks which then collapse at single-stranded nicks (exacerbated by R-loop-induced RTCs) causing an increase in mutation and amplification rates [128,129].

The alteration of replication process by R-loops has been associated with the incomplete replication of portions of DNA, since the removal of factors involved in R-loop homeostasis leads to UFBs and micronuclei accumulation. In this regard, it has already been discussed that BRCA2, TOP1 and RNaseH2 alterations cause genome instability followed by micronuclei increase. Additionally, TDP-43, a hallmark of amyotrophic lateral sclerosis, has been shown to regulate R-loop accumulation preventing replication hindering and the consequent increase in DNA damage. When cells are depleted of TDP-43, they exhibit a significant increase in both UFBs and micronuclei [130]. Similarly, TOP3B null cells or cells depleted of DDX18 or DDX17 helicases show higher levels of UFBs and/or micronuclei compared to control cells, probably because they are less effective in preventing replication stress consequences [131–133]. It has also been shown that the alteration of R-loop homeostasis by drugs, such as Top1 poisons or G4 binders, can cause UFBs and micronuclei increase and that RNaseH overexpression reverses this phenotype [17]. However, if such increase is due to the impairment of replication needs to be further verified. The discovery that R-loop modulation can lead to micronuclei production or cytoplasmic hybrids accumulation is very interesting as the atypical activation of immune response may be implicated in the onset of several diseases and, at the same time, opens new perspectives for the development of effective and personalized strategies especially for cancer treatment.

2.3. I-motifs

i-motifs are four-stranded DNA secondary structures that can form in cytosine-rich sequences. They consist of two parallel-stranded DNA duplexes held together in an antiparallel fashion by intercalated cytosine-cytosine base pairing (Fig. 4A). Given the complementarity of their sequences, G4s and i-motif may form in opposite strands at the same location of a duplex DNA. There is conflicting evidence regarding the fact that G4 and i-motif structures can be folded simultaneously or whether they are mutually exclusive. Recently, it has been demonstrated that the simultaneous formation of G4 and i-motif structures in the two complementary strands seems to depend on the length of the related sequences [2,3]. In short sequences, G4 and i-motif formation is mutually exclusive due to steric hindrance, while longer sequences allow the formation of both structures [2,3]. Until now, i-motif structures have been strictly associated with G4s and their characterization has mostly been performed with *in vitro* experiments. Only recently, the *in vivo* identification of i-motif structures provided evidence that they form in regulatory regions of the human

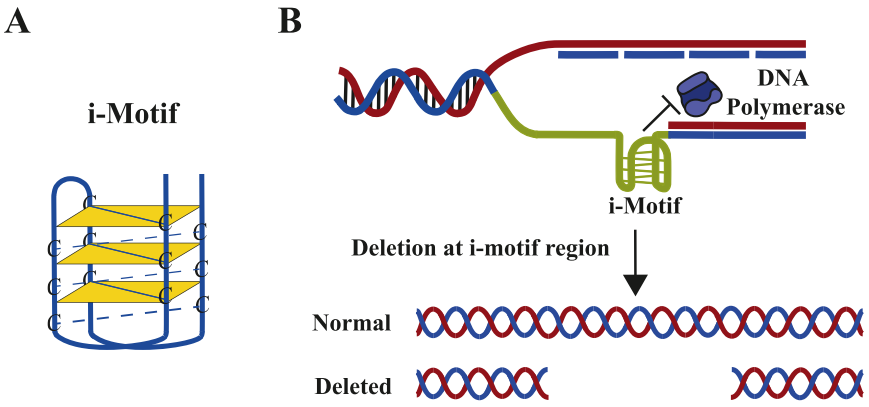

Fig. 4. i-motif secondary structure. (A) Schematic representation of i-motif characterized by cytosine-cytosine base pairing and **(B)** i-motif-induced deletions model. During DNA replication, i-motif formation can inhibit the progression of DNA polymerase, provoking replisome uncoupling and fork arrest. Non-resolution of i-motif before the end of replication causes deletions that occur in i-motif forming sequence (green DNA region). Created with Biorender.com.

genome, including promoter and telomeric regions, suggesting that they have key roles in the regulation of main biological processes [134]. For example, i-motif formation has been detected *in vivo* in the promoter region of *E2A* gene which encodes a transcription factor known to be essential for immunoglobulin gene recombination and early B cell development [135]. Interestingly, a dynamic i-motif structure in the HIV-1 long terminal repeat (LTR) seems to negatively regulate HIV-1 transcription, suggesting a role for i-motif structure in modulating viral transcription [146]. i-motif forming sequence has also been found in the promoter region of oncogenes, such as *HRAS*, *VEGF*, *c-Myc* and *Bcl2* [25], and i-motif folding in the promoter region of these genes has been demonstrated to modulate their expression level by recruiting different regulatory binding proteins [25,136]. Dysregulation of these genes is known to trigger a dynamic process of genomic instability that is linked to tumour initiation. Indeed, Myc deregulation can affect copy number of certain genes and induce karyotypic instability, which results in a variety of chromosomal changes [137]. Bcl2, instead, is known to prevent genome instability by down-regulating the Non-Homologous End Joining pathway involved in DSB repair and V(D)J recombination [138]. Besides oncogenes, i-motif structure is also located in the insulin minisatellite region [139], which is strongly linked to the genetic susceptibility of insulin dependent diabetes mellitus [140]. Therefore, alteration in i-motif formation can have a negative impact on genome stability and lead to the development of diseases.

2.3.1. *i-motif related genome instability*

How i-motifs promote genome instability is still under investigation. However, recent in *vivo* and in *vitro* data provide evidence that i-motifs can impair DNA replication leading to DNA breaks and deletion events that can ultimately affect genome stability [21,141,142]. Martella and colleagues discovered that deletion events at $d(TCCC)_n$ elements in the human PC3 cell line could be due to i-motif formation at these repetitive sites [141]. They propose that i-motif stabilization *in vivo* provides a replication impediment that, if not resolved before the end of the replication process, can lead to deletions in the subsequent replication cell cycle [141] (Fig. 4B). A more recent study better elucidates how i-motif structures can affect the eukaryotic replisome inducing replication fork arrest and DNA breakage in *vitro* [142]. They demonstrated that i-motifs arising during DNA replication can induce fork arrest by impairing DNA synthesis leading to helicase-polymerase uncoupling and ssDNA exposure. Excessive replisome uncoupling is known to induce an accumulation of ssDNA which can lead to RPA exhaustion and massive DNA breakage, resulting in irreversible fork collapse and DNA replication arrest [143]. Consistent with this, the authors also observed that i-motifs, if not promptly unwound by Pif1 helicase activity during replication, can lead to the breakage of nascent DNA [142]. DNA breaks, along with unstable exposure of ssDNA can also contribute to recombination and mutation events that affect genome stability. Overall, these findings provide insight into the mechanistic understanding of how i-motif structures can contribute to genome instability and impair DNA replication. Moreover, a direct correlation between i-motif stabilization and genome instability can be proved by data obtained from Qu and co-workers [144]. They demonstrate that i-motif stabilization in human telomeres with single-walled carbon nanotubes (SWNTs) ligand [145] inhibits telomerase activity in living K562 and HeLa cells, resulting in telomere-binding proteins displacement, telomere uncapping, DNA damage and apoptosis [144]. SWNTs inhibit telomerase favouring duplex dissociation and a further subsequent i-motif stabilization that, in turn, facilitates the induction of G4 structure [144,145], which is already known to inhibit telomerase [146]. Further investigations are needed to clarify the biological roles of i-motif structures *in vivo* and their interplay with G4 structures. However, the evidence that i-motif structures have regulatory functions suggests that targeting these structures with small molecules could have potential therapeutic application for genetic disease.

2.4. *Hairpins, cruciforms and slipped strand DNA structures*

Hairpin structures form when a single-stranded nucleic acid (either DNA or RNA) folds back on itself resulting in a stem-loop structure [147] (Fig. 5A). During biological processes, such as replication and transcription, the formation of hairpins can occur at perfect or non-perfect inverted repeats, also called perfect-palindrome or quasi-palindrome [148–150]. RNA hairpins form as a consequence of RNA folding during the transcription of an inverted-repeat DNA template [151]. On the other hand, DNA hairpins originate during both replication and transcription as the presence of a single strand facilitates the folding of the structure. When two hairpins form on opposing DNA strands, this results in more complex structures similar to Holliday junctions and called cruciforms (Fig. 5A) [152–154]. While hairpin formation is energetically favourable, the formation of cruciform requires energy and negative super-helicity can provide it [9,152,155]. Both hairpins and

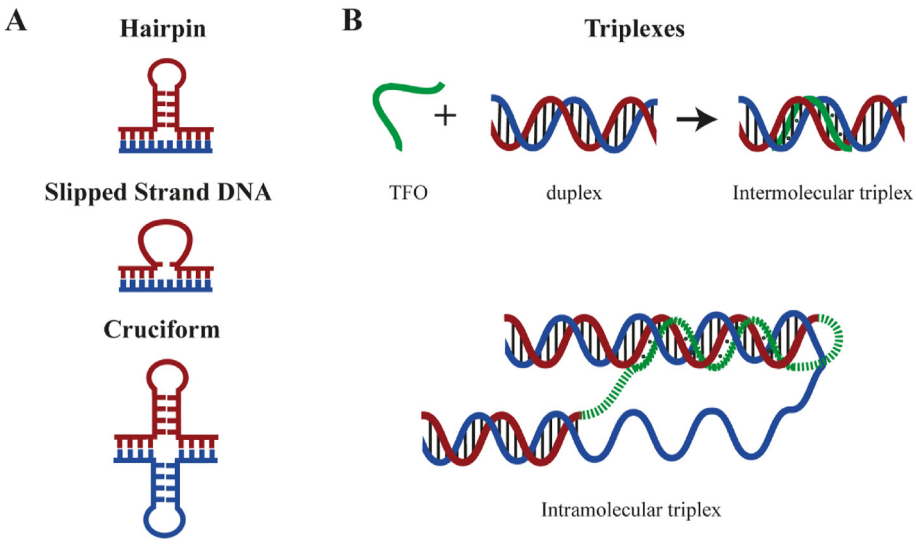

Fig. 5. Schematic representation of DNA loop structures and triplexes. (A) DNA loop structures include Hairpin, Slipped strand DNA and Cruciform structures. **(B)** Intermolecular triplexes form when an independent triplex-forming oligonucleotide (TFO) anneals to the double strand nucleic acid (Top). Intramolecular triplexes form when the same DNA or RNA strand folds back binding to the duplex of origin (Bottom). DNA folding-back strand in green.

cruciforms have several structural elements that can be recognized by different proteins contributing to various physiological functions [156–158]. For example, hairpin structure forming at 3′UTR of histone mRNA is recognized by the stem-loop binding protein (SLBP), allowing mRNA post-transcriptional processing [158], while hairpin recognition and processing by Artemis:DNA-PKcs is a fundamental step of non-homologous end joining and V(D)J recombination [159,160]. Brázda and colleagues also described that junction-resolving enzymes, DNA repair and transcription factors as well as replication and chromatin associated proteins can specifically recognize the DNA stem-loop structure or the four-way junction conformation with a much higher affinity for cruciform structures than for linear sequences [157]. Although these structures have physiological functions, their uncontrolled formation can hinder biological processes resulting in DSBs and genome instability which have been linked to certain diseases [161–164].

2.4.1. DNA loop structures related genome instability

To better understand DNA hairpin and cruciform related genome instability, several studies have been carried-out *in vitro* as well as in *Escherichia coli*, *Saccharomyces cerevisiae* and in mammalian cells. These studies have shown that hairpin and cruciform related instability is induced by both replication-dependent and replication-independent mechanisms. In the first case, the formation of hairpins, more than cruciforms, causes DNA polymerase stalling [165–167] and the most common outcome is the deletion or the expansion of the hairpin forming sequence. In particular, if hairpins form at the template strand, deletions are more likely to occur as DNA polymerase can bypass the hairpin structures (Fig. 6A). On the other side, when the secondary structures form at the newly synthesized fragment, expansion of the DNA sequence will occur, as the slippage of DNA polymerase causes the backward realignment of nascent strand [168–171] (Fig. 6B). During the DNA polymerase shifting, which is characterized by the misalignment of DNA strands, a loop-structure defined Slipped strand DNA may form [169,172,173] (Fig. 5A). Slipped strand DNA structures are known to form at direct repeats [174,175], more specifically at (CTG)n:(CAG)n, (CGG)n:(CCG)n and (CCTG)n:(CAGG)n sequences. When the loop sequence of Slipped strand DNAs is partially self-complementary, they can fold forming hairpin structures. The coexistence of hairpins and Slipped strand DNA structures in the same DNA region increases their stability favouring the occurrence of replication errors [175,176]. When these structures impair replication, it has also been observed the increasing of fork collapse chances and DNA damage due to mechanical breakage or nuclease activity of repair/resolvase enzymes which operate to promote a complete DNA synthesis. For example, several studies reported that MSH2-MSH3 proteins, members of the mismatch repair complex, bind hairpins and/or Slipped strand DNA structures involved in trinucleotide repeat expansion [177–179], already associated with neurological diseases. Additionally, it has been demonstrated that the SbcCD complex cleaves the hairpins formed during lagging- and leading-strand DNA synthesis [171,180,181] generating DSBs [182]. The same function might be exerted by Mre11/Rad50/Xrs2 and Mre11/Rad50/Nbs1 in yeast and human cells, respectively [183–185]. Interestingly, Zhang et al. proposed a hairpin-bypass mechanism to complete DNA synthesis which depends on the template switching activity. This process leads to the formation of an intermediate cruciform structure which is subsequently cut by specific endonucleases [186] contributing to DNA damage formation and replication-related genome instability.

While in *E. coli* these structures are known to cause genome instability exclusively in relation to the replication process [171,180,181], in eukaryotes additional mechanisms are involved [166]. Indeed, DNA breakage can also be the consequence of replication-independent cruciform cleavages. For example, it is known that there are structure-specific nucleases such as ERCC1-XPF [182] or the Holliday-junction resolvase GEN1 [187] which cleave cruciform structures in a context indendent from replication. Moreover, it has been shown that the formation of hairpins along mRNA can interrupt the transcription process [188–190] or pause/stall ribosome motion [191] with the consequent accumulation of incomplete products and endo-nucleolytic cleavage of mRNA [192,193]. Until now, these molecular processes concerning RNA hairpins have only been studied *in vitro*, hence, further experiments are needed to fully understand the mechanism and its consequences *in vivo*.

To date, the involvement of hairpins/cruciforms in the onset of human diseases is still under investigation. Researchers' efforts

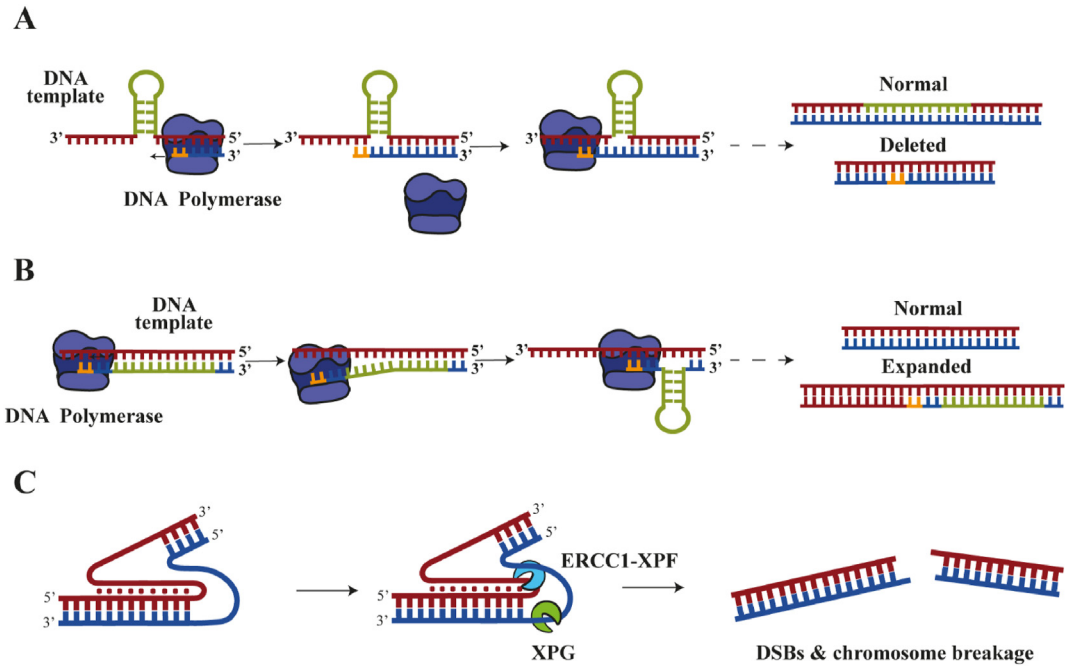

Fig. 6. Hairpin structures and triplexes as a source of genome instability. (A, B) The formation of hairpins during the replication process can cause deletions or expansions of the hairpin forming sequence (in green) as a consequence of DNA polymerase slippage. **(A)** When the DNA polymerase encounters a stable hairpin on the template strand, it can detach from the template and the 3′ end of the newly synthesized DNA (orange DNA region) can anneal to another complementary sequence downstream the hairpin structure. The deletion of the hairpin forming sequence will be the result of the next replication cycle. **(B)** Alternatively, when replication fork encounters an obstacle, the DNA polymerase, together with the nascent strand, can slide backwards. The reannealing to a different complementary region gives rise to a hairpin in the newly synthesized strand causing expansions at the next replication cycle. **(C)** Intramolecular triplexes can be recognized by repair enzymes like ERCC1-XPF and XPG. XPF cleaves the loop between the strands annealed through Hoogsteen bonds while XPG cleaves the ssDNA at the 5'end of Watson-Crick base-paired duplex. Triplex cleavage by XPF and XPG causes an increase of DNA breakage and genome instability. Created with Biorender.com.

have allowed us to discover that HER2-positive breast tumours are characterized by the duplication of palindromic sequences in the ERBB2 oncogene [194] and that hairpins are involved in the expansion of triplet repeat in Friedreich's Ataxia [164]. We also know that DSBs caused by hairpin and cruciform structures, if not further processed and repaired, result in gross chromosomal rearrangements such as translocations [195]. Until now, only few studies have directly determined the involvement of such structures in the occurrence of translocations [187,196,197]; however, breakpoint regions have been found to be located in palindromic DNA portions that are prone to form hairpins/cruciforms in both somatic and germ cells [163,182,197–199]. An example is the palindrome-mediated t(11:22) reciprocal translocation found in Emanuel syndrome which is characterized by mental disability, microcephaly, heart defects and genital anomalies in males [199]. Despite the progress, little is known about these specific structures and additional studies are necessary to understand how they contribute to human diseases.

2.5. Triplexes

Triple helical DNA (Triplexes) are structures composed of three strands of only DNA or RNA as well as a mix of both (Fig. 5B). Triplexes can form when a polypurine (R) or polypyrimidine (Y) single strand, called triplex-forming oligonucleotide (TFO), binds to the major groove of a R:Y duplex in a sequence specific manner [200,201]. TFO pairs specifically to the purine strand through Hoogsten (Y:R*Y) or reverse Hoogsten (Y:R*R) bonds, according to the direction of the TFO with respect to the duplex. Depending on the origin of the third strand, we distinguish between intermolecular or intramolecular triplexes (Fig. 5B). Intermolecular triplexes form when an independent TFO pairs to the double strand nucleic acid, whereas intramolecular structures form when the same DNA/RNA strand folds back on itself and binds to the duplex. Intramolecular DNA triplexes are called H-DNAs (hinged DNA) or *H-DNAs depending on whether the third strand is rich in pyrimidine or purine, respectively. While H-DNAs form exclusively at R:Y regions containing mirror repeats [202], *H-DNA can also form at non-mirror repeats and at non-homopurine/homopyrimidine regions, even with some mismatches [203–206]. Different triplex conformations depend on bonds direction and stability, which are given by the base identity, pH of the surrounding environment, backbone distortion adopted by the triplex, negative super-helicity and presence of divalent cations [156,173,182]. Conditions as negative super-helicity and presence of divalent cations in (GAA•TTC) > 59 repeats allow the formation of more complex structures called sticky DNA. The exact conformation of these structures has not yet been elucidated, although they were initially described as bi-triplex structures [207] and subsequently redefined as long and stable Y:R*R triplexes [208].

2.5.1. Triplexes related genome instability

The biological function of DNA triplexes is not yet clear, however, it is well established that they represent a hot-spot for DNA mutations [209] and that they hinder biological processes causing human diseases. Indeed, together with the other NCSs, triplexes can block ongoing DNA polymerases especially at repeated regions such as mirror repeats or trinucleotide repeats [210,211]. Fork stalling due to the presence of these structures has been linked to the development of the Autosomal dominant polycystic kidney disease [211], a disorder characterized by the growth of cysts within the kidneys and caused by the occurrence of mutations in the *PKD1* and

PKD2 genes. Indeed, *PKD1* contains a very large R:Y tract able to form triplex structures [212,213] which can block DNA replication causing DNA damage and gross deletions [214,215].

The presence of triplex structures during replication processes can also lead to the expansion of the replicated region caused by polymerase slippage, as already described for hairpins and Slipped strand DNAs. To this extent, Gacy and co-workers demonstrated that the formation of triplex structures impairs the replication process thus causing triplet expansion in the *FXN* gene, which is associated with Friedreich's ataxia disease [216]. More in detail, the autosomal disease is caused by the expansion of a (GAA)*n* repeat contained in the *FXN* gene [217,218]. The additional formation of non-B DNA structures inhibits the transcription process. While secondary structures such as triplexes or R-loops act as pausing sites for RNPII, sticky DNA inhibits transcription by sequestering RNA polymerase which directly binds the structure [219–221]. About transcription impairment, both *in vitro* and *in vivo* experiments have already shown that the presence of triplexes, which can be further stabilized by the hybridization of the RNA to its template (R-loop), can block the transcription process in the presence of Mn^{2+} or high concentrations of K^{+} and Li^{+} [222,223]. The inhibition of transcription by triplex structures has also been associated with cancer occurrence as the regulation of certain cancer-associated genes is altered when they form at the promoter of such genes. For example, it has been shown that H-DNAs represent an obstacle for transcription machinery at the *c-Myc* promoter [224,225] and that triple helices forming near the TATA box of the ATP1A2 gene can cause its downregulation [226] which has been associated with breast cancer [227].

It has also been suggested that NER and other repair enzymes contribute to triplex related mutagenesis since they recognize and cleave triple helices as if they were “DNA damage” [215,228–230]. Interestingly, Zhao and colleagues recently demonstrated that ERCC1-XPF and XPG not only cleave H-DNAs during replication but also in a replication independent context causing genome instability (Fig. 6C), while triplex cleavage by FEN1 endonuclease, protects DNA during replication. XPF cleaves a specific loop of the H-DNA suggesting that the cleavage is structure specific (Fig. 6C). Interestingly, its deficiency results in a reduction of mutation rate and alters the distribution of H-DNA related breakpoints [231]. In line with other published evidence [231,232], the paper also reports that long triplex forming sequences are particularly enriched at translocation breakpoints in human cancer cells implicating triplexes in cancer etiology. In support of this, it has been shown that H-DNAs may form in the promoter of c-*Myc* gene nearby the t(8; 14) translocation breakpoint associated with lymphomas [209,233] while the t(14; 18) translocation at the B*cl*-2 major breakpoint region detected in follicular lymphoma is caused by RAG complex cleavage induced by triplexes [234,235]. Moreover, Zhao and colleagues demonstrated that H-DNA forming sequences at breakpoint hot-spot are longer than in other tracts, which means that they can fold into more stable H-DNA causing DNA damage and translocation events [231].

In parallel with the study of DNA triplexes, the formation of RNA and RNA-DNA triplexes, previously reviewed in Refs. [200,230,236], has also caught the interest of the scientific community. RNA triplexes turned particularly interesting as it has been demonstrated the existence of non-coding RNAs which are stabilized in triplex structures consequently accumulating into cells and leading to cancer development [200,237–239]. On the other side, there are non-coding RNAs forming miRNA–DNA:DNA triplexes which can modulate gene expression by inducing DNA methylation [240] or adopting a still unknown mechanism independent from chromatin modifiers [241]. It has also been suggested that microRNAs synthesized from retroviruses or transposons may form triple helices with the DNA region that gave them rise, thereby inhibiting its replication [242]. The fact that TFOs can target genes leading to the formation of triplexes with the ability to control gene expression and other biological processes, revealed their therapeutic potential. Several studies have been conducted to evaluate the usage of synthetic TFOs in Duchenne muscular dystrophy, HIV infection or cancer treatment by downregulating the expression of target genes or by carrying drugs [243]. To date, such TFOs have not yet been utilized in a therapeutic context and further studies are needed to properly exploit that tool.

2.6. Z-DNA

Z-DNA structure, first described in the late 1970 [244], is a left-handed structure that occurs most frequently in alternating purine/pyrimidine (d(Pu/Py) or d(Py/Pu)) repeat sequences [245] such as $CG_{(14)}$ repeats (Fig. 7A). Sequences with the potential to adopt Z-DNA are abundant in eukaryotic genomes and occur approximately once every 3000 bp in the human genome [246]. B-DNA to Z-DNA transition *in vivo* is a dynamic process and can occur in certain conditions such as in the presence of negative supercoiling, Z-DNA binding proteins and base modifications [247]. During transcription, the movement of RNA polymerases generates negative supercoiling behind the polymerase that stabilizes Z-DNA conformation at permissive regions. DNA unwrapping from nucleosomes also induces negative supercoiling that can lead to Z-DNA formation [248,249], and its subsequent stabilization contributes to maintain the chromatin in an open state, positively regulating transcription initiation. If not resolved, Z-DNA represents a barrier for the DNA to be re-wrapped into nucleosomes leaving this DNA region in an open chromatin state for a longer period compared to normal conditions [250]. Several proteins have the ability to recognize and bind Z-DNA making this structure a *cis*-element in gene regulation [247,251]. Among these proteins the double-stranded RNA adenosine deaminase ADAR-1, the tumour-related protein DML-1 and the vaccinia virus EL3 protein have been extensively studied for their binding affinity to Z-DNA/RNA (as also dsRNA can adopt a Z conformation) [247]. In a study conducted by Oh et al. [251] it was demonstrated that the human ADAR-1 protein can modulate transcription by stabilizing Z-DNA structure at a promoter region. In addition, the binding of ADAR-1 to Z-DNA/RNA has been shown to enhance its efficiency in A-to-I editing, a process that involves the conversion of adenosine (A) to inosine (I) in RNA molecule [252]. These results suggest that ADAR-1 interaction with Z-DNA/RNA is important to exert its role in gene regulation also at a post-transcriptional level. Interestingly, the RNA-editing activity of ADAR-1 plays an essential role in suppressing the innate immune activation, since it marks the dsRNA as self through A-to-I editing thus preventing autoimmune-related diseases [253,254]. Accordingly, mutations in the sequence encoding the Z-structure binding domain of ADAR-1 cause the autoimmune disorder Aicardi-Goutieres syndrome, by inducing an up-regulation of interferon stimulated genes [254]. In a recent article it has been revealed that the immunopathology caused by ADAR-1 mutation in mice is dependent on the activity of another Z-DNA binding protein, known as DML-1 (also called ZBP1) that activate the innate immune response by acting as a cytosolic DNA sensor [255]. Similarly, in the context of viral infection, the ability to bind Z-DNA is essential for the EL3 protein to exert its activity in promoting viral pathogenicity in mice [256]. Overall, these data provide evidence that the Z-structure binding domain of ADAR-1, DML-1 and EL3 proteins is important for their proper function in the immune pathway suggesting that Z-DNA conformation may have a crucial role in regulating the innate immune response. Therefore, a better understanding of the molecular mechanism underlying the

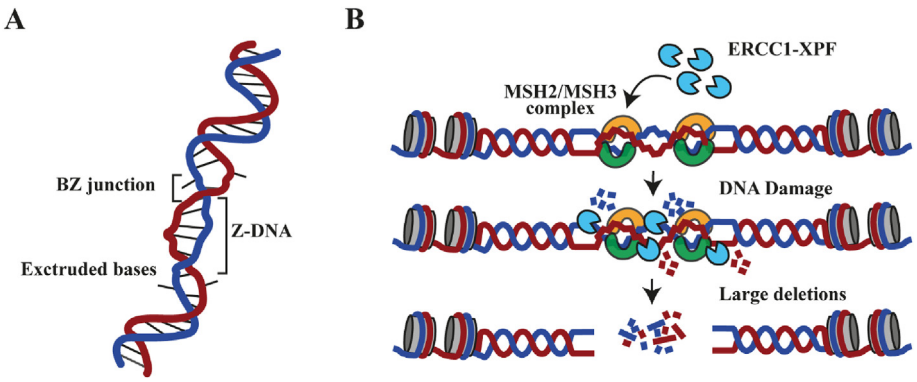

Fig. 7. Z-DNA secondary structure. (A) Schematic representation of the left-handed "zig-zag" nature of the Z-DNA backbone with canonical B-DNA on the ends. B-Z junction and extruded bases are underlined. **(B)** Molecular model through which Z-DNA induces DSBs and large-scale deletions, features of Z-DNA mediated genome instability. Z-DNA can be recognized by MSH2-MSH3 repair complex which recruits specific nucleases such as ERCC1-XPF leading to the processing of Z-DNA.

interplay between Z-DNA/RNA and these proteins *in vivo* could provide insight into the pathogenesis of autoinflammatory disease potentially leading to the development of targeted therapies for these pathologies. Evidence supporting that Z-DNA structure may have key biological roles in human cells is also provided by Shin and co-workers [257]. They performed a ChIP-seq analysis demonstrating that Z-DNA-forming sites are enriched in promoter regions that are also occupied by RNAPII and, through a reporter assay system, they associated Z-DNA with active transcription [257]. Several disease-related genes, including oncogenes, are transcriptionally regulated by Z-DNA [258]. This is the case of *c-Myc*, *CSF-1* and *HO-1* genes, whose expression levels increase after Z-DNA formation and stabilization in their promoter regions [259,260]. Conversely, the *ADAM-12* promoter contains a Z-DNA-forming sequence (ZFS) that negatively regulates *ADAM-12* expression in normal cells [261]. Loss of this ZFS element leads to overexpression of *ADAM-12*, which is commonly observed in various human cancers that are usually associated with a poor prognosis, such as the pancreatic ductal adenocarcinoma and triple-negative breast cancer [262,263]. Taken together, the formation of Z-DNA in the promoter region of these genes can modulate their expression levels, potentially affecting cellular behaviour and genome stability. The molecular mechanism through which Z-DNA structure mediates the expression levels of these disease-related genes is still not fully defined. However, current studies suggest that Z-DNA formation in the promoter region may induce changes in the chromatin structure that in turn can modify the accessibility of the DNA, affecting the recruitment and binding of proteins or complexes involved in the regulation of the transcriptional process [258].

2.6.1. Z-DNA related genome instability

It is known that the modified physical characteristics of a region surrounding the Z-DNA structure may increase its susceptibility to DNA damage and enzymatic cleavage, leading to increased localized genetic instability. This can result in a variety of outcomes, including deletions, rearrangements, and mutations [264]. The molecular mechanism of Z-DNA-induced genomic instability is still not defined. However, it is currently known that Z-DNA induces genomic instability in terms of large-scale deletions and gross chromosomal rearrangements in both yeast and human cells with a mechanism independent from DNA replication and in which are involved components of repair processing [265]. Wang et al. demonstrated that Z-DNA forming sequences in human cancer cells are hot-spots of chromosomal breakpoints, responsible of genetic instability as they cause gene translocation in leukemias and lymphomas [247]. Multiple Z-DNA motifs have also been found near breakpoints in the *c-Myc* P1 promoter, in the breakage hot-spot of *Bcl-2* gene and nearby chromosomal translocation that occur in DNA regions rich in immunoglobin-related genes, that are closely related to blood cancers [258]. Z-DNA has also been linked to the generation of DBSs along the human and mice genomes [265,266]. Indeed, DSBs have been found at Z-DNA forming sequence, suggesting that large-scale deletions and breakpoints could originate from DNA cleavage that occurs near the Z-DNA structure [173]. Recently Mc Kinney et al. demonstrated that ERCC1-XPF and MSH2-MSH3 interact with Z-DNA and are required for Z-DNA-induced genomic instability in yeast and human cells [267]. In their proposed model Z-DNA is recognized by MSH2-MSH3 repair complex as "a damage event" and recruits the structure-specific nucleases ERCC1-XPF which then process Z-DNA (Fig. 7B). This DNA processing can lead to DSBs generation, probably resulting in large-scale deletions or translocations near or at the site of Z-DNA forming sequences. Interestingly, the interaction of these proteins with Z-DNA appears to be outside of their canonical role in NER and Mismatch repair pathways and differs from their role in triplex-induced genomic instability, which requires the canonical NER mechanism [173,267].

3. Conclusion

Non-canonical DNA and RNA structures play an important role in the regulation of biological processes; however, their unscheduled formation and/or stabilization triggers mutagenic mechanisms consequently leading to human diseases. In this review, we described the principal non-canonical DNA and RNA structures focusing on the mechanisms through which they alter genome stability. Among the proposed mechanisms, impairment of replication and transcription processes seems to be particularly relevant, even if published data underline that repair proteins may have a significant role in genome instability induced by non-canonical structures. There is still much to know about these structures, even if remarkable progress has been made in the last decades thanks to the development of specific experimental and bioinformatic tools [201]. The growing interest of the scientific community in these structures lies in the possibility of using them as therapeutic targets by regulating gene expression or hampering the replication process. In this vein, researchers have already tested the usage of exogenous TFOs allowing the formation of sequence specific triplexes that inhibit the expression of *c-Myc* [268] and other disease related genes [9]. Interestingly, experiments conducted in rats showed that triplex-mediated inhibition of MET (protein associated with several human cancers), leads to cell death

and tumour regression in hepatoma [269]. Several studies have also provided much insight into G4 stabilizing compounds with therapeutic activity, particularly anticancer effects. How G4 binders work has already been extensively reviewed [9,18,19] with particular attention to their immunostimulatory activity. Indeed, despite efforts, only a few G4 binders have reached early phases of clinical trials and none of them have shown good efficacy in cancer patients. Looking for non-cytotoxicity effects of G4 binders, researchers have shown that they can activate the innate immune gene response suggesting that such compounds can be used in the context of immunotherapy [18]. Even if not directly targeted, R-loops are also important in the development of strategies for the treatment of human diseases. In this regard, inhibition or removal of R-loop binding proteins increases tumour sensitivity to chemotherapy [92,270] while drugs that alter R-loop homeostasis such as CPT analogues are already used in the standard treatment of human ovary, colon, and lung cancers. Interestingly, recent discoveries revealed R-loop dependent mechanisms which stimulate innate immune genes [17,101,124]. In particular, Top1 poisons trigger immune gene expression depending on the presence of the cGAS/STING pathway suggesting the potential use of anticancer drugs to improve precision medicine strategies [17]. These and other findings highlight the importance of understanding the specific properties and related functioning of non-B DNA structures in order to develop new therapeutic strategies for the treatment of human diseases.

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