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Perspective

Structural Motifs at the Telomeres and Their Role in Regulatory Pathways

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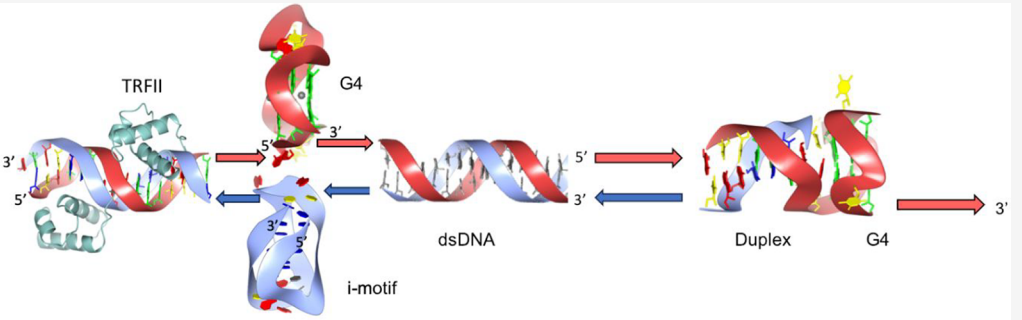

ABSTRACT: Telomeres are specialized structures, found at the ends of linear chromosomes in eukaryotic cells, that play a crucial role in maintaining the stability and integrity of genomes. They are composed of repetitive DNA sequences, ssDNA overhangs, and several associated proteins. The length of telomeres is linked to cellular aging in humans, and deficiencies in their maintenance are associated with various diseases. Key structural motifs at the telomeres serve to protect vulnerable chromosomal ends. Telomeric DNA also has the ability to form diverse complex DNA higher-order structures, including T-loops, D-loops, R-loops, G-loops, G-quadruplexes, and i-motifs, in the complementary C-rich strand. While many essential proteins at telomeres have been identified, the intricacies of their interactions and structural details are still not fully understood. This Perspective highlights recent advancements in comprehending the structures associated with human telomeres. It emphasizes the significance of telomeres, explores various telomeric structural motifs, and delves into the structural biology surrounding telomeres and telomerase. Furthermore, telomeric loops, their topologies, and the associated proteins that contribute to the safeguarding of telomeres are discussed.

Eukaryotic cells contain linear chromosomes that are encased by a nucleoprotein complex at each end called a telomere.[1] The hexanucleotide repeats found within telomeric DNA are linked to various proteins that bind to both telomeric double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA), through direct or indirect interactions. These proteins come together to form the protective protein telomere cap.[2] Telomeres play a crucial role in stabilizing the ends of chromosomes, and their protective function may hinge on whether they are in an "uncapped" or "capped" state.[2,3] For an extended period, it was believed that the telomeres were transcriptionally inactive. Nevertheless, recent findings have revealed that telomeric DNA is often transcribed into telomeric repeat-containing RNA (TERRA).[4] Telomeric RNA is a newly emerging component in telomeric function that could be an important element of telomere machinery.[2] Previously, studies focused on telomeric DNA and its linked proteins. However, the identification of TERRA RNA at the chromosome ends has the potential to provide fresh perspectives and enrich our existing understanding.[5] The proteins present at the telomeres possess a unique structure that allows them to oversee and safeguard DNA, making them integral to numerous biological processes. Additionally, telomeres play a role in regulating gene expression and function as a molecular timer, governing the replicative capacity of human cells.[6] In proliferating cells that lack functional telomerase, telomeres shorten with each mitotic division, and the cells finally die. The telomeric DNA and TERRA RNA structural motifs are discussed in detail in this Perspective along with their roles in regulatory pathways.

STRUCTURE AND FUNCTION OF TELOMERES

Telomeric DNA is composed of repetitive sequences located at the termini of chromosomes. This characteristic is observed in a diverse range of eukaryotic species. The guanine-rich (G-

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Table 1. A Summary of the Telomere-Associated Proteins Discussed in This Review

Telomere-associated proteins	Role at telomeres	PDB id (if available)	Reference
Telomerase	Extension of telomeric DNA	7QXB	17, 24, 27
The shelterin complex	Protects and regulates telomeres. Consists of six proteins TPP1-POT1-TRF1-TRF2-TIN2-RAP1		34, 35
TPP1 (POT1 interacting protein)	Interacts with PO1 and TIN2. Recruits telomerase to telomeres and is in direct contact with telomerase	5UN7,5H65,7TRE,5XYF	26, 36−38
POT1 (protection of telomeres)	Recognizes the 3′ single strand and binds to ss-ds DNA junction, prevents telomere instability	8SH1,7S1O,7S1T,7S1U	27, 30, 32, 33, 39
TRF1 (telomere repeat binding factor)	Recognizes dsDNA TTAGGG sequences	8OX1,1W0T	29, 40−43
TRF2 (telomere repeat binding factor)	Binds to and promotes the development of T-loops	1W0U,5XYF	29, 37, 41, 43, 44
TIN2 (TRF interacting nuclear)	Interacts with TPP1, TRF proteins and acts as a bridging unit	5XYF	37, 45, 46
RAP1 (repressor/activator protein)	Dependent on TRF2 for telomere binding, inhibits DNA repair	3K6G	47−50
ssDNA binding CST complex	Consists of three proteins CTC1-STN1-TEN1	8SOK	25, 51, 52
CTC1	Controls access of telomerase, prevents G-overhang extension, involved in telomere length homeostasis	6W6W	25, 53, 54
STN1	Binds to ssDNA and protects telomeres from DNA degradation	4JOI	25, 53, 55
TEN1	Required for DNA polymerase α-mediated C-strand synthesis	4JOI	25, 51, 52, 55, 56
DNA2 helicase	Interacts with TRF1/2 in shelterin complex, removes telomeric G4	5EAX,5EAN	57−59
Pif1 helicase	Unwinds G4, inhibits telomerase activity at telomeres	6HPT,6L3G,7OAR	60−65
FANCJ (Fanconi anemia complementation group J) helicase	Involved in homologous recombination, DNA damage repair, G4 resolution, and maintaining genomic stability		66, 67
StyRecQL (stylonychia RecQ-like) helicase	Resolution of telomeric G4		68
TLS (translocated in liposarcoma)/FUS (fused in sarcoma) proteins	Binds to telomeric G4 DNA and TERRA		69
HMGB1 (high mobility group B1) protein	Binds to noncanonical DNA structures like G4, hemicatenated DNA loops, and four-way junctions	4QR9	70−72
Gen1 (genetic endonuclease)	Resolves HJ at T-loops	5T9J	73, 74
SLX1/4 (structure-specific DNA binding protein required for maintenance of genome stability X) endonuclease	Resolves HJ at T-loops	7CQ4	75, 76
RTEL1 helicase	Unwinds T-loops, promotes telomere replication	7WU8	77−79
RecQ helicase complex	Consists of SGS1-TOP3-RMI1-MPH1-SRS2 proteins. Resolves D-loop, unwinds G4	2WWY,6CRM	80−83
ATM (Ataxia Telangiectasia mutated) kinase	Involved in dsDNA breaks	8OXP	84−86
ATR (Ataxia Telangiectasia and RAD3) kinase	Involved in ssDNA damage	5YZ0	84, 85, 87
MRN complex	Consists of MRE11-RAD50-NBS1 proteins. Recognizes ds breaks, primes DNA ends for repair, activating ATM, implicated in nonhomologous end joining and homologous recombination	8BAH,3AV0,3QKU	88−91
RAD51	Facilitates strand exchange during HR, involved in D-loops and recruitment of TERRA via R-loops	5H1B	80, 92−94
RNase H1 and H2	Prevents R-loop accumulation	2QK9,3P56	95−97
ATP-dependent DNA helicase senataxin	Prevents R-loop accumulation		98, 99
DHX9 helicase	Unwinds R-loops and G4	8SZP	100, 101
EST1A (ever shorter telomeres)/SMG6	Regulates telomerase via TERRA, involved in nonsense-mediated decay process	2HWW	102−105
RAD51AP1 (RAD51-associated protein)	Involved in R-loop and D-loop formation, role in ALT pathways		106
BRCA1 (p220)	Deals with ssDNA damage at R-loop termination sites		107, 108

rich) sequences consist of tandem repeats of (TTTTGGGG)n in lower eukaryotes such as Oxytricha, or (TTGGGG)n in Tetrahymena and (TTAGGG)n in vertebrates.[7,8] Telomeric sequences in human somatic cells usually range in length from 10 to 15 kb.[9] Contrary to the rest of the double-stranded telomeric DNA, the G-rich strand has a 5′ to 3′ strand orientation, and the 3′ end strand extends past the complementary C-rich stand in a single-strand overhang.[10] This overhang length varies depending on the species and is typically 50−200 nucleotides long in humans.[11] Recent studies have confirmed the presence of several proteins that are essential in maintaining the integrity of the telomeric DNA (Table 1). Telomeres can therefore be defined as ribonucleoprotein complexes present at the ends of the chromosomes.

In the process of lagging strand DNA synthesis, the terminal regions of linear DNA are unable to achieve complete replication. This arises from the intrinsic asymmetry inherent in semiconservative DNA replication, a phenomenon commonly referred to as the "end-replication problem."[12] As normal somatic cells divide, telomeric repeats gradually shorten with every replicative cycle at a rate of 50−200 bp per cell division. Soon after, the cells enter a state of irreversible growth inhibition and eventually die via cellular apoptosis.[8] The shortening of telomeric DNA can be viewed as a molecular clock that marks the beginning of cellular senescence.[9] This biological phenomenon has been linked to cellular immortality and aging because immortalized cells' telomeric DNA does not shrink following division.[13]

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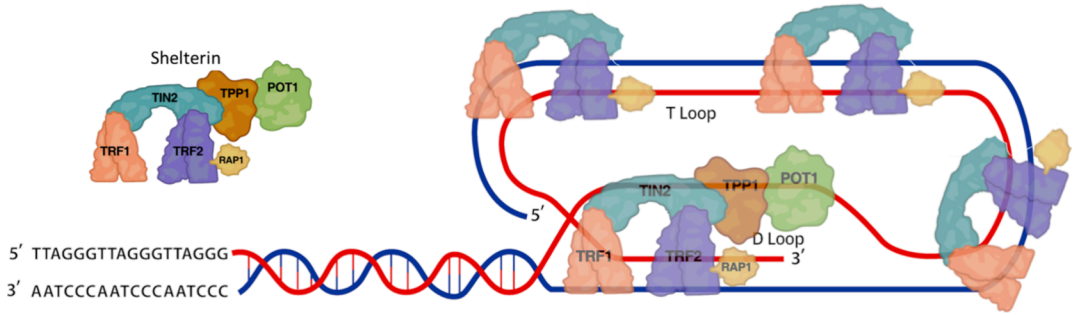

Figure 1. Human telomeres associate with the six-protein shelterin complex. A homodimeric protein called TRF1 interacts with the double-stranded DNA (top strand: TTAGGG). TRF2 aids in the T-loop formation. RAP1, a protein connected to TRF2, prevents DNA repair. POT1 interacts with ssDNA. When TPP1 is lost, POT1 function is hampered. A protein called TIN2 interacts with the TPP1-POT1 complex, TRF1, TRF2, and TIN2.

As a result, it is essential to preserve both the length of the telomeres and the presence of the single-stranded overhang for the stability of chromosomes and the viability of cells. Failure to do so results in end-to-end fusion events and erosion that lead to genomic instability and cell senescence.[14] Furthermore, telomeres have also been linked to sister chromatid pairing during mitosis, homologous meiotic synapses, and the development of nuclear regions that may be critical for transcriptional regulation.[15] When DNA breaks are detected, DNA damage surveillance proteins activate enzymes that break down DNA or fuse chromosomal ends. These biological processes highlight the importance of telomeres in the protection of chromosomal ends.

In addition to protecting the ends of DNA strands, telomeres carry out additional vital tasks such as controlling the expression of genes that are either close to the telomeres (called TPE) or far from them (called TPE over long distances, or TPE-OLD).[16] The minimal length of telomeric DNA repeats and the efficiency of the related protein complexes are two factors that strictly control the function of telomeres.[17] Furthermore, it is believed that appropriate telomere function is aided by higher-order DNA conformations like the G-quadruplexes (G-rich, four-stranded nonhelical structures) and T-loop.[12] Moreover, telomeric chromatin plays a crucial role in signaling, maintaining telomeres, and controlling telomere function; nevertheless, many of the specific molecular mechanisms and structures of human telomeric chromatin remain unclear. Additionally, RNA polymerase II transcribes a lengthy noncoding RNA called telomeric repeat-containing RNA (TERRA) from telomeric DNA in telomeric regions.[18] TERRA has been linked to the regulation of telomerase, the arrangement of heterochromatin at telomeres, the control of gene expression, and the DNA damage response (DDR) that is brought on by telomere malfunction.[19]

MAINTENANCE OF TELOMERES

Recombination and retrotransposition are two examples of the diverse processes that have evolved in various cells to stop the progressive degradation of telomeres.[20,21] Telomerase, a specialized enzyme present in eukaryotes, is an RNA-dependent DNA polymerase complex that helps in the maintenance of telomere length by synthesizing telomeric DNA sequences.[8] Besides maintaining telomeric length in the germline or rapidly dividing cells, telomerase also plays a key role in tumorigenesis and is a hallmark of cancer.[14]

Telomerase is a ribonucleoprotein multicomplex composed of a catalytic protein subunit (hTERT), also known as the TERT reverse transcriptase, and an RNA moiety (hTR). The expression of hTERT protein is not typically observed in normal somatic cells, whereas TER is not only present in telomerase negative cells but is effectively recruited into a fully functional ribonucleoprotein complex upon the introduction of hTERT *in vitro*.[14] hTR is a ubiquitously expressed RNA component that serves as a template for the insertion of TTAGGG repeats to the ends of chromosomes, thereby aiding in the catalysis, localization, and assembly of the telomerase.[22] Telomerase almost universally provides the molecular basis for unlimited proliferative potential. Telomerase is present in ∼85% of all cancer cells and absent in normal somatic cells.[23]

Telomerase differs from other reverse transcriptases in that it carries its template RNA for telomeric DNA synthesis. The RNA component consists of 451 nucleotides. This RNA contains a sequence that is complementary to about 11 bps in humans, acting as a template for telomere replenishment.[21] The template region is longer than the telomeric repeat that it encodes. The longer template plays an important role in both alignment and elongation. By using base-pairing, a piece of the template aligns with the primer 3′ section, and elongation replicates it to the 5′ end. No matter where synthesis begins within the template area, the length of the template guarantees complete replication of the telomeric repeat sequence. The elucidation of telomerase, TER, and its associated protein complexes by the use of cryo-EM places the individual elements in a wider structural context.[24−27] The structures are now available of the human telomerase with telomerase RNA (TER) bound to the shelterin protein TPP1 (PDB ID 7TRE)[26] and a larger complex of telomerase-DNA-TPP1-POT1 (PDB ID 7QXB)[27] that spatially places the key telomere binding proteins in context for recruitment of telomerase to the telomere and its processivity (Figure 1).

TELOMERE BINDING PROTEINS

Telomerase can bind ssDNA *in vitro* and extend primer sequences in the presence of NTPs; but *in vivo*, the appropriate substrate for telomerase activity is not naked DNA repeats but the shelterin complex, which is a collection of six proteins (Figure 1).[28] Telomeric DNA sequences are directly recognized by the three proteins, TRF1, TRF2, and POT1. However, a shelterin complex is created when three more proteins, TIN2, TPP1, and RAP1, associate together. These core proteins act to recruit additional components to the telomere to create multiprotein complexes that help regulate telomere maintenance.[28]

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Telomeric repeat-binding factors 1 and 2 (TRF1 and TRF2) directly attach to dsDNA TTAGGG sequences through a DNA binding domain located at the C-terminal region of the protein.[29] This provides a shielding effect on the chromosomal ends, thereby preventing an incorrect DNA damage response. TRF1 has acidic amino acids close to its N-terminus, while TRF2 has a basic region rich in Gly/Arg. They bind DNA as homodimers or oligomers by homotypic interactions in the TRF homology (TRFH) domain. These proteins connect to arrays of the telomeric sequence TAGGGTTAG with remarkable sequence selectivity. Nonetheless, the protection of telomeres 1 (POT1) protein recognizes the 3′-single-stranded overhang at the end of the chromosomal dsDNA.[30] POT1 protein is present in organisms such as the microsporidia, plants, mammals, and fission yeast.[31] It is a highly conserved protein that is essential for the regulation and maintenance of telomerase. Additionally, POT1 structural domains have been identified either alone or in complexes, such as POT1a bound to dsDNA with a GTTAGG repeat 3′-overhang (PDB ID 8SH1).[32] Another is the c-terminal domain (POT1c) (PDB 7S1O, 7S1T, and 7S1U).[33] The complex describes how POT1a functions to safeguard the key interaction site known as the ds-ssDNA junction. This structural configuration emphasizes how essential it is to cap the phosphorylated 5′ C-rich strand of the junction, inhibit RPA (replication protein A) loading, and restrict ATR (Ataxia telandiectasia and RAD3) recruitment (Figure 2A). Furthermore, the interactions between the POT1 OB/TPP1 binding domains with the shelterin complex bind the ssDNA overhang to the telomeric dsDNA.

Moreover, TRF1- and TRF2-interacting nuclear protein 2 (TIN2) are used by TPP1-POT1 heterodimers to link the duplex section of the telomeres to the single-stranded overhang.[45] TIN2 interacts with TRF1 at its C-terminus, and it binds to a hinge domain at TRF2's N-terminus. Using a third protein interaction site located at its N-terminus, TIN2 binds to TPP1 and forms a complex. Thus, via binding to TRF1, TRF2, and TPP1, TIN2 plays a crucial part in the shelterin complex.[28,34] Repressor/activator protein 1 (RAP1) in humans is dependent on TRF2 for telomere binding, since it is not capable of binding DNA.[48] Through its C-terminal domain, RAP1 interacts with a tiny helical area in TRF2's hinge domain to form a complex.[47]

TIN2 and TPP1 (POT1 interacting protein 1) interacts with POT1 and TIN2 via the POT1-binding domain and the C-terminal of TIN2.[46] POT1's association with telomeres is contingent upon its correlation with TPP1, which functions as the principal route for POT1's recruitment to telomeres. POT1 is known to disrupt the telomeric G-quadruplex (see below), enabling telomerase extension.[109] However, it is believed that RAP1 in yeast binds to telomeric DNA to promote the synthesis of G-quadruplexes,[110] whereas RAP1 in humans is recruited to telomeres by TRF2.[49] It has been shown that TRF2 binds to T-loops and promotes their development (Figure 1).[25] The shelterin complex as a whole controls signaling cascades from chromosomal ends and protects and regulates telomeres.[34]

Synthesis of the complementary C-strand by DNA polymerase α is also connected to telomere maintenance and cannot bind to ss telomeric DNA without the assistance of CTC1-STN1-TEN1 proteins known collectively as the ssDNA binding complex CST.[25,51,52] Subunits of CST control access of telomerase, preventing G-overhang extension, while TEN1

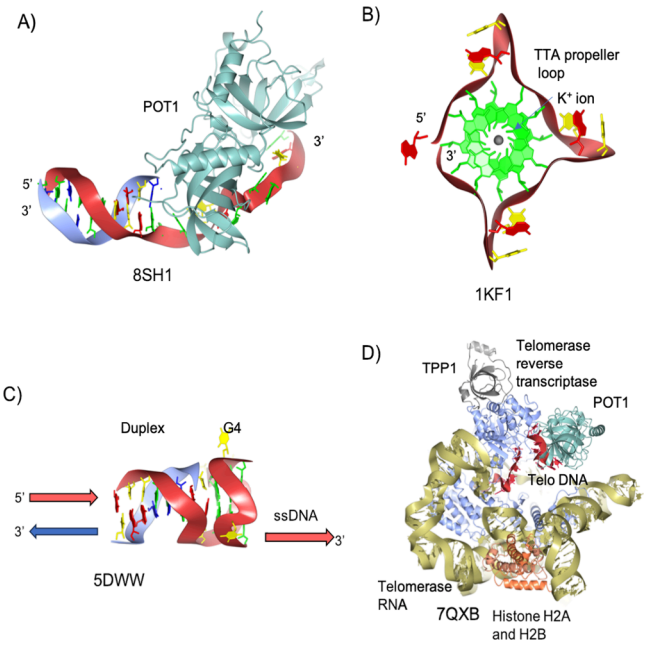

Figure 2. Structures found at the human telomeres. (A) Structure of POT1 (cyan) bound to duplex/single-stranded interface and the 3′ end of unfolded telomeric ssDNA; (B) the crystal structure of the propeller topology of human telomeric DNA G4; (C) structural model of the G4-duplex DNA interface; (D) structure of telomerase holoenzyme illustrating the reverse transcriptase (ice blue)-telomerase RNA (khaki)-TPP1 (gray)-POT1 (cyan)-histone H2A/B (orange)-telomeric DNA (red). Red arrows show the G-rich DNA direction, and blue arrows show the C-rich DNA direction. The corresponding PDB identifiers are listed below each subfigure.

is required for DNA polymerase α-mediated C-strand synthesis. In the cryo-EM structure (PDB ID 8SOK) with and without telomeric ssDNA, the CST complex can be observed to be interacting with POT1/TPP1 revealing how CST recruitment to the telomere is regulated by POT1 and its phosphorylation state.[51]

According to a recent study, human cells may include 200 telomere-associated proteins that interact with and may have an impact on telomeric structure.[111] These were identified via the biochemical purification of the telomeric complexes. Given the wide variety of telomere components, it is possible that human telomeres are extremely malleable in their organization.

HIGH-ORDERED NUCLEIC ACID STRUCTURES AT THE TELOMERES

G-Quadruplexes. Complementary DNA is associated with a double-stranded arrangement; however, it can also form multistranded structures beyond this duplex arrangement by either unzipping and then independently refolding of the two strands into alternative topologies or through the self-association of multiple strands.[81] In regions containing telomeric DNA, the repetitive G-rich sequences can refold into a G-quadruplex (G4) (Figure 2B). Here the core structure consists of stacked G-quartets where the guanines associate with one another via Hoogsteen hydrogen bonding in a coplanar cyclic array, stabilized by eight hydrogen bonds. Through $\pi-\pi$ stacking interactions, G-quartets can successfully stack on top of one another to create four-stranded G4 DNA structures.[112,113] This arrangement results in a negatively charged central channel lined by carbonyl oxygen groups along

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the central axis of the structure. The charge repulsion in the channel is stabilized by the presence of monovalent cations. Each G4 consists of two distinct features: the centrally stacked G-stem and the unpaired bases that connect the guanine stretches to form the loops. The extended G-rich 3′ single-strand overhang at the ends of telomeres lacks the complementary C-rich strand and so provides an opportunity for these motifs to readily form and modulate access of POT1 to the ssDNA ends of the chromosomes.[32] Structurally, it has been observed that G4s formed from human telomeric sequences can exist in multiple topologies.[114] The several kb of single-stranded repeating G-rich sequences present at the telomeric ends can also form multiple G4 units adjacent to each other. These units can then combine to form complex multimeric G4 structures.[115−117] The structure of the interface between dsDNA and G4 has been determined showing G4s stacked externally to the dsDNA providing a model for the 3′ end of a linear chromosome in the absence of POT1 (Figure 2C). The G4s are thermodynamically stable with melt temperature above dsDNA and can be further stabilized by the presence of small molecule ligands.[114] It is worth mentioning that a G4 targeting clinical candidate—QN302—has been approved for Phase 1 clinical trials by the FDA for pancreatic ductal adenocarcinoma.[118,119]

Crucial to the discussion of G4 is the presence of G-quadruplex binding proteins (G4BPs) other than POT1 that perform several important functions like providing stability to the G4 complex as well as facilitating its unfolding.[120] The basic categories of G4BPs are based on the regulatory mechanisms and functional interactions these proteins have with G4s. First, these proteins are classified as G4-folding proteins, which alter G4 structures, and G4-interacting proteins, which are functional proteins recruited by G4. Another way to categorize G4BPs is based on the distribution of G4s in the genome, i.e., DNA and RNA G4BPs.

G4BPs carry out a number of biological tasks like telomere homeostasis, which occurs at the site where telomere-binding proteins form a ternary complex with the G4 telomeric DNA structures.[66] G4BPs such as helicases must resolve the G4s that arise during replication in order for the replication machinery to function properly. DNA2 is a helicase/nuclease protein that was initially identified in yeast but also isolated in mammalian DNA that localizes at telomeres and interacts with shelterin components TRF1 and TRF2. Using the helicase-dead DNA2 mutant protein, it was demonstrated that mammalian DNA2 nuclease identified and cleaved telomeric G4 DNA in a helicase-independent manner *in vitro*, leading to the nucleolytic elimination of both the G4 generated in 5′ flap structures and the telomeric G4 created in template DNA. The standard ssDNA repair apparatus could then probably close the resultant DNA gap in the template.[57]

The 5′−3′ DNA helicase FANCJ (Fanconi anemia complementation group J) is involved in a number of biological activities, including homologous recombination, DNA damage repair, G4 resolution, and maintaining genomic stability.[121] In order to facilitate effective DNA replication, FANCJ may unfold and remove G4 structures; in contrast, lack of it will halt replication at G4s and ultimately result in DNA damage.[122] It has been demonstrated that *S. cerevisiae's* RecQ helicases Sgs1p and BLM preferentially unwind G4s over Holliday junctions. Researchers have also isolated a RecQ-like helicase called the StyRecQL, and it is evident that this helicase is linked to telomerase in the replication band, is drawn to replicating telomeres by telomerase, and plays a role in the unfolding of the G4.

TLS/FUS has also been identified that binds to G4 telomeric DNA and TERRA simultaneously. *In vitro*, a fold in the G4 Htelo and TERRA is the particular target of the C-terminal Arg-Gly-Gly (RGG) domain in TLS, which forms a ternary complex with them. Additionally, TLS binds G4 TERRA *in vivo* and G4 DNA in the telomere double-stranded region.[69] Recently, researchers also isolated a nuclear protein that is highly prevalent in vertebrates called nuclear protein high mobility group B1 (HMGB1). HMGB1 shows a high affinity to bind to noncanonical DNA structures such as hemicatenated DNA loops and four-way junctions in addition to G4 DNA. Furthermore, it exhibits nonsequence selectivity in binding to B-form DNA, resulting in DNA helix deformation and promoting DNA interaction with other nuclear proteins.[71]

To aid in DNA replication, Pif1 helicase, another protein present in yeast cells, may attach to and unfold G4 structures.[60] Pif1 prefers to attach itself to G4 forming sequences in the S-phase of the cell cycle.[61] Pif1 helicase then unwinds any G4 structures, thereby reducing double strand breaks during the cell cycle.[123] In the absence of Pif1, these sites are susceptible to double breaks.[124] Furthermore, Pif1 is also negative regulator of telomerase.[62]

T-Loop Structures. The main function of the long 3′ ssDNA overhang in mammalian telomeres may be to load POT1 and the shelterin complex at the end of the telomere, allowing them to interact with other shelterin complexes attached to other loops around the telomere.[25] When the 3′ ssDNA overhang (TTAGGG in mammals) loops back and is tucked into the double-stranded component of the telomeric DNA molecule, lasso-like three-stranded DNA displacement loops known as T-loops or telomere loops are formed.[125] These loops aid in concealing and protecting the single-strand overhangs of chromosomal DNA.[126] According to a recently suggested T-loop concept,[125] both terminal strands are annealed to their corresponding strands in the form of a bubble. Because this structural configuration possesses traits of both a replication fork and a Holliday junction, the T-loops are more stable within this configuration than with merely paired dsDNA with an ssDNA overhang. To further reinforce the stability of these T-loops, shelterin complexes loaded at the telomeric end preferentially loop back (Figure 1). As stated earlier, loading of POT1 and the shelterin complex at the terminal ends of the telomere allows them to interact with other shelterin complexes attached to different loops along the length of the telomere. This structural configuration, which is observed in naturally isolated T-loops, allows for a wider distribution of sizes for the circular part of the loop.[126]

The recently suggested model of the T-loop bubble states that the resolution of the Holliday junction (HJ) at the site where the two ssDNA cross over one another would result in a covalently closed ssDNA circle that anneals to the strand bearing the free 3′ terminus (Figure 3C,D).[126] A rolling circular replication template that employs regular chromosomal replication components can significantly extend the preceding DNA by employing resolvases such as GEN1 or SLX1/4 to resolve the HJ at the T-loop. This theory offered an alternative mechanism by which the telomere may be extended by the T-loop. As was already established, the involvement of the T-loop in DNA transactions supports their function in telomere homeostasis. The origins of linear chromosomes and

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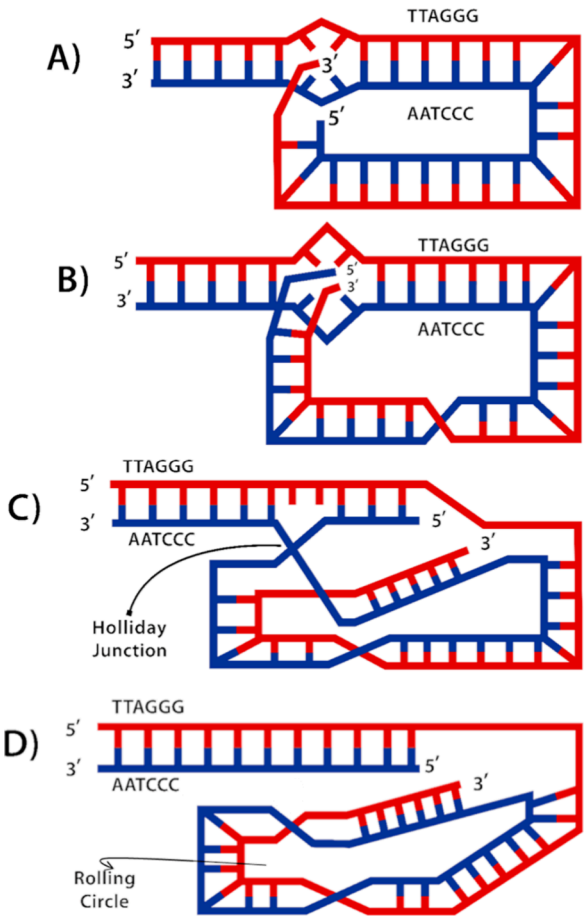

Figure 3. T-loop structures: (A) The integration of the 3′ terminal, which can serve as a replication origin, in both the structures depicted in A and B. Here, only the 3′ single-stranded overhang from the G-rich strand, annealed into the double-stranded DNA, is depicted in the conventional T-loop junction. (B) Terminal strands from a DNA molecule with blunt ends can be introduced to form a more stable T-loop structure. (C) Within the T-loop, an embedded replication origin and a Holliday junction can be observed. The presence of a classical Holliday junction is indicated by the topological equivalence between the structures in B and C. (D) Following resolution of the Holliday junction with resolvases like Gen1 or SLX1/4, a rolling circle replication template is generated.

telomeres as well as how they overcome the difficulties associated with end-protection and end-replication, are still unknown. A recent study identified a potential CDK2 phosphorylation site at Ser365 in human TRF2, which may be mutated to alanine (Myc-tagged TRF2(S367A)) or treated with λ-protein phosphatase to eliminate it.[77] This suggests a mechanism of unwinding the T-loops to facilitate telomere replication. The cell cycle analysis showed that this alteration is significantly less in the S phase but prevalent in the G1, G2, and M phases. This provides a brief window to the RTEL1 helicase in the S-phase during the PP6R3 phosphatase dephosphorylation to temporarily access and unwind T-loops and promote telomere replication.[77]

D-Loops and Their Structures. During the formation of a D-loop, the dsDNA coils around in a lasso-like fashion.[47] It is then possible for the single-stranded 3′ terminus overhang of telomeric DNA to re-enter the dsDNA and produce a displacement loop in the process (D-loop; Figure 3B).[81] Once formed, D-loops are dynamic structures. A DNA polymerase that is committed to using donor DNA as a template during repair can expand D-loops with an annealed 3′-OH end. SGS1-TOP3-RMI1, MPH1, and SRS2 are proteins that dissolve D-loops by the use of helicases or topoisomerases.[80] In the process of heteroduplex rejection, mismatch repair proteins improve the D-loop disruption mechanism as mismatched DNA. Sequence mismatches or differences could occur when DNA strands from different origins, such as sister chromatids or homologous chromosomes, construct a D-loop structure for repair or recombination. These discrepancies may result from DNA mistakes or genetic variances. By identification of these differences and mistakes within the D-loop, the mismatch repair proteins attach to the D-loop. The D-loop is then broken down, ensuring that only correctly matched DNA strands are used for repair or recombination, maintaining the integrity of the genetic material, and halting the spread of mutations.[127] Due to the enzyme's dynamic nature, two broken ends cannot invade the same donor molecule at the same time, resulting in the formation of a double-Holliday junction, or for a single end to invade two different donors at the same time, resulting in multi-invasions (MI). This prevents structure-selective endonucleases from modifying the donors in the covalent downstream covalent process.

D-loops are also important intermediaries during homologous recombination and a crucial step in the DNA double-strand break repair (DSBR) pathway.[84] DSBR is a highly intricate process organized by a sophisticated interplay of enzymes and proteins. Key players in this regulatory network include ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and RAD3 (ATR) related kinases, vital checkpoint enzymes finely attuned to detect DNA damage and activate the ensuing DNA damage response (DDR).[19] ATM primarily addresses double-strand breakages, while ATR is specialized in responding to single-strand DNA damage. Both kinases phosphorylate downstream targets, instigating the initiation of repair processes.[85] Another critical enzyme in this repair cascade is the MRN complex, comprising MRE11, RAD50, and NBS1 (also known as NBN).[88] This multifaceted complex assumes a pivotal role in recognizing DSBs, processing DNA ends, and activating ATM. It serves to prime DNA ends for repair and is implicated in both nonhomologous end joining (NHEJ) and homologous recombination.[89] RAD51, another essential enzyme, operates as a recombinase protein pivotal to homologous recombination (HR). It orchestrates the formation of nucleoprotein filaments on single-stranded DNA, facilitating the intricate process of strand exchange during HR.[92]

Beyond DSBR, several proteins, such as telomerase and shelterin complex components, rely on the structural properties of the telomeric DNA, including the D-loop, to carry out their functions.[81] The D-loop within the telomeric DNA provides a structural feature that guides the telomerase to the appropriate location on the chromosome ends. It serves as a recognition site or a platform for telomerase to bind and accurately extend telomeric repeats. Without the D-loop, telomerase might have difficulty accessing and lengthening the telomeres effectively. The D-loop contributes to the proper assembly and stability of the shelterin complex. The D-loop provides structural cues that assist in the recruitment and positioning of shelterin proteins, ensuring the protection of telomeres from unwanted DNA damage responses.[25,81]

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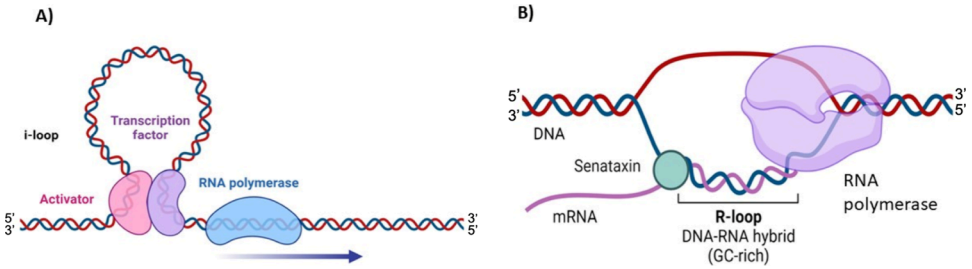

Figure 4. (A) DNA i-loop formation is a crucial step in gene transcription regulation. The DNA double helix serves as the backdrop, with RNA polymerase representing transcription initiation. Surrounding the DNA, transcription factors influence gene expression, and a specific activator protein (pink) triggers transcription in a precise DNA region. (B) The process of transcription with the formation and resolution of an R-loop is facilitated by the presence of RNA polymerase and the enzymatic action of senataxin. RNA polymerase initiates transcription by unwinding a section of the DNA, synthesizing a complementary RNA strand, and temporarily forming an R-loop as the nontemplate DNA strand is displaced and hybridized with the RNA. Senataxin (Sen1/SETX), depicted in purple, plays a critical role in resolving the R-loop, separating the DNA-RNA hybrid, allowing transcription to continue, and maintaining genomic stability. This process ensures the accurate synthesis of RNA transcripts and the proper functioning of transcription machinery.

The length of the D-loops in human telomeric DNA may become longer, making it more difficult to break the D-loop. Therefore, it is possible that the DNA strand invasion machinery (which is by definition a propulsion mechanism for the route forward) is already inducing the reverse reaction by dictating the shape of the D-loop.[81] If that is the case, it may be a component of the regulatory branch, which is responsible for promoting genome stability, and to prevent errors, maintain genomic integrity, safeguarding against mutations and diseases like cancer and act as a quality control system for DNA repair.[81,128]

i-Loop Structures. Damage-induced loops are key intermediates (termed i-loops) that link telomere damage to telomere erosion and the generation of extrachromosomal telomeric t-circles (Figure 4A).[129,130] The development of t-circles might be viewed as a result of chronic telomeric damage brought on by long-term chemotherapeutic actions that promote telomere shortening. Elements that prevent the exchange of chromosomal strands or cause inappropriate single-strand annealing at telomeres hinder the development of i-loops at the site of damage and eventually resist the development of extra-chromosomal t-circles. Additionally, damage to the telomeres would explain why many mutant genes involved in telomere maintenance have t-circles. Branch migration of i-loops, facilitated by specialized helicases such as RTEL1, BLM, and WRN, is self-induced when i-loops act as a substrate for these proteins at telomeric repeats.[130] This process affects i-loop excision, which may reduce the probability of telomerase loss. Noncanonical telomeric repeats present in cells can also cause hindrance to telomerase loss in ALT (alternative lengthening of telomere) cells.[131] Moreover, in yeast to humans, the development of circular DNAs may occur due to i-loops being produced from telomere damage, which occurred in other tandem repeats. Therefore, the i-loop rate would produce more repetitive elements with shorter repeated motifs due to the exposed complementary sequences after telomere damage. Extrachromosomal circles are most likely to be produced by telomeres with a repeat unit of 6 nt as compared to most other lengthy repeat units. The strong proclivity of telomeric repeats to form i-loops that may be excised as circles, resulting in continuous and random variations in the number of repeats, is one explanation for the diversity in the amplitude of telomere length across different chromosomes and cells.[130]

R-Loop Structures. R-loops are unique nucleic acid structures generated when a newly transcribed RNA strand intrudes into the double-stranded DNA region following RNA polymerases, establishing an RNA-DNA hybrid (depicted in Figure 1). This process leads to the displacement of the nontemplate DNA strand, resulting in the formation of a single-stranded DNA (ssDNA) region. In the context of gene transcription, R-loops conventionally stem from regions rich in guanine clusters, also known as G-clusters (as illustrated in Figure 4B).[132] Newly produced RNA is more likely to anneal with complementary ssDNA when these clusters are present.[133] After the formation of the R-loop, stabilization and extension of the RNA-DNA hybridization are achieved, extended by the addition of successive guanine-rich (G-rich) regions. Elongation loses benefits as the structure breaks and the G-rich content decreases.[133]

The necessity for G-rich sequences is diminished, and R-loop formation is facilitated by additional variables. For example, interactions between the template strand and newly transcribed RNA are more likely when there is more negative supercoiling on the transcription bubble's following fork. Moreover, even when the G-rich area is distant from the original G-cluster, nicks in the nontemplate strand can encourage DNA-RNA hybridization of developing RNA to the template strand.[133] Among the many processes that contribute to maintaining the integrity of the transcription bubble and preventing R-loop accumulation are nuclease activity and topological stress reduction. These mechanisms are thoroughly described in recent review papers.[132−135] To summarize, many enzymes work in tandem to prevent the accumulation of R-loops. RNase H1 and RNase H2 use 5′-3′ exonuclease activity to remove RNA from the loop. Since RNA-specific ribonucleases are the only enzymes that are known to break down hybridized RNA, these have been preserved throughout evolution in both prokaryotes and eukaryotes.[95] In addition to RNase H1/2, cells include helicases like mammalian DHX9 and Aquarius that "untangle" DNA-RNA lesions (AQR). Two more helicases that are known to demolish R-loop structures are ATP-dependent DNA helicase senataxin and PIF1, an ortholog of the yeast Sen1p.[98]

Recently, structural motifs with cooperative relationships between G4s, TERRA (see below), and R-loops have been reported.[136] These unique structures, termed G-loops, have been observed in ALT cells, where G4 and R-loop form on

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opposing strands[137] (Figure 5B). The high G-loop levels in ALT cells suggest a plausible role these structural motifs play in

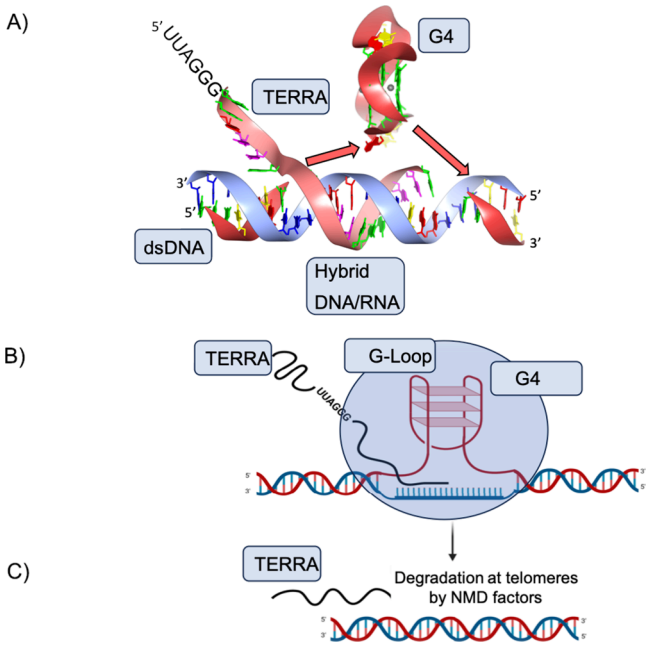

Figure 5. TERRA is a crucial component of telomeric heterochromatin, which also contains UUAGGG repeats formed from the transcription of telomeric regions and subtelomere-derived sequences. (A) A composite image highlighting known structural elements along with 3D spacial arrangements with the necessary rotational alignment and resulting topological complexity of these arrangements, R loop with insertion providing the framework for G4 formation. (B) The formation of the G-loop where the R-loop and G4 occur on opposing strands. (C) The NMD factors that interact with telomeric chromatin physically displace or degrade TERRA at telomeres.

the ALT maintenance mechanism. Moreover, the presence of G-loops is being presented as ALT biomarkers and potential therapeutic targets.[137]

TERRA. Eukaryotic telomeres are transcribed into telomeric repeat-containing RNA (TERRA) despite the fact that they are heterochromatic structures.[4,18] From several, if not all, chromosomal ends, TERRA molecules are translated in the direction of the telomeric DNA. They have UUAGGG repeats formed from transcription of telomeric regions and subtelomere-derived sequences (Figure 5A).[138] It has been demonstrated that an increase in TERRA—the G-rich RNA—during the transcription of telomeres may also result in an increase in homologous recombination.[139] Moreover, it has also been proposed that TERRA interferes with telomerase activity, which is responsible for lengthening the telomeric DNA at the ends of the chromosomes. Mammalian TERRA molecules have 5′-UUAGGG-3′ sequences that complement the hTR component of the telomerase enzyme's template region.[104] Since the TERRA and hTR components complement one another, it is possible that they will attach to telomerase directly and influence its activity. This theory is supported by the discovery that in cell extracts TERRA molecules are linked to human telomerase. Independent of hTR, researchers provided compelling evidence that TERRA molecules engage in interactions with hTERT.[104]

A cellular quality control system called NMD (nonsense-mediated decay) recognizes and breaks down aberrant RNA molecules with early stop codons, halting the synthesis of truncated or dysfunctional proteins.[103] It helps ensure the accuracy and integrity of gene expression by eliminating potentially harmful or nonfunctional RNA transcripts. NMD factors are crucial in maintaining genomic stability, especially at telomeres. These factors physically interact with telomeric chromatin, the DNA−protein complex at telomeres. When they do so, two important actions take place regarding TERRA. First, NMD factors can displace TERRA from its telomeric position by competitively binding to it and in the process effectively removing it from the telomeric region. Second, NMD factors can contribute to the degradation of TERRA, thereby marking it for disposal when it is displaced or targeted by these factors (Figure 5B).[104]

In complex eukaryotes like humans, the recruitment and activation of telomerase at chromosomal ends is not well-known, and the function of EST1A (Ever Shorter Telomeres 1)/SMG6 in this process is unclear although human EST1A/SMG6 physically interacts with telomerase in a manner similar to yeast Est1.[104] Though its effects on TERRA displacement at telomeres imply that EST1A/SMG6 may regulate telomerase through TERRA, its relationship with telomerase is consistent with a role in telomerase regulation.[18] It has been hypothesized that TERRA may control telomerase in a telomere-length-dependent way, since TERRA is more prevalent when telomeres are long and the TERRA-mimicking RNA oligonucleotide $(UUAGGGG)_3$ suppresses telomerase activity *in vitro* as evaluated by the TRAP assay.[140] The discovery that some cancers had lower levels of TERRA than the equivalent normal tissue is also consistent with the idea that TERRA regulates telomerase negatively.[18,138]

By physically interacting with telomerase, hEST1A/SMG6 plays a significant function among the many proteins of the NMD process.[103] Thus, it is possible to hypothesize that human EST1A/SMG6 may affect telomerase via TERRA regulation. Studies both *in vivo* and *in vitro* have demonstrated that TERRA at least partially controls the telomerase.[141] Researchers have shown that there should be a sufficient equilibrium between TERRA formation, telomerase availability, RNA binding protein hnRNPA1, as well as free 3′ overhang of telomeric DNA at a specific period.[142] When TERRA production exceeds hnRNPA1 abundance, hnRNPA1 can access the 3′ end of telomeric DNA and prevent telomerase from extending it, preventing it from binding to the telomeres.[18,138]

Direct correlations between TERRA and homologous recombinant (HR) factors such as RAD51, BRCA1, and RTEL have been shown in recent studies.[93,108,143,144] It was demonstrated that TERRA stimulates R-loop formation at telomeres and starts strand invasion, which is reliant on RAD51. Similarly, RNA binding activity is shown in RAD51-associated protein 1 (RAD51AP1), a component that plays important roles in ALT pathways. Through specific HR intermediates known as DR-loops, RAD51AP1 uses RNA as part of a system that creates R-loops and displacement (D)-loops. The HR-driven DSB repair (HR-DSBR)18 factor BRCA1 (p220) also deals with ssDNA damage at the R-loop termination sites. A recent study has also shown the role of TERRA and RAD51API to the ALT pathways in RAD52 knockout cells by promoting telomeric R-loop formation that leads to G4 formation in telomeres.[106] The dynamic telomeric R-loops generated by TERRA and RAD51AP1 activate the RAD52-independent ALT pathway, which in turn triggers G4

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to orchestrate an R-to-D-loop transition at telomeres to drive break-induced replication of telomeres.[106] These mechanisms are being widely studied for their role in cancer therapies.

i-Motif. i-Motifs are four-stranded DNA structures that are formed in sequences rich in cytosines in the adjacent unpaired DNA strand, just like the G-rich sequences form G4s.[145] The two parallel stranded duplexes associate in a head-to-tail orientation within the i-motif upon the intercalation of the CC + base pair (Figure 6A).[146] At low pH values, this structure is

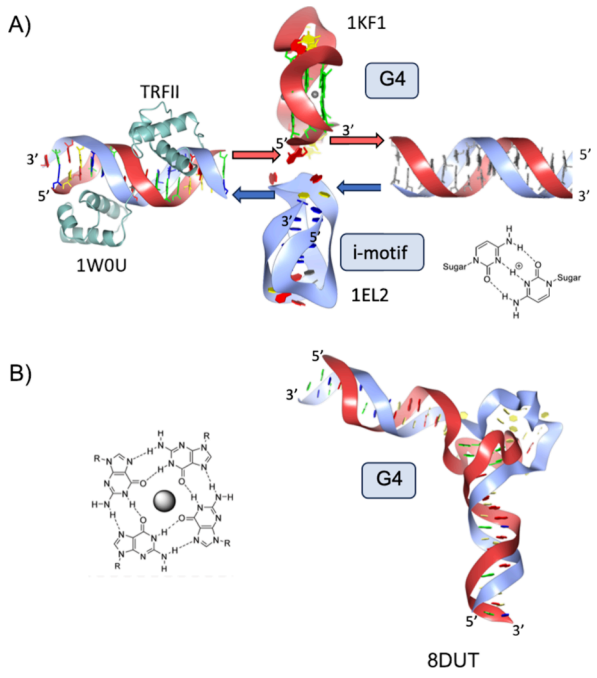

Figure 6. Models of motifs formed from human telomeric sequences. (A) Telomeric dsDNA bound by TRFII and TRFI (PDB 1W0U) adjacent to a bubble allowing the formation of G4 on the G-rich strand (PDB 1KF1) and an i-motif on the C-rich strand (PDB 1EL2). (B) The cryo-EM model of a G4 within the context of an open bubble containing a central G4 was determined (PDB 8DUT). Red arrows show the G-rich DNA direction, and blue arrows show the C-rich DNA 5′-3′ direction. A hemiprotonated cytosine-cytosine (C−C+) base pair and a G-tetrad have also been shown to highlight the hydrogen bonding patterns. The corresponding PDB identifiers are also listed below each subfigure.

stabilized by the protonation of cytosines. It has been proposed that this structure may affect the dynamics of the telomeric DNA duplex and promote its opening.[147] The i-motifs may be classified into two main intercalated topologies: the 3′-E topology, where the outermost base pair (C−C) is located at the 3′-end, and the 5′-E topology, where the outermost base pair (C−C) is located at the 5′-end. Between these two topologies, the 3′-E topology is also more stable.[148] The interactions between sugar−sugar contacts along the tiny grooves, which encourage optimal backbone twisting and the formation of stacking bases, are responsible for the molecule's stability.[149] The overall stability of i-motif structures, however, is determined by the number of cytosine residues that interact with one another. This suggests that when more cytosine residues form hydrogen bonds, the molecule will be more stable. In addition, other factors that affect the stability of the i-motif include temperature, salt content, and environmental pH.[150] A great deal of research has been conducted to understand how i-motif structures behave in various environments. This includes examining the effects of altering the lengths of the cytosine tract and loop, utilizing cytosine analogs that have been epigenetically modified, and changing the DNA backbone as well as other modifications.[151] Since pH plays a crucial part in regulating folding, its impact on the i-motif has been well studied, including how stable it is at various pH levels and how it affects the structure's kinetic and thermodynamic characteristics.[152−154] It should be noted that the thermodynamic and thermal stability are related but not identical properties of i-motifs.[155] Conversely, the impact of the temperature on the structure has received far less attention. Unusual effects related to temperature have been previously noted in the i-motif while investigating its pH-responsiveness.[148,156,157] This includes the isothermal hysteresis in pH transitions[158] and the hysteresis that is frequently seen between thermal melting and annealing curves for the structure.[159] It has also been discovered that kinetic partitioning occurs when a pH drop causes the i-motif to fold quickly into one conformation at first, but over time, it unfolds and refolds to a slower-forming, more stable conformation. As a result, the i-motif structure was described as residing in an equilibrium at a specific pH and temperature where conformers were slowly interconverting.[159] The influence of the temperature is a significant variable that warrants careful examination because of its dynamic character.

The most stable pH range for human telomeric i-motif complexes is ∼6.0.[159] The use of a free proton by the nucleic acids during the folding process has been found to allow certain i-motifs to form at neutral pH.[148] To detect the i-motif complexes, certain parameters must be met, including molecular crowding, negative superhelicity, and a temperature of 4 °C.[160] Furthermore, for i-motifs to be stable at a neutral pH, the superhelicity must remain negative.[161]

HOLLIDAY JUNCTIONS AND TELOMERES

Holliday junctions (HJ) reflect branched nucleic acid structures that consist of two pairs of double-stranded arms joined together.[162] HJs are intermediates of homologous recombination (HR).[163] HR is critical during meiosis because it promotes genetic variety by allowing the flow of genetic material across cells. HJs form a covalent bond between DNA molecules that are undergoing recombination during mitosis, and as a result, they must be removed before the chromosomes are segregated.[164] The inability of the HJ to resolve results in severe mitotic repercussions resulting in the creation of DNA breaks and chromosomal abnormalities.[165] Although high-density lipoproteins are produced to aid in the effectiveness of DNA repair, they are also believed to be toxic, because they have the ability to interfere with proper chromosome segregation (chromosome separation). The primary aim of the HJ is to allow the exchange of distinct portions of genetic information. Three structural arrangements of telomeric HJs have been reported including single, double, and protein-associated HJs (Figure 7A).[166]

In vivo, HJ exhibits different structural variants. When the HJ is free in solution, it acquires a variety of different interconvertible configurations (Figure 7B).[167] The junction expands to an open form in the presence of low salt conditions and absence of multivalent ions, which lessens the repulsion between the negatively charged phosphates concentrated at the junction.[166,168] When either a large concentration of monovalent cations or a high concentration of multivalent

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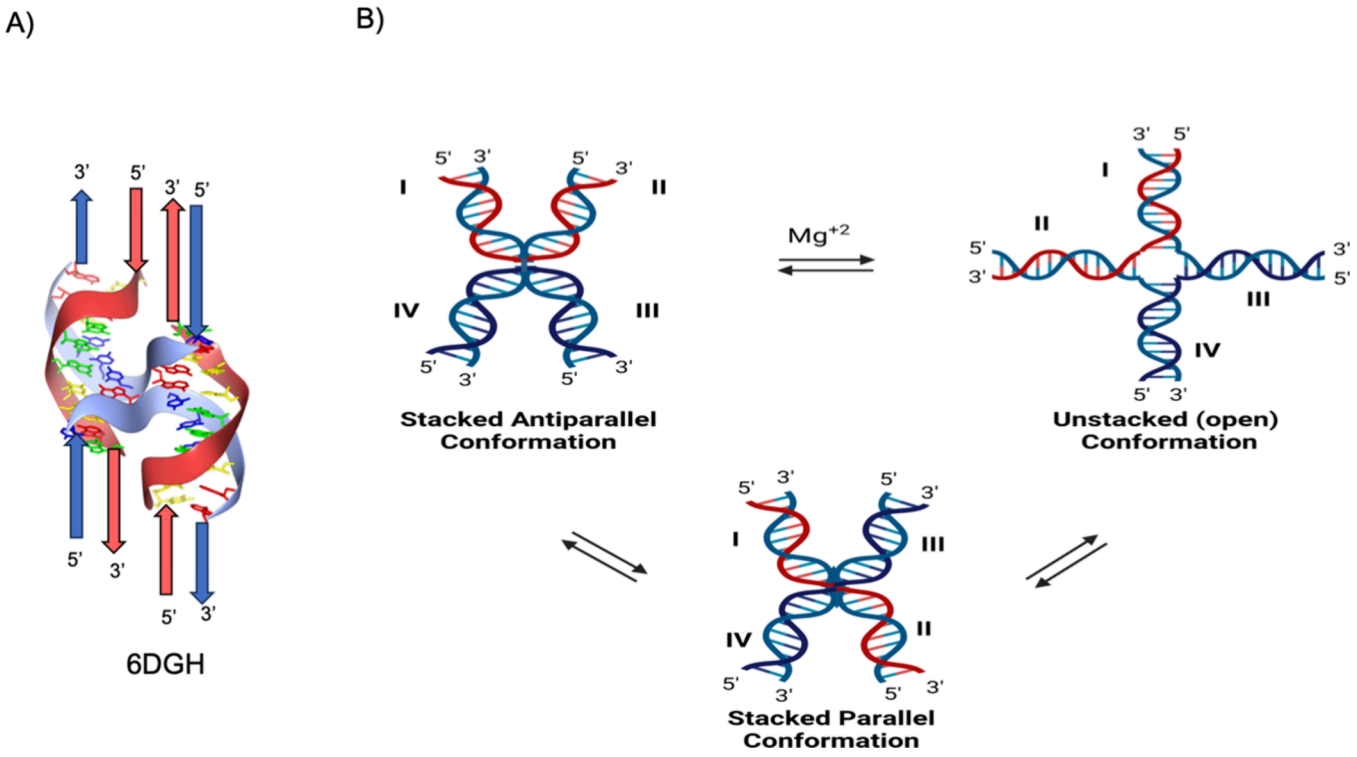

Figure 7. Potential Holliday junction conformations. (A) Crystal structure of a single Holliday junction formed from telomeric DNA folded in the stacked X-conformation (PDB 6DGH). Strand exchange occurs via the C-rich strand. Red arrows depict the G-rich strand; blue arrows depict the C-rich strand. (B) Three conformations of a Holliday junction parallel, open, and stacked-X antiparallel HJs. In the presence of magnesium ions, the HJ formation undergoes a transition from an open to a stacked antiparallel topological state.

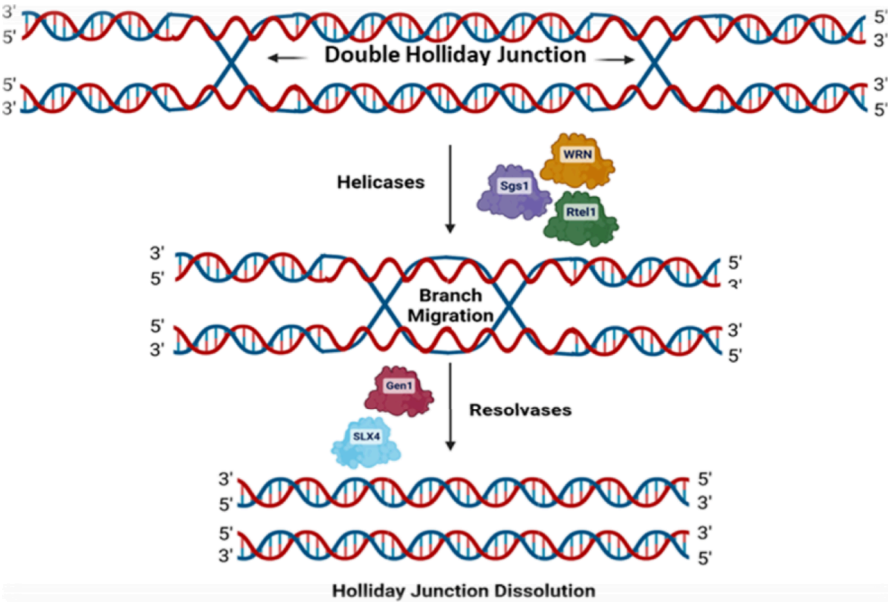

Figure 8. Regulation of double HJ resolution. Several helicase molecules can bring double-Holliday junctions very near together, and then, the Holliday junction can be resolved by resolvases such as Gen1 or SLX1/4.

cations is present, the junction overcomes electrostatic repulsion and folds into one of two stacked conformers.[167,168] Although HJs are produced to aid in the effectiveness of DNA repair, they are also regarded to be toxic because they have the potential to interfere with normal chromosome segregation.[169] The ability to recognize distinct physical and geometric features of the junctions is necessary to comprehend how the HJ is processed by proteins.

Single HJs represent structures that occur independently. In mitotic cells, single HJs are resolved by structure-selective endonucleases known as HJ resolvases.[170] This type of junction typically consists of a symmetrical sequence that allows them to move freely, which means that the four single arms can slide in a particular pattern through the junction depending on the base-pairing.[171] The main goal of single HJs is to facilitate the repair of the breaks encountered in double

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strands. A study initiated by Haider et al. revealed that the C-rich lagging strand contains structural features that constrain crossover geometry and allow the formation of the telomeric HJs.[166]

When two single HJs are topologically linked within shared proximity, they are defined as double-Holliday junctions (dHJ; Figure 8). Like single HJs, dHJs also reflect the critical intermediates of the homologous recombination process.[172] The components consist of separate links that can be cleaved via DNA structure-selective endonucleases, popularly referred to as HJ resolvases.[173] In other instances, dHJ can undergo processing via a reaction known as "the dissolution of double-Holliday junction", which requires the cooperative action of several enzymes.[172] dHJs play the critical role of ensuring the migration of different HJs toward each other to establish a hemicatenated intermediate, which can undergo decatenation via topoisomerase during the dissolution procedure.[172]

At the telomeres, the dHJ helps in restructuring the telomeric proteins within the cells so that the potential chance of cancer can be reduced. Single and double HJs are key intermediates of the ALT pathway.[174] This recombination-mediated telomere maintenance method progresses via the formation of HJ intermediates and is central to telomerase-negative maintenance of telomeres.[174]

CONCLUSION

The ends of chromosomes of eukaryotes are preserved by the telomere, which are structurally composed of nucleoprotein complexes. The telomere is especially exposed to continuous shrinkage as the DNA replicates during the continuous regeneration of tissue, thereby conferring a high risk of chromosomal instability. Telomeric erosion has been observed in aging tissues and hyperproliferative disease states—both of which are associated with an elevated risk of cancer. Telomere protection failure can result in either degenerative aging or malignancy, with the specific outcome determined by the integrity of DNA damage checkpoint responses. It is important to note that the reactivation of telomerase helps to preserve telomere length in many of the advanced cancers, such as epithelial cancers. Numerous regulatory pathways for telomere length regulation have been reported, and genome-scale investigations have aided in the identification of genes involved in telomere length management. These observations underpin the premise that the degradation of telomeres identified in aging people may support the various aging phenotypes. Consequently, a degraded telomere is a representation of a significant genotoxic indicator, which can trigger DNA damage indicating pathways with the ability to speed up aging. It is essential to secure the chromosome ends from the reaction to the triggers due to damage of DNA. The mending of DNA pathways is accomplished by the activity of particular proteins that produce telomeres at the ends of chromosomes. Telomeres must be regulated and maintained because they are heterochromatic and fold into certain configurations (T-loops), or with the formation of G4s at the 3′ ssDNA ends, which might obstruct DNA replication. Telomeres with altered shape or chromosome ends that are severely short generate defective telomeres and eventually result in replicative senescence or chromosome instability. Emerging data suggest that TERRA, a type of long noncoding RNA transcribed at telomeres, is involved in the mechanisms governing telomere preservation and chromosomal end-protection.

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Notes

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