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The human telomere and its relationship to human disease, therapy, and tissue engineering

Ian K. Moon, Michael B. Jarstfer

University of North Carolina, School of Pharmacy, Division of Medicinal Chemistry and Natural Products Chapel Hill, North Carolina 27599-7360

TABLE OF CONTENTS

1. ABSTRACT

The chromosomes of eukaryotes end in a specialized complex of proteins and repetitive DNA called the telomere. Telomeres form a protective cap that prevents chromosome fusions, protects chromosome ends from degradation, and assists in positioning chromosomes in the nucleus. In the absence of replenishing mechanisms, telomeric DNA is lost during each cell cycle owing to incomplete replication, oxidative damage, and nucleolytic degradation. The ribonucleoprotein complex telomerase offsets this loss of telomeric DNA, but its activity is absent in most differentiated human cells. Thus, the aging process results in ever shortening lengths of telomeric DNA. Related to this is the requirement for a mechanism of telomeric DNA maintenance in tumors, leading to telomerase expression in >85% of all cancers cells. The integral roles of telomere biology in these pathophysiological states have substantially motivated its investigation. Here, the literature on the human telomere will be reviewed with an emphasis on the relationship to human health.

2. THE TELOMERE

Linear chromosomes present a significant challenge to cellular life. Left unattended, the ends of linear chromosomes, called telomeres, appear as DNA strand breaks leading to untoward repair, and they are susceptible to erosion by incomplete replication and nucleolytic degradation (1-3). These issues require a significant amount of extra energy to ensure proper telomere maintenance. Despite these problems, all higher organisms, some viruses and some bacteria maintain their DNA as linear chromosomes (4). The results of several studies suggest that telomeres, and therefore linear DNA, are essential for successful meiosis in eukaryotes and are therefore a requirement for sexual reproduction (5, 6). Thus the advantages inherent in sexual reproduction provided by linear chromosomes offsets the penalty inherent in stabilizing and maintaining their ends, at least in higher organisms.

Telomeres, the physical ends of linear chromosomes, are composed of non-coding DNA and

Table 1. Telomere sequences and lengths among telomerase-dependent organisms

Group	Organism	Telomere length, dsDNA	Telomere Length, G-overhang	Telomere sequence (3' strand, orientated 5'-3')
Vertebrate	Human	5 - 15 kb	60-600 nt	TTAGGG
Vertebrate	Lab mouse	~30 to 120 kb	150-200 nt	TTAGGG
Filamentous fungi	*Neurospora crassa*	~150 bp	ND	TTAGGG
Filamentous fungi	*Didymium*	100-400 bp	ND	TTAGGG
Kinetoplastid protozoa	*Trypanosoma brucei*	3 - 20 kb	< 30 nt	TTAGGG
Ciliate	*Tetrahymena*	250-400 bp	14-15 or 20-21 nt	TTGGGG
Ciliate	*Euplotes*	exactly 28 bp	exactly 14 nt	TTTTGGGG
Higher plant	*Arabidopsis thaliana*	2.5 - 5 kb	>20-30 nt	TTAGGG(T/C)
Green algae	*Chlamydomonas*	300-350 bp	ND	TTTAGGG
Insect	*Bombyx mori*	6 - 8 kb	ND	TTAGG
Roundworm	*C. elegans*	2-4 kb	ND	TTAC(A)(C)G(1-8)
Budding yeast	*Saccharomyces cerevisiae*	~300 ± 75 bp	12-16 nt (50 - 100 nt at the end of S phase)	G(2-3)(TG)(1-6)T
Budding yeast	**Candida albicans**	0.5 - 2.5 kb	ND	GGTGTACGGATGTCTAACTTCTT

specific telomere-associated proteins. In the vast majority of eukaryotes, the telomeric DNA is composed of short repetitive sequences that are rich in guanosine. Specifically, telomeric DNA is G-rich in the strand pointing toward the 3'-end of the chromosome, and contains a double stranded region followed by a single strand of the G-rich sequence forming a 3' overhang. The length of the double stranded region, the length of the single strand overhang, and the sequence of the repeat unit is species dependent. For example humans have 5,000-15,000 base pairs of telomeric DNA, depending on tissues and donor age, with the sequence 5'-TTAGGG/CCCTAA followed by a 60-600 nucleotide overhang (Table 1) (7, 8). In this chapter, the structure of the telomere will be discussed with an emphasis on human telomeres and the role that telomere structure and its constituent parts play in human disease, therapeutics and tissue engineering.

3. BIOLOGICAL ROLES OF THE TELOMERE

Telomeres have several roles in ensuring chromosome integrity. The primary role of the telomere is to mask the chromosome ends from the DNA repair machinery that would otherwise recognize them as double strand breaks or homologous recombination intermediates (1, 3, 9). This end capping is the result of several pathways including repression of non-homologous end joining, DNA damage signaling and homologous recombination, and control of telomere processing after DNA replication. Loss of telomeric DNA, a change in the telomeric DNA sequence, or the loss or mutation of telomere proteins prevents proper end capping leading to rapid growth arrest, genetic instability, or cell death. Another role of the telomere involves DNA replication. Normal DNA polymerases cannot efficiently replicate the ends of linear DNA molecules, resulting in the loss of telomeric DNA during each cell cycle. To overcome the loss of DNA, the telomere serves as a substrate for a specialized DNA replication enzyme called telomerase (10), which replenishes telomeric DNA by synthesizing the G-strand. The C-strand is synthesized by the normal DNA replication machinery. Aside from the specific role of the telomere in chromosome-end maintenance, telomeres also function in a broader architectural manner (11-13). Telomeres are associated in the nuclei of human cells, which is increased in quiescent cells and appear to have a cell-cycle dependence (12). Though yet to be proven, it has been proposed that these associations are involved in promoting genetic stability.

Telomeres also play an important role in meiosis by functioning in the meiosis-related nuclear re-organization that facilitates homolog recognition, owing in part to their role in forming the telomere bouquet during zygotene. In the yeasts *Schizosaccharomyces pombe* (14) and *Saccharomyces cerevisiae* (15) and in maize (16), for example, mutations that alter telomere structure and prevent telomere bouquet formation also cause inefficient homologous chromosome pairing, induce aberrant chromosome segregation, and in *S. pombe* cause defective sporulation (17, 18). Similarly, in the organism *Caenorhabditis elegans*, telomere shortening leads to failed segregation of meiotic chromosomes (19). The end result is the formation of infertile gametes. Such effects have not been directly observed in humans; however, the telomerase negative mouse model becomes sterile after several generations suggesting a tie between telomere function and proper meiosis in mammals (20).

The cellular roles of the telomere are clearly manifested in human physiology with ramifications on development (21), aging (22), and tumorigenicity (3). Cellular replication is controlled in part by telomere structure, which is affected by telomere length. Thus, telomere length serves as a mitotic clock that regulates replicative senescence. In healthy individuals, replicative senescence is hypothesized to serve as a mechanism to protect against uncontrolled cell growth related to neoplastic growth. However, the telomere-related mechanisms governing replicative senescence also affect aging and age-related disorders, as evidenced by the direct correlation between telomere length and geriatric-associated disease states. The relationship between telomere maintenance and human health will be explored in detail below with specific attention to the telomere-associated proteins and the structure of telomeric DNA.

4. REPLICATION OF TELOMERIC DNA

Two issues regarding the complete replication of telomeric DNA must be overcome by eukaryotes. First, telomeres contain 3' overhangs, which cannot be produced by leading strand DNA synthesis (Figure 1) (23). Second, the lagging strand synthesis cannot fully replicate the very 5' ends, the so called "end-replication problem" (24, 25). Replication of linear chromosomes is characterized by the progression of a replication fork and coordinated synthesis

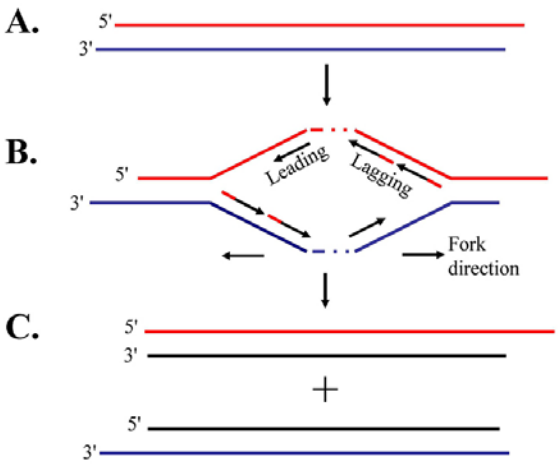

Figure 1. Chromosome end replication must overcome two problems. Lagging strand DNA synthesis does not complete the replication of the 5' end resulting in a 3' overhang, which could contribute to loss of telomeric DNA. Complete leading strand DNA synthesis results in a blunt end though the mature structure contains a 3' overhang. Parental DNA is indicated as either red or blue, and replicated DNA is black. Arrows show the direction of the replication fork.

of the leading and lagging strands. Since replication can only occur in a 5'-3' direction, only the leading strand synthesis moves in the direction of the fork and can continue until the end of a chromosome is reached. Unless the polymerase recognizes the end and stops, leading strand synthesis gives rise to a blunt-ended double stranded product, disallowing the production of the 3' overhang, which is an integral component the telomere structure. The lagging strand, by contrast, is synthesized in a discontinuous fashion, in which RNA primers synthesized by DNA primase are used as substrates to synthesize short Okazaki fragments. Due to the requirement of a template, it is impossible for an Okazaki fragment to be synthesized using the very 3' end of the chromosome as the template, as there can be no RNA primer produced, thus the incomplete replication of the 3' end of the chromosomes. Even if the RNA primer were produced exactly at the extreme 3' end, removal of the primer would result in the loss of 8-10 nucleotides. In addition to this end replication problem, oxidative damage and nucleolytic processing result in the shortening of eukaryotic telomeres by 50-200 nt during each cell cycle, and the rate of telomere shortening is proportional to the size of the 3' overhang (7).

4.1. Telomere maintenance by telomerase

The ribonucleoprotein complex telomerase helps solve both the lagging and leading strand problems associated with reproduction of telomeric DNA (10). Telomerase functions as a reverse transcriptase by using a portion of its integral RNA subunit as the template and the 3' end of the chromosome as a primer for the synthesis of the G-rich strand of the telomeric DNA. Thus, the loss of telomeric DNA can be overcome by extension of the 3' end by telomerase. Furthermore, telomerase synthesizes single stranded DNA, thus it can produce a 3' overhang. However, the biochemical requirement of telomerase for a single strand DNA primer suggests that any blunt end produced by leading strand DNA synthesis must be processed to provide a proper telomerase primer. While the details of processing the leading strand have yet to be revealed, it is apparent that the synthesis of the G-strand and C-strand of the telomeric DNA are coupled in yeast and ciliates and is presumed to be similarly coupled in humans. Recently, it was observed that lagging-strand synthesis generates longer G overhangs at the telomeres of normal telomerase negative human cells than leading-strand synthesis (105-110 nt compared to 60-65 nt on average) (26). Further investigation found that telomerase expression results in elongated overhangs at the leading daughter telomeres suggesting that overhangs are generated differently at the leading and lagging daughter telomeres in human cells.

4.2. Telomere maintenance by ALT

Although most human cancers use telomerase as the mechanism for telomeric DNA maintenance, some use an alternative lengthening of telomeres (ALT) mechanism (27, 28). ALT is characterized by a high rate of telomeric recombination (29) and the presence of ALT-associated PML (promyelocytic leukemia) bodies (30). These are subnuclear structures containing the PML protein, linear and circular forms of telomeric DNA, telomere-binding proteins and several proteins involved in DNA synthesis and recombination. Two limiting mechanisms for ALT maintenance of telomeres can be considered. In the first, homologous recombination occurs between telomeres using telomere sister chromatid exchange (31). In this scenario, sister chromatids asymmetrically align their telomeres allowing for homologous recombination to transfer unequal portions of telomeric DNA, resulting in one sister chromatid with a net gain in telomere length and one sister chromatid with a net loss. Upon cell division, the sister chromatids are separated into two daughter cells, one daughter cell inheriting the telomere depleted chromosome and the other inheriting the telomere elongated chromosome. The daughter cell that inherits the telomere depleted chromosome will reach senescence earlier, and will subsequently perish first, while the one that inherits the telomere elongated chromosome has an increased proliferative potential. It has been proposed that telomere sister chromatid exchange would need to be coupled with unequal segregation at mitosis, leading to segregation of lengthened telomeres to one cell and dispensing of too short telomeres in the other, which would be sacrificed (27). Asymmetrical segregation has been observed in adult stem cells (32), and there is evidence that telomeres may regulate this process (33). A separate model is that the telomeric DNA circles present in ALT-associated PML bodies are expanded by rolling circle replication and recombined with telomeres using the standard mechanisms of sister chromatid exchange (34, 35). In this scenario, the large extrachromosomal telomeric DNA circles abundant in ALT cells may be generated by an incorrect processing of the Holiday junction structure formed by the t-loop (see section 5), where the single-stranded terminus of the telomere inserts itself into the double-stranded telomeric region. After processing, multiple copies of the resulting

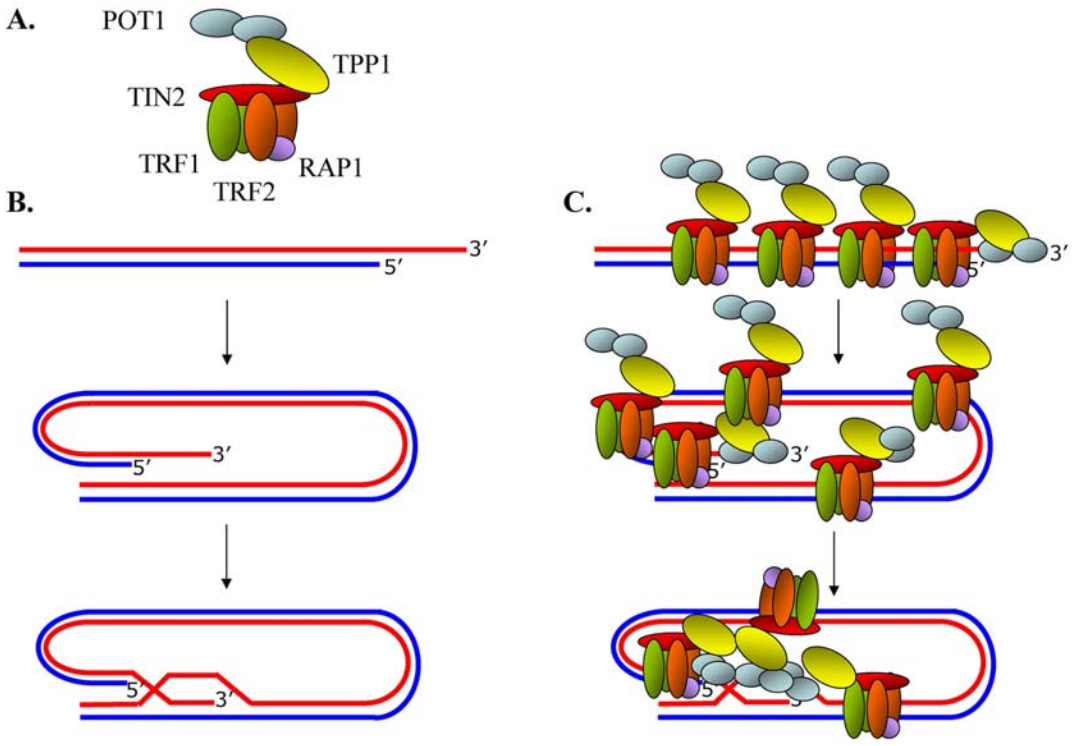

Figure 2. The telomere forms a lasso-like loop structure under the direction of the shelterin complex. A. Six proteins, TRF 1, TRF2, TPP1, POT1, TIN2, and RAP1 form a dedicated telomere-protection protein complex in humans. B. Human telomeres end in a t-loop characterized by invasion of the 3' overhang into a region of double stranded telomeric DNA. C. Shelterin caps the telomere in part by inducing t-loop formation.

telomeric DNA circle could be rapidly synthesized using the DNA replication process known as "rolling circle replication". The multitude of telomeric DNA circles could, in turn, serve as a source of telomeric DNA for maintaining telomeres through recombination with chromosomes via the mechanisms of sister chromatid exchange.

Interestingly, there is some correlation with tumor origin and telomere maintenance mechanism with telomerase activation often favored in tumors arising from the epithelial compartment and ALT more common in tumors that arise from tissues of mesenchymal origin (36). Furthermore, ALT arises more frequently in cultured cells than in human tumors, but is particularly common in specific subtypes of soft tissue sarcomas with complex karyotypes, including astrocytic brain tumors and osteosarcomas, and telomere lengths in ALT cells are more heterogeneous than telomerase positive cancer cells with some very long telomeres (~50 kb) commonly found.

5. THE STRUCTURE OF THE TELOMERE

The telomere is a nucleoprotein complex consisting of telomeric DNA, specific telomere-associated proteins and nucleosome arrays that are characteristic of constitutive heterochromatin (37), which is typical of transcriptionally inactive regions of the genome (38). Telomere-associate heterochromatin is composed of heterochromatin protein 1 (HP1) isoforms HP1α, HP1β, and HP1γ and is enriched with histone 3 trimethylated at lysine 9 (H3-K9) and histone 4 trimethylated at lysine 20 (H4-K20) (38). Several proteins that are associated with telomeric DNA are unique to the telomere. These form a complex defined as "shelterin", and include the double stranded telomeric DNA binding factors 1 and 2 (TRF1 and TRF2), the single stranded telomeric DNA-binding protein Protection of Telomeres 1 (POT1), and three interconnecting proteins RAP1, TIN2, and TPP1 (Figure 2) (9). Together these proteins maintain proper telomere structure and therefore its function.

The higher order structure of the telomere has been examined by electron microscopy and has been observed to form a lasso-like structure termed t-loops (Figure 2) (39). T-loops were first observed in telomeric restriction fragments from human and mouse cells (39). These protein-free DNA samples were psoralen cross-linked to avoid dissociation of the loop structure. Subsequently, isolates of intact telomeric chromatin were also found to form a lasso-like structure (40). T-loops are proposed to form by the invasion of the 3' telomeric overhang into the double stranded region of the telomeric DNA, forming a displacement loop of single stranded DNA that was detected by binding to *E. coli* SSB, a single stranded DNA binding protein (39). Several structures can be envisioned for the knot at the telomeric lasso, but the presence of the displacement loop rules out the possibility of a triplex-type structure. The most likely structure is

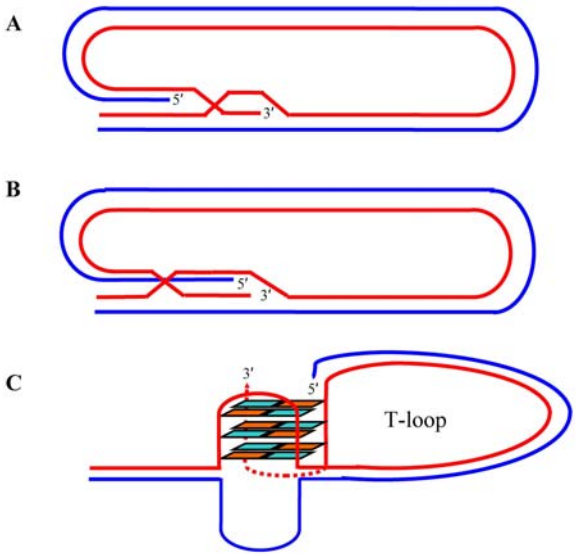

Figure 3. The t-loop can be tied with three different knots. A. The 3' overhang inserts into the double stranded DNA to form a new duplex and a displacement loop. B. The strand invasion structure resembles a Holliday junction after branch migration of the 3' overhang insertion point. C. The 3' overhang associates with the G-strand of the insertion point via G-quadruplexes. The C-strand could either be single stranded or form an I-motif.

simple base-pairing of the invading strand with the C-strand, resulting in displacement of a G-rich strand. A similar, but more stable structure could also form by branch migration to yield a Holliday junction-like structure. However, alternative structures can be considered. One is the formation of a G-quadruplex-like structure between the invading G-rich strand and the G-rich strand at the point of insertion, resulting in displacement of the C-rich strand (Figure 3).

The fine structure of the telomere has also been examined. The lengths of the G-overhang and double stranded DNA of the telomeres found in the macronucleus of ciliates are precisely defined in contrast to the heterogeneous lengths of human telomeres. Thus, it was somewhat a surprise when the terminal nucleotides of C-rich telomere strands were found to precisely terminate in CCATC in 80% of all examined telomeres (41). In the same study, the G-terminal nucleotide was found to be less precise and had a telomerase expression-dependent bias. One contributor to the C-strand end sequence has been identified as POT1 (42). A 10-fold reduction in POT1 by RNAi resulted in a change of the sequence of the recessed 5' end to a random position within the AATCCC repeat.

6. TELOMERE-ASSOCIATED PROTEINS

Proteins associated with the telomere perform several functions including end capping, synthesizing and processing telomeric DNA, and communicating the state of the telomere to other cellular pathways. Telomere-associated proteins can be categorized by their biochemical activity and by their functional specificity. The single telomere-specific DNA polymerase is telomerase, which produces the G-rich DNA strand. A specific telomere-associated protein complex containing TRF1, TRF2, POT1, TPP1, TIN2, and RAP1 is essential for telomere stability. This complex is commonly referred to as shelterin (9) or the telosome (43), and deletion of any of these proteins leads to disruption of telomere-length regulation and end protection. Subcomplexes of shelterin that contain either TRF1 or TRF2 have been observed by several techniques. Several other proteins with major roles elsewhere in the cell moonlight at the telomere, and their deletions have a variety of telomere-maintenance phenotypes.

6.1. Telomerase

Telomerase is a unique DNA polymerase that reverse transcribes a small portion of its integral RNA subunit using the chromosome end as a primer to synthesize the G-rich strand of telomeric DNA (10). A protein subunit that functions as the reverse transcriptase, called hTERT for human TElomere Reverse Transcriptase (44, 45), and a RNA subunit, hTR for human Telomerase RNA (46), are the only components required for reconstitution of telomerase activity *in vitro* (Figure 4). However, other proteins are required for proper assemblage and nuclear localization of cellular telomerase, both *in vitro* and *in vivo* (2, 47).

The endogenous assemblage pathway of human telomerase involves several steps. Generally, hTERT is the limiting factor for telomerase assemblage whereas hTR is actively transcribed in most cells. Transcription of hTR is conducted by RNA polymerase II (Pol II) (46), and the nascent transcript undergoes several modifications that are required for hTR stability. Two separate reports demonstrated that hTR contains a 5'-2,2,7-trimethyl guanosine (TMG) cap (48, 49). The 3'-end of hTR dictates further maturation owing to a box H/ACA domain that is conserved in vertebrate telomerase RNA (Figure 4) (50-52). The hTR box H/ACA domain is absolutely required for the cellular assemblage of telomerase (51) though it is dispensable for *in vitro* reconstitution (53). The box H/ACA motif guides the post-transcriptional maturation of non-coding RNAs and the association of four proteins, dyskerin (Cbf5), NHP2, NOP1 and GAR1 are known requirements for the accumulation of box H/ACA RNAs, including hTR (54-56). The biogenesis of hTR appears to be more complex than other box H/ACA RNAs as the presence of a loop between the box H/ACA motifs is required for cellular accumulation and assemblage. It is proposed that this motif binds to a yet to be defined ribonucleoprotein assemblage factor (51, 54). Further insight into telomerase assemblage has been provided by a series of studies examining the subcellular localization of hTERT and hTR by fluorescence microscopy. Both hTR and hTERT have been observed to accumulate in the nucleolus (57) and in Cajal bodies (58). These structures are known sites of ribonucleoprotein assembly and post-transcriptional regulation, respectively, suggesting that they are sites of telomerase assemblage. Consistent with its Cajal body localization, hTR shares a common Cajal body-specific localization signal with scaRNAs (48). Most of the

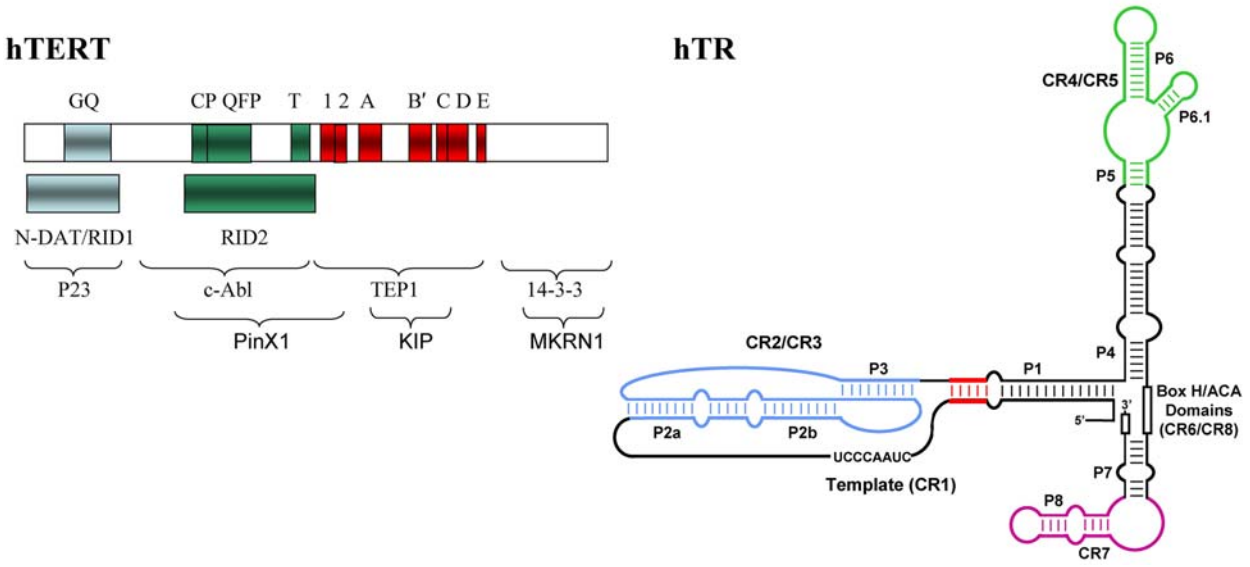

Figure 4. Domain structure of hTERT and hTR. Features of the domain structure of hTERT include the RT motifs (1, 2, A, B', C, D, and E) and telomerase specific T- GQ, CP, and QFP motifs, which were identified by sequence analysis. The RNA interaction domain 1 (RID1), N-terminal dissociates activities of telomerase (N-DAT), and RNA interaction domain 2 (RID2) domains were defined functionally. Positions that have been identified as hTERT binding sites are indicated. Domains of hTR that are conserved among vertebrates are indicated.

evidence points to the Cajal-body as the site of telomerase assemblage. Specifically, the accumulation of hTR in Cajal bodies only occurs in hTERT-positive cells,(58) but hTERT can accumulate in the nucleolus even in the absence of hTR (57). Perhaps unassembled hTERT accumulates in the nucleolus awaiting the signal to assemble with hTR in Cajal bodies.

Purified hTR and hTERT can not assemble into an active complex, but require the presence of crude eukaryotic cell extracts such as rabbit reticulocyte lysate (RRL) to generate active telomerase. It has subsequently been shown that the heat shock protein hsp90 and its partners are the required activity in these crude lysates, and hsp90 is demonstrably required for telomerase activity in cultured cells (59, 60). The role of hsp90 seems to be complex. *In vitro*, hsp90 activity is required to maintain the stability of the hTERT polypeptide and to keep telomerase in a conformation that is competent in primer-binding (60). Within cells, hsp90 inhibition results in proteosome-catalyzed degradation of hTERT, which could be a direct effect, or it could be mediated through signal transduction pathways reliant on hsp90 (61). The finding that hsp90 has not been observed in the nucleolus, but has been seen in the nucleus is consistent with the suggestion that the nucleolus is not the site of telomerase assemblage, assuming that hsp90 is involved in telomerase assemblage *in vivo*. Several other proteins interact with hTERT and many have been shown to regulate its assembly, posttranslational modification, localization, and enzymatic activity (Table 2).

The biochemical activity of human telomerase has been the subject of several studies and has been extensively reviewed (2, 10, 47, 62, 63). Human telomerase appears to be a functional dimeric complex containing two hTR and two hTERT molecules. The role of dimerization, however, is not clear. Human telomerase can bind and extend a variety of primers, even those with only partial telomeric sequences. After binding, primer extension occurs until the end of the template is reached. This template boundary is set in part by the P1 pairing region of hTR. The nascent DNA product can realign with the template allowing further extension owing to a repeat addition processivity activity that is unique to telomerase and allows production of products that contain multiple copies of the telomeric repeat. The importance of this repeat addition processivity *in vivo* is not clear. Human telomerase, like telomerase from other organisms, also has a nuclease activity that seems to mirror flap-endonucleases in its activity (64). The *in vivo* role of this activity awaits separation of function mutants as well as a robust assay.

Human telomerase activity can be reconstituted by adding both the pseudoknot (nts 1-209) and the CR4/CR5 (nts 241-330) domains to hTERT *in vitro* and thus are the only hTR domains required for catalytic activity (53). These domains can also bind independently to mammalian TERTs (65, 66). Accordingly, they have been the focus of several structural studies, and the separate efforts of the Feigon and Varani groups have led to solution structures of portions of these domains (Figure 5) (67-69). As these represent the first three dimensional structure of a human telomerase component, they are critical in understanding the mechanism of telomerase, for targeting telomerase in anticancer drug discovery, and in understanding the role of hTR-related mutations in human disease, which will be elaborated on below.

Table 2. Telomerase-associated proteins

Protein(s)	Role in telomerase function	Other cellular roles
Ku70/Ku80	Binds hTR and hTERT	DNA repair
hEST1a/hEST1b	Positive regulator, binds hTERT	
TEP1	hTR-dependent binding to hTERT	Component of vault particle
KIP	Positive regulator, binds hTERT	Ca^{2+}-binding protein
PinX1	Negative regulator, bind hTERT and hTR (in the presence of hTERT?)	
14-3-3	Regulates cellular location, binds hTERT	Protein localization
MKRN1	Ubiquitinates hTERT leading to its degradation	E3 Ubiquitin ligase
PKCa, PKCg, Akt, c-Acl	Phosphorylates hTERT	Cellular kinases
Hsp90/p23/Hsp70, Hop	Assemblage	Chaperon protein folding and protein-ligand interactions
Stau	Proposed in hTR biogenesis, binds hTR	Transport
L22	Proposed in hTR biogenesis, binds hTR	Ribosome protein
dyskerin	hTR biogenesis, binds hTR	RNA processing

6.2. TRF1 controls telomere length

TRF1 was first identified by biochemical purification using telomeric-DNA affinity chromatography (70). Biochemical characterization of TRF1 revealed highly specific DNA-binding to double stranded telomeric DNA and the ability to remodel DNA including pair telomeric repeats and bend DNA tracts upon binding (71-73). TRF1 is a dimeric protein that utilizes its C-terminal Myb-type DNA binding domain to bind double stranded telomeric DNA. Immunofluorescence and chromatin immunoprecipitation analysis of the interaction between TRF1 and the telomere show a direct and positive correlation between telomere length and telomere-associated TRF1 levels, suggesting that TRF1 binds along the double stranded region of the telomere as opposed to just the very termini (74, 75). Cell-based assays using inducible expression of TRF1 and TRF1 mutants in telomerase positive cells revealed its direct involvement in regulating the length of telomeric DNA. Overexpression of TRF1 led to a telomere shortening phenotype whereas expression of a dominant negative mutant of TRF1, that lacked the Myb domain but retained the dimerization and nuclear localization domains, resulted in telomere lengthening (75, 76). Furthermore, the rate of telomere erosion in telomerase negative cells is not affected by TRF1 inhibition, suggesting that TRF1 exerts its effects on telomere extension (77). Taken together, these data suggest that TRF1 regulates telomere length in *cis* by counting the number of telomeric repeats and inhibiting telomerase activity in a dose-dependent fashion.

TRF1 interacts with several other proteins associated with telomere maintenance. The poly(ADP-ribose) polymerase enzymes tankyrase 1 and 2 ADP-ribosylate TRF1 leading to inhibition of its telomere association (78). As a result, overexpression of tankyrase 1 leads to telomere elongation. The ability of tankyrase to inhibit TRF1 is modulated by the protein TIN2, which protects TRF1 (79). As a result, knockdown of TIN2 results in telomere elongation. TRF1 has also been shown to associate with TPP1, POT1, and the endogenous telomerase inhibitor PINX1 (74, 80, 81). These interactions underscore the reality that several mechanisms can account for the ability of TRF1 to regulate telomere length.

6.3. TRF2 guards and shapes the end

TRF2 was first identified based on sequence homology to TRF1, and like TRF1 it binds to double stranded telomeric DNA as a homodimer that can also form higher order oligomers (82). Also like TRF1, it uses a Myb-type DNA binding domain to bind specifically to the sequence 5'-YTAGGGTTR, where Y is a pyrimidine and R is a purine, and localizes to the telomere (82, 83). The known biochemical activities of TRF2 were expanded when the basic N-terminal domain was shown to bind DNA junctions in a structure dependent fashion. As expected for a shelterin component, several reports have established that TRF2 is essential for telomere protection and telomere-length regulation. TRF2 inhibition leads to end-to-end chromosome fusion, loss of the G-overhang (83), and induction of apoptosis mediated by p53 and ATM (ataxia telangiectasia mutated) kinase (84). The TRF2 inhibition-dependent loss of the G-overhang involves the ERCC1/XPF flap endonuclease (85), and results in the induction of DNA-damage induced foci at telomeres (86). The so called TIFs (for Telomere Dysfunction-Induced Foci) involved several DNA damage response proteins including 53BP1, γ-H2AX, Rad17, ATM, and Mre11. TRF2 also plays a role as a negative regulator of telomere length as its overexpression results in shortened telomere phenotype (77). Interestingly, TRF2 overexpression changes the telomere length that causes senescence in telomerase negative cells to a shorter length (77). This suggests that excess TRF2 can establish a functional telomere structure even under the pressure of reduced telomere length and further suggests that telomere structure and not telomere length initiates telomere-based replicative senescence.

A role for TRF2 in establishing proper telomere structure was revealed by studying the interaction of TRF2 with telomeric DNA using electron microscopy (87). It was found that that TRF2 can remodel model telomeres into t-loop structures. The remodeling activity was sensitive to the presence of telomeric sequence at the junction between the single strand overhang and the double stranded region of the model DNA suggesting that TRF2 recognizes this junction during the remodeling process. The N-terminal basic domain was recently demonstrated to allow structure specific binding of TRF2 to DNA junctions (88). The precise role of TRF2 in t-loop formation *in vivo* has yet to be studied, and will be difficult to establish as

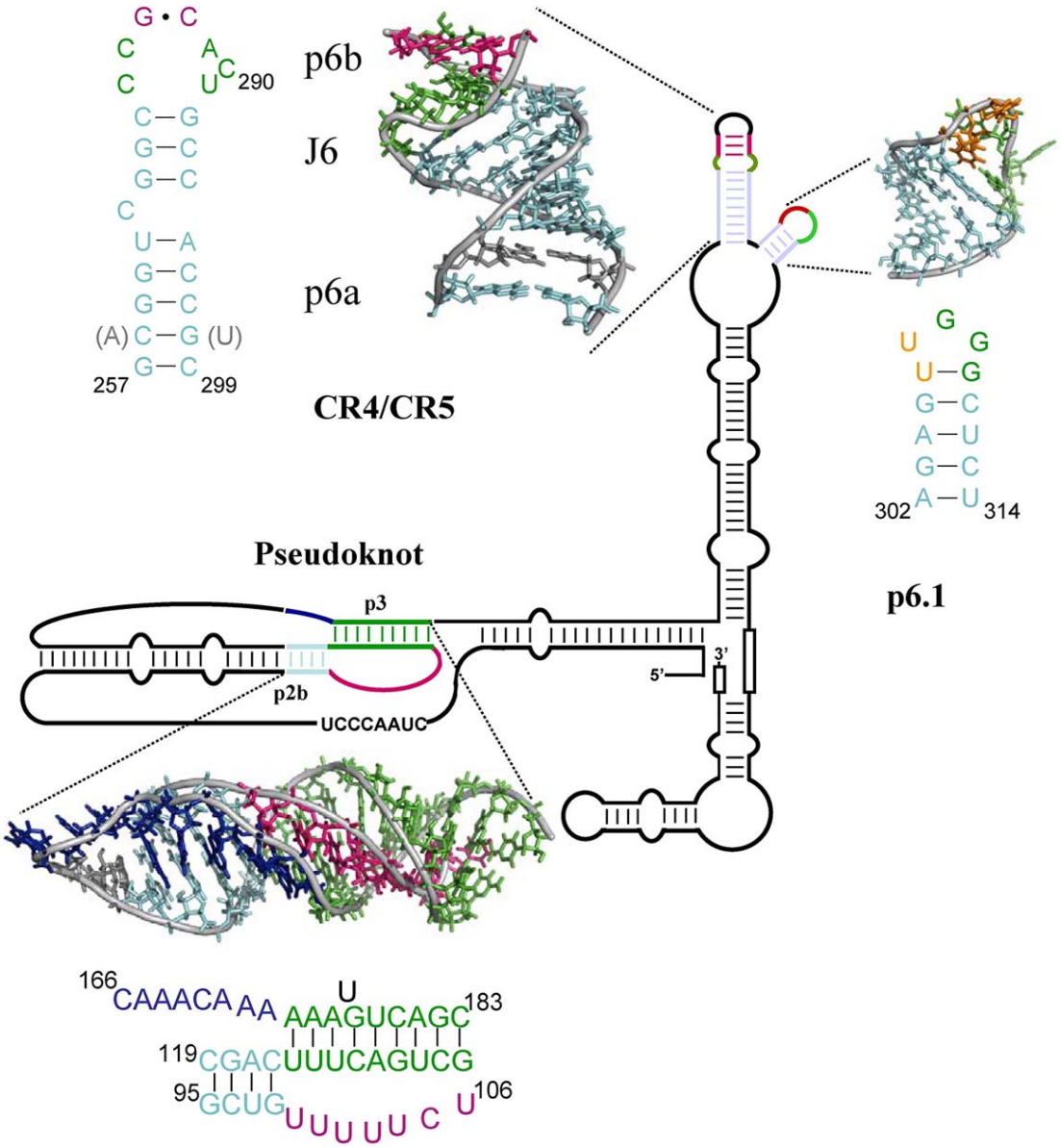

Figure 5. Solution structures of human telomerase RNA domains. Black dashed lines indicate the location of the individual structures in the full-length RNA. The full-length RNA and sequence of the individual structure elements are colored to match the ribbon diagrams. For the pseudoknot, the p2b helix is cyan, the p3 helix is green, the J2a/3 loop is blue, the J2b/3 loop is pink, and U177, which was deleted in the construct used for structure determination is black. For the CR4/CR5 domain, the p6a helix is cyan, the J6 bulge is green, the p6b helix is pink, the p6.1 helix is cyan, uridines in the loop are orange and guanosines in the loop are green. Residues in the NMR structures that are not native to human TR are colored grey. Coordinates for all structures were rendered using PyMol (http://www.pymol.org).

analysis by electron microscopy is currently the only t-loop assay available.

6.4. POT1 is the human single stranded telomeric DNA binding protein

A human single stranded telomeric DNA-binding protein resisted identification until weak sequence homology between a previously uncharacterized *S. pombe* protein and the α subunit of the *Oxytricha* telomere end binding protein was used to establish an evolutionarily conserved family of telomere proteins termed POT1 for Protection Of Telomeres 1 (89). The roles of POT1 in telomere maintenance have been investigated by several groups. Two studies revealed that POT1 can be a telomerase-dependent positive regulator of telomere length by observing an increase in telomeric DNA upon ectopic expression of POT1 (90, 91). Expression of a N-terminally truncated mutant of POT1 that lacks its DNA-binding

domain also resulted in telomere lengthening, leading to the suggesting that POT1 is responsible for mediating the TRF1-effect on telomere length (74). Partial POT1 inhibition (10 fold reduction) by RNAi caused a reduction in the 3' overhang DNA, a transient DNA damage response in G1, and a change in the sequence of recessed 5' end of the telomere, which normally ends on the sequence ATC-5', to a random position within the AATCCC repeat (42). In two separate studies, POT1 inhibition resulted in telomere instability leading to apoptosis or senescence, though at a lower frequency than in TRF2 inhibition studies, in support of a role for POT1 in telomere capping (92, 93). Interestingly, POT1 overexpression was found to rescue telomere instability resulting from expression of a dominant negative mutant of TRF2 (93). Perhaps, POT1 can block the nucleolytic degradation observed when TRF2 is inhibited.

With respect to its biochemistry, human POT1 binds as a dimer with specificity for the sequence 5'-TAGGGTTAG-3' at both the 3' end and at internal sites (94). The structure of POT1 in complex with DNA has been solved by X-ray crystallography (95). Because it binds to the same substrate as telomerase, single stranded telomeric DNA, it might be expected to affect telomerase activity. In fact, the effect of POT1 on telomerase is complex and depends on the specific experimental setup. For example, if the DNA primer used for telomerase assays was the consensus POT1 binding sequence, 5'-(GGTTAG)-$_3$, than POT1 inhibited telomerase-catalyzed primer extension. However, inhibition was decreased by adding two G's to the 3' end of the primer, 5'-$(TTAGGG)_3$ (96, 97). Telomerase was more processive when using the POT1-bound 5'-$(TTAGGG)_3$ as a primer, perhaps as a result of disrupting G-quadruplex structures that form in the nascent DNA product. This model is consistent with the observation that POT1 can disrupt intramolecular G-quadruplex structures, allowing telomerase to extend these otherwise inert primers (98).

6.5. TIN2 and TPP1 anchor the telomere-binding proteins in a functional complex

TIN2 and TPP1 are telomere-associated proteins that do not bind DNA but instead function as scaffolding for the shelterin complex (80). TIN2 was originally identified through a screen for TRF1-binding proteins (99), and TPP1 (formerly named PTOP/PIP1/TINT1) was identified through screens for TIN2- and POT1-binding proteins (100-102). TIN2 binds TRF1 with its C-terminus and alters its activity in at least two ways. *In vitro* DNA-binding assays demonstrated that TIN2 augments the ability of TRF1 to catalyze telomeric DNA clustering, that is the ability of TRF1 to bring together and align separate strands of telomeric DNA (103). As mentioned above, TIN2 also protects TRF1 from tankyrase activity, despite bridging the interaction of tankyrase to TRF1 (79). These effects are presumably a result of a TIN2-induced conformational change of TRF1. Because TIN2 mutants that abrogate this TRF1-induced telomere clustering also increase telomere length in cultured cells, it seems that this activity is in part responsible for the role of TIN2 as a negative regulator of telomere length. TPP1 also functions as a negative regulator of telomere length. TPP1 binds directly to POT1 and TIN2 and helps to stabilize the interactions between TRF1, TIN2, and TRF2 (80).

6.6. RAP1

RAP1 was originally identified by a yeast two-hybrid screen for TRF2 interacting proteins and associates with the telomere through the interaction of its C-terminus with TRF2 (104). Overexpression of RAP1 resulted in telomere shortening whereas expression of dominant negative mutants of RAP1 gave rise to telomere lengthening, consistent with the conclusion that RAP1, like several other shelterin components, is a negative regulator of telomere length maintenance (105). The association of RAP1 to other shelterin components seems to be bridged completely by TRF2 (80).

6.7. Telomere-associated proteins not uniquely associated with the telomere

In addition to these telomere-specific proteins, several others function at the telomere. These proteins can be classified either as DNA processing proteins or enzymes involved in post translational modification, such as the poly(ADP-ribose) polymerase enzymes tankyrase 1 and 2 that regulate TRF1 (78). Several nucleases have been indicted in telomere maintenance. The ERCC1/XPF flap endonuclease associates with TRF2 and the telomere and mediates the telomere-length effect associated with TRF2 inhibition (85, 106). The absence of ERCC1/XPF results in extrachromosomal circles containing telomeric DNA. The authors proposed that these originated from recombination of a telomere with an interstitial, telomere-like sequence. Thus, the role of ERCC1/XPF at the telomere may be to prevent such recombination events. The Mre11 complex, Mre11/Rad50/Nbs1, regularly functions as a DNA repair complex and it accumulates at the telomere after the onset of telomere damage. A role of the Mre11 complex in normal telomere end processing is suggested by several lines of evidence. Reduction of the Mre11 complex by RNAi resulted in a transient shortening of G-overhang length in telomerase-positive, but not telomerase negative cells (107). In a separate study, the Mre11 complex was reported to be recruited to the telomere at G2, when telomeres were also found to be normally uncapped (108). The targeted degradation of the Mre11 complex resulted in the telomere being exposed throughout the cell cycle concomitant with an increase in telomere fusions. These results suggest that the Mre11 complex is directly involved in telomere end processing, perhaps to recruit the other processing machinery required. The Mre11 complex has been found associated with telomeric DNA in ALT-associated PML bodies (APBs), and is required for the ALT phenotypes in some ALT cells (109).

The Bloom's (BLM) and Werner's (WRN) helicases, which are associated with diseases involving genetic instability, have been implicated in telomere maintenance (110). WRN, a RecQ helicase with 3' exonuclease activity, interacts with TRF2 and localizes at telomeres (111). A critical observation regarding the role of WRN in telomere maintenance was made by the Kalseder lab. They showed that the loss of WRN resulted

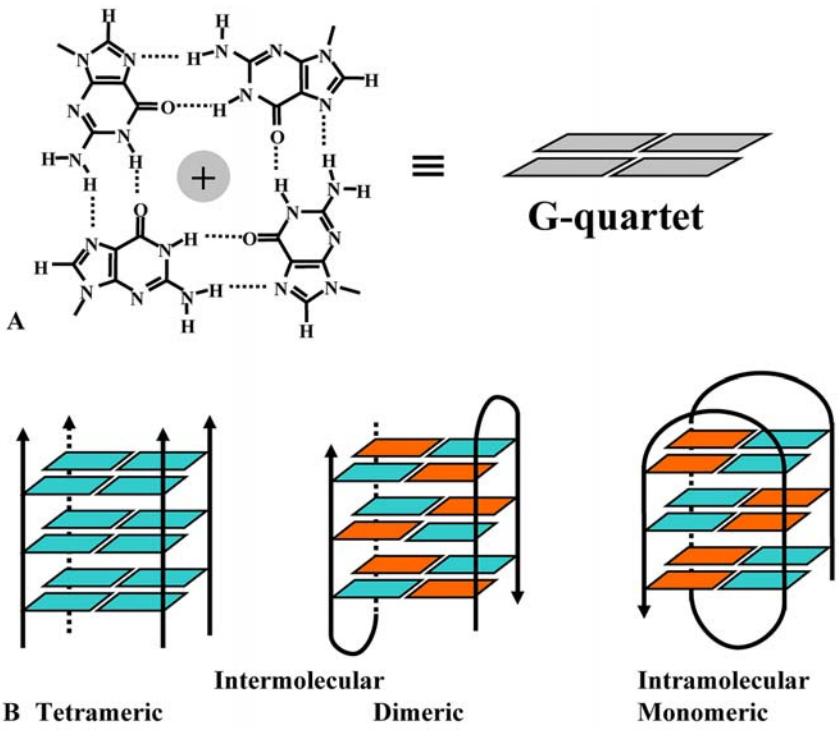

Figure 6. G-quadruplex structures. A. The chemical structure of the G-quartet forms a network of Hoogsteen base pairs with a monovalent cation at its core. B. DNA with runs of guanosine rich sequences can form several quartets that stack to form a variety of G-quadruplex structures. A single DNA strand can fold upon itself to form an intramolecular G-quadruplex, while two and four DNA strands can assemble into dimeric and tetrameric structures, respectively. The arrows indicate the parallel or anti-parallel orientation of DNA strands. Guanosine residues are colored according to the configuration of the glycosidic bind. *Anti* guanosines are colored aqua and *syn* guanosines are colored orange.

in deletion of telomeres from single sister chromatids, but only telomeres replicated by lagging strand synthesis were affected (112). Possible roles of WRN in telomere replication are suggested by two of its biochemical activities. In biochemical assays, WRN has been shown to unwind G-quadruplex DNA and to resolve telomeric D loops (113). Furthermore, its activities are stimulated by TRF2. Thus, a model for WRN at the telomere is that WRN assists in the resolution of t-loops to allow telomeric DNA extension and removes G-quadruplexes, which may hinder DNA replication. BLM is also a RecQ helicase and has been established as a telomere associated protein through its interaction with telomeric DNA in the presence of TRF1 and TRF2, and its ability to associate with telomeric DNA by chromatin immunoprecipitation. The ability of BLM to unwind duplex telomeric DNA is stimulated by TRF2 but inhibited by TRF1, consistent with a role in replication of telomeric DNA (114). POT1 also stimulates the ability of BLM and WRN to unwind telomeric DNA, further drawing a connection between these helicases and telomere maintenance (115). BLM has a unique effect in ALT cells, where its overexpression results in increased telomeric DNA, whereas BLM overexpression has no reported effect in telomerase positive cells (116). This result suggests that the role of BLM in telomere maintenance is related to the nature of the repetitive telomeric DNA, and separate from a telomerase-related function.

7. THE STRUCTURE OF TELOMERIC DNA

7.1. Single-stranded telomeric DNA forms G-quadruplexes

Because single stranded telomeric DNA contains repetitive tracks of G-rich DNA, it has a propensity to fold into structures known as G-quadruplexes (Figure 6) (117, 118). These unique nucleic acid structures are stabilized by a tetrad of guanosine residues that are connected by a network of Hoogsteen base pairs centered on monovalent cations, typically K^+ or Na^+. The guanosine quartets stack onto each other in folded molecules to form the higher order structures known as G-quadruplexes. A variety of folding topologies have been observed for G-quadruplex structures, and their variance depends on several factors including strand stoichiometry, the pattern of the strand orientation, the sequence intervening between the runs of guanosine, the identity of the associated monovalent cation, and the annealing conditions. Sequences with single runs of guanosine can form four stranded intermolecular structures. These are characterized by both a parallel strand orientation, i.e. all strands are aligned in the same 5' to 3' orientation, and by the anti configuration of the glycosidic bonds of each guanosine residue. DNA strands with two runs of guanosine can form a variety of dimeric, trimeric, and tetrameric structures. Single DNA strands with four runs of guanosine can fold into intramolecular structures as

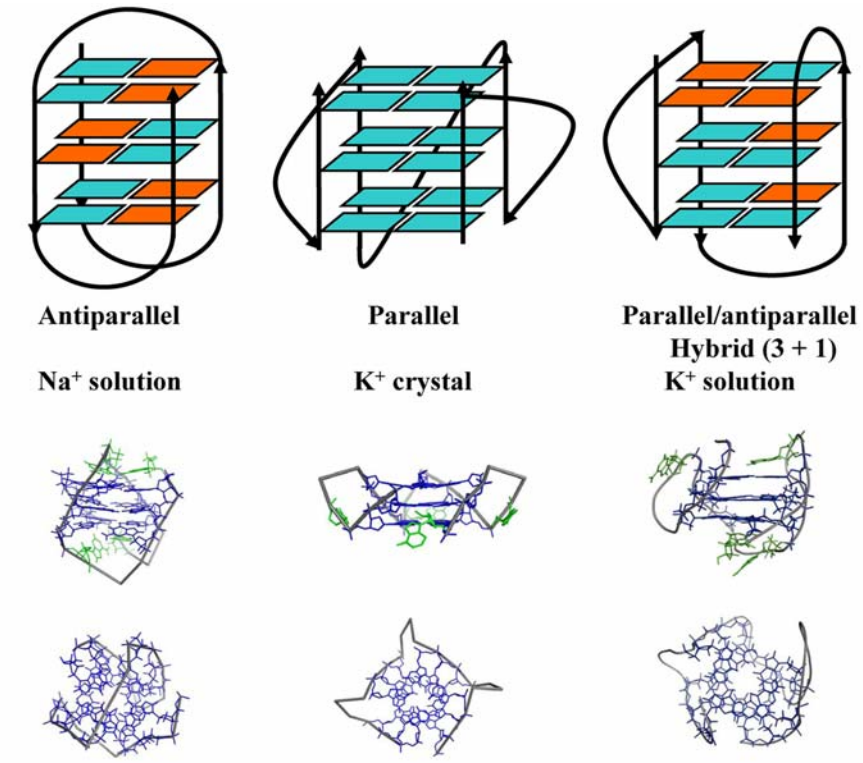

Figure 7. The structures observed for the intramolecular quadruplex formed by the human telomeric repeat sequence. A. The solution structure in Na^+. B. The crystal structure in the presence of K^+. C. The solution structure in K^+. Schematic structures are colored as in Figure 6.

well as various intermolecular structures, depending on the folding conditions.

The structures of G-quadruplexes formed by DNA strands with one, two or four repeats of the telomeric DNA sequence have been examined by several biophysical techniques including NMR and X-ray crystallography. The sequence $d(T_2AG_3)$ anneals to form a four stranded, parallel quadruplex with all guanosines in an anti configuration, as expected for a strand with a single repeat of the telomeric sequence (119). Dimeric structures formed by strands with two repeats of the human telomeric sequence are much more complex. In the presence of K^+, the two repeat sequence d(TAGGGTTAGGGT) forms both parallel and antiparallel dimeric structures that can interconvert in solution (120). In a K^+-containing crystal, the sequence was observed as a parallel dimer (121). An interesting structure was observed for the three repeat sequence $d(G_3T_2A)_2GGGT$, which was observed as a dimeric complex with all the guanosine from one strand forming three of the corners of the quartet and one set of guanosine from the other strand forming one side of the structure. The overall folding topology has been classified as a (3 + 1) assembly because three strands are parallel, and one strand is antiparallel (122). The intramolecular G-quadruplex formed by oligonucleotides containing four repeats of the telomeric sequence has been observed as a parallel propeller-like assembly in K^+-containing crystals (121), an antiparallel cross-over basket in solution in the presence of Na^+,(123) and as a mixed (3 + 1) assembly in solution in the presence of K^+ (Figure 7) (124, 125). The diversity of possible structures is highlighted by single molecule fluorescence studies that suggested the coexistence of at least two interconverting structures (126, 127). These structures are likely (3 + 1) structures, as two separate folding topologies of the (3 + 1) assembly have been observed by NMR (128).

7.2. *In vivo* relevance of G-quadruplex structures of telomeric DNA

The recognized ability of telomeric DNA to fold into G-quadruplex structures has sparked a significant level of interest into the biological significance of these structures, and despite the ability to form *in vitro*, the *in vivo* importance of G-quadruplex DNA at telomeres is still remarkably controversial. The best evidence for the presence of G-quadruplex DNA at the telomeres of an organism comes from work with the ciliate *Stylonichia lemnae* (129, 130). In a series of experiments, synthetic single-chain antibody fragments specifically targeted against the intermolecular G-quadruplex assembled from the ciliated protozoan telomeric sequence were used to examine the telomere structure. The antibody Sty 49, which recognizes both parallel and anti-parallel quadruplexes but not Sty 3, which recognizes only the parallel quadruplex, provided a strong signal in the macronucleus, but not micronucleus, of vegetative *Stylonichia*. The authors deduced that only an anti-parallel

dimeric G-quadruplex exists *in vivo*. Though, the existence of parallel G-quadruplexes can not be dismissed. The evidence for G-quadruplex structures at human telomeres comes from the use of G-quadruplex ligands to detect this structure on human chromosomes. A novel fluorescence ligand, 3,6-bis(1-methyl-4-vinylpyridinium)carbazole diiodide (BMVC), gave a G-quadruplex-specific signal when incubated with human chromosomal DNA (131). In a separate study, detection of a G-quadruplex structure was achieved by isolating metaphase spreads of normal human and tumor cells cultured in the presence of the radiolabeled G-quadruplex ligand ^{3}H-360A followed by autoradiography (132). Both studies report that their respective ligands bound preferentially to terminal regions of the chromosomes. One caveat from these experiments is the possibility that the G-quadruplex ligand is trapping G-quadruplex structures that normally would not exist in the absence of ligand.

Despite the lack of an established functional role for G-quadruplex DNA at the telomere, the incontrovertible fact of the inherent ability of quadruplex formation by telomeric DNA suggests that their presence can arise within the cellular environment. This led to investigation of selective G-quadruplex stabilizing and binding ligands, which is explored further below. Furthermore, it suggests that proteins that recognize G-quadruplex DNA should exist, as is indeed the case.

7.3. Proteins that interact with G-quadruplex structures of telomeric DNA

The growing list of proteins that bind to, cleave, resolve or promote the formation of telomeric G-quadruplexes lends support to the view of the quadruplex as a biologically significant entity. Human Topoisomerase I can bind to pre-formed inter- and intramolecular G-quadruplexes and catalyze G-quadruplex formation from single stranded DNA (133). Notably, the G-quadruplex binding inhibits normal topoisomerase I DNA cleavage activity (134). As noted in section 6.7 above, the helicase BLM and WRN unwind telomeric DNA. This activity occurs with a 3'–5' polarity and requires ATP, Mg^{2+} and at least one single-stranded 3' tail (135, 136). Zaug and colleagues showed that POT1 is able to disrupt an intramolecular telomeric G-quadruplex, thus allowing telomerase-catalyzed primer extension in vitro (98). Based on the experiments performed, it appeared that POT1 may be simply trapping the single stranded G-quadruplex DNA resulting in a shift of the equilibrium to the unfolded state, as opposed to a catalytic process. Several recent reports on heterogeneous nuclear ribonucleoproteins A1 (hnRNP A1) and D (hnRNP D) have demonstrated the ability of these single strand telomeric DNA binding proteins as telomeric G-quadruplex resolvases (137-139). hnRNP D, alias AUF1, uses its C-terminal BD2 domain to reduce the presence of a G-quadruplex DNA signal when incubated with folded human telomeric DNA, as measured by circular dichroism and chemical shift perturbation studies. Based on these results, it was postulated that BD2 can actively unfold the quadruplex (137). However, the results do not rule out the possibility that that BD2 simply traps the dissociated form of the quadruplex in a passive manner as shown with POT1. hnRNP A1 has also been shown to disrupt telomeric G-quadruplexes leading to a model for the stimulatory effect of hnRNP A1 on telomerase activity (138). In the model, hnRNP A1 stimulates telomerase by resolving G-quadruplexes present in the telomerase product facilitating the translocation step of telomere elongation. hnRNP A1 expression has also been shown to lengthen telomeres in mice, consistent with a role as a positive regulator of telomerase (140). Replication protein A (RPA) unwinds G-quadruplex DNA in an ATP independent reaction that does not require a 3' tail (141). RPA accelerated the rate of G-quadruplex dissociation by ten fold compared to the complementary DNA sequence for one population of G-quadruplexes reported to exist in the reaction mixture. Using biochemical purification guided by tetrameric G-quadruplex resolving activity, the gene product DHX36, a DEXH –type helicase, was identified from human cell lysates as a G-quadruplex resolvase (142). Studies examining telomeric substrates are apparently underway.

8. THE RELATIONSHIP OF THE TELOMERE TO CANCER AND AGING

Intact telomeres are essential for genetic stability. As a consequence, loss of telomere function has a profound physiological effect. Generally, the most common occurrence leading to dysfunctional telomeres is short telomeres. However, it is possible for a cell to have normal telomeric DNA lengths and still not form a properly capped telomere. A simple mechanism to overcome a deficiency in telomere maintenance is to express telomerase or activate the ALT pathway. These provide a means to replenish the telomeric DNA. However, telomerase is down regulated in almost all terminally differentiated human cells and ALT appears to be latent in normal cells (27). As a result, cultured somatic cells have a limited replication capacity. Cellular replication leads to loss of telomeric DNA. When the telomeres have reached a critically short length, cells irreversibly arrest growth. This senescence pathway requires cell cycle and DNA damage checkpoints controlled by Rb/p16 and p53. In the absence of the Rb/p16 pathway, telomere dysfunction can lead to apoptosis, and in the absence of both Rb and p53, genetic instability ensues, which may produce surviving cells typically characterized by chromosomal rearrangements (143). There are two significant ramifications of telomere biology on human health. First, telomere erosion attending progression of human lifespan establishes the correlation between telomere length and age. Second, the requirement of a cellular mechanism for telomeric DNA maintenance has consequences for cancer cell biology (Figure 8).

8.1. Implications of telomere biology on cancer

Telomeres are important components of the initiation and progression of neoplastic growth in two key ways. Most obvious is the inescapable requirement for a mechanism to maintain telomeric DNA that makes telomerase so commonly expressed in cancer cells. Though less common, some cancer cell types (10-15%) use the ALT pathway, which is prevalent in astrocytic brain

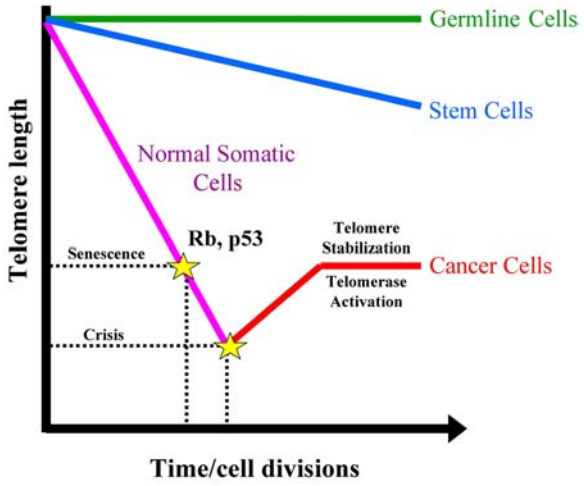

Figure 8. The relationship between telomere length and cellular aging. Telomere length, represented on the ordinate, is progressively lost during successive rounds of cellular replication, represented on the abscissa, in telomerase negative somatic cell lines. This leads to Rb- and p53- mediated growth arrest, marked as senescence. Inactivation of p53 and Rb function bypasses senescence leading to further cell growth and further telomere erosion. Telomere shortening leads to genetic instability and crisis. Rare survivors reactivate a telomere-stabilization pathway, either telomerase or ALT. Germline cells are telomerase positive and maintain the lengths of their telomeres. Pluripotent stem cells express some telomerase activity, but not enough to fully maintain the full length.

tumors and osteosarcomas (28). Second, the overall length of telomeric DNA and the actions of telomere-associated proteins affect genetic stability and can activate the senescence pathway, which suppress neoplastic transformation. In the absence of the Rb/p16 and p53 pathways, however, senescence is not activated, leading to genetic instability and potential transformation. Evidence for these pivotal roles of telomere maintenance in tumorigenicity is provided by the ability to inhibit tumor growth by inhibiting telomerase activity in telomerase positive cancer cells (144) and by the ability to convert primary human cells to cancer cells using only constitutive telomerase expression coupled to oncogene expression and/or loss of a tumor repressor gene, for example the simian virus 40 large-T oncoprotein and an oncogenic allele of H-ras in combination with ectopic expression of hTERT has been demonstrated to transform a variety of cell types (145).

The critical role telomere maintenance plays in cancer biology makes it important to note the affects of transformation on the expression of the telomere-associated proteins and to describe the roles of telomere proteins in establishing and maintaining tumors. As noted before, hTERT and the concomitant telomerase activity is observed in 85-90% of all cancers, but is absent in most differentiated tissues (146). Inhibition of telomerase in cancer cells results in gradual loss of the telomeric DNA followed by senescence or apoptosis, depending on the genetic background of the cell (147). In addition to its roles in maintaining the length of telomeric DNA, several recent reports highlight alternative roles for telomerase in protecting telomeric DNA, maintaining the proliferative capacity of tumor cells, and inhibiting apoptosis. One of the first indications that telomerase functioned beyond DNA synthesis came from experiments with human fibroblasts that were transformed with SV40 T-antigen and were immortalized by ectopic expression of hTERT (148). Surprisingly, these cells did not engage in telomere elongation, suggesting that telomerase promoted immortality without synthesizing DNA in this case. Subsequently, it was demonstrated that a mutant of hTERT, a C-terminal HA-tagged hTERT version that was catalytically active but unable to maintain telomeres in cells, conferred a tumorigenic phenotype (149). Interestingly, one recent experiment using a catalytically inactive hTERT mutant (D788N) demonstrated that telomerase inhibits apoptosis independent of its reverse transcriptase activity through a pathway involving poly(ADP-ribose) polymerase and p53 (150). The findings were substantiated by the observation that p53 induced apoptosis is inhibited by constitutive expression of hTERT (151), the effect of ectopic expression of hTERT in telomerase negative non-small cell lung carcinoma cells (152), and the finding that hTERT inhibits the mitochondrial death pathway (153) and other recent results (154). These findings lead to several important questions including what are the alternative functions of telomerase, and how can they be affected for the benefit of human health? Possible roles for telomerase beyond DNA synthesis include end-capping, regulation of telomeric structures such as the t-loop, mitotic spindle function, inhibition of apoptosis by inhibiting pro-apoptotic proteins such as Bax (153) or other proteins (155), and regulation of DNA repair (156). Expression of telomerase and telomere length have also been examined as prognostic indicators for several cancer lines. High levels of both hTERT mRNA levels and/or telomerase activity are an indicator of poor prognosis in most tumor types including, but not limited to, gliomas (157), non-small-cell lung cancer (158), metastatic hepatic cancer (159), breast cancer (160, 161), and various leukemias (162-164). Additionally, some cancers are associated with an increased incidence of ALT and in malignant liposarcomas, the ALT phenotype was better at predicting disease outcome with ALT associated with increased mortality (165).

The expression levels of the shelterin members in tumors have also been examined, but few consensus correlations can be found between their levels and tumor progression across different cancer types. One of the most consistent findings is an inverse correlation of TRF1 expression and tumor progression in human primary tumors, which has been observed in gastrointestinal (166), and astroglial brain tumors (167), non-small cell lung cancer cells (168), and acute leukemia cells (169). These data suggests that TRF1 down-regulation provides a growth advantage for cancerous cells, and this idea is corroborated by the observation that TRF1 expression directly correlated with prognosis (170). This correlation is not universal

among cancer as primary adult T-cell leukemia (ATL) cells from patients were shown to overexpress TRF1, TRF2 and TIN2 (171). These cells also displayed abnormally short, but stable telomere lengths. Perhaps the high TRF2 expression levels were required to maintain telomere structure in these cells and therefore prevent senescence associated with the short telomeres. A similar correlation between increased TRF2 and short telomere was found in gastric carcinomas (172), and during hepatocarcinogenesis (173). Moreover, in one study on gastric cancers the expression of TRF2 correlated to multidrug resistance (174). Expression of telomere-associated genes was also examined in patients with non-small cell lung cancer cells. The level of TRF1 was significantly lower in the tumor samples than in adjacent normal tissues, but no significant differences were found for TRF2, POT1, and RAP1. There were differences in RAP1 and TRF2 expression between patients. Notably, the expression levels of RAP1 correlated with overall survival, suggesting that RAP1 would be a useful prognostic indicator, and higher levels of TRF2 expression correlated to lower tumor grade (175). POT1 levels have been reported to directly correlate with tumor stage and telomere length in some primary gastric carcinoma cells, suggesting some growth advantage for increased POT1 in some situations (176).

8.2. Implications of telomere biology on aging

Most human somatic cells are devoid of telomerase activity, and telomerase positive cells from proliferative tissues do not express telomerase at levels high enough to completely maintain telomere length. As a consequence, telomeres shorten during the aging process. This has been established for humans by examining telomere lengths from a variety of tissues samples from patients representing a range of ages. Telomere length reduction has been observed in peripheral blood cells, colon, fibroblasts, liver, pancreas (177), kidney (178, 179), and lung of both males and females, but not in brain samples (179). This correlation between shortened telomeres and age has led to the examination of telomere length as a prognostic indicator of age related conditions. Several reports have now documented the correlation between shortened telomeres in peripheral blood cells and a wide variety of age-related diseases including Alzheimer's disease, atherosclerosis, myocardial infarction, and vascular dementia. Significant or borderline inverse correlation was observed between leukocyte telomere length and diabetes, diastolic blood pressure, and myocardial infarction, with each kilobase pair of telomere lost corresponding with a threefold increased risk of myocardial infarction and stroke (180). Dementia is commonly associated with aging. However, no association between telomere length in old age with age-related physical and cognitive decline or mortality was observed in one published report (181). However, telomere length was found to predict poststroke mortality, dementia, and cognitive decline (182). Of note, telomere length appears to be a good indicator of morbidity in older populations (60-74 years) (183) but the correlation decrease with very old (>79) populations (184). Perhaps, aged telomeres are unstable irrespective of length. The data so far only demonstrate that telomere length correlates with aging, but these studies do not demonstrate a causative relationship between telomere length and aging. The best indication of such a relationship comes from the several progeria related disorders that have telomere maintenance defects associated with their etiology. These are discussed further in section 9 below.

What causes the erosion of telomeric DNA and the differences in telomere length between people? Even though the end-replication problem seems to directly affect telomere length and appears in part responsible for senescence in cultured cells, oxidative stress and the attending loss of the oxidation sensitive, G-rich telomeric DNA also can contribute significantly to telomeric loss *in vivo*, especially in post mitotic cells (185, 186). Thus, environmental and lifestyle conditions could affect telomere length. This idea is supported by a few reports. A study of healthy premenopausal women showed that levels of perceived stress correlated with shorter telomeres, on average by the equivalent of at least one decade of additional aging compared to low stress women (187). Both obesity and cigarette smoking are also associated with increased oxidative stress and inflammation. A large cohort study consisting of 1,122 white women aged 18-76 years was used to investigate the effects of obesity and smoking (188). Telomere length decreased with age at a mean rate of 27 bp per year, and obesity was associated with a decrease of 240 bp of telomeric DNA compared to lean women. A dose-dependent relation with smoking was noted, and each pack-year smoked was equivalent to an additional 5 bp of telomere length lost compared with the rate in the overall cohort. Thus obesity and smoking caused 8.8 and 4.6 years, respectively, of extra aging as measured by loss of telomeric DNA.

9. DISEASES ASSOCIATED WITH DEFICIENCIES IN TELOMERE MAINTENANCE

In addition to the relationship with aging and cancer, several genetic disorders have telomere maintenance defects as a central contributor to the disease associated phenotype. Two major classifications of telomere-associated disorders are those that result in a telomerase deficiency, including dyskeratosis congenita (DC), and those that affect telomere DNA processing, such as the Werner's and Bloom's syndromes.

9.1. Telomerase deficiency and deregulation in human disease

Patients with one of a variety of diseases associated with bone marrow failure have been found to carry mutations in a component required for generating telomerase activity. Mutations in hTR, hTERT, and hTR processing proteins have been found and all correlate with decreased telomerase activity and shortened telomeres in these patients. The most common abnormality is hTR sequence variance, which is found in approximately 10% to 15% of patients with acquired hematopoietic failure syndromes (189). DC is a rare inherited multi-system disorder characterized by abnormal skin pigmentation and morbidity associated with bone marrow failure (190). Autosomal dominant DC patients are heterozygous for mutations in either hTR (191) or hTERT (192, 193) and

display haploinsufficiency of their telomerase activity. Other diseases associated with bone marrow failure, including aplastic anemia, paroxysmal nocturnal hemoglobinuria, and myelodysplastic syndrome have also been attributed to acquired mutations in hTR (189). The predominant X-linked form of DC results from mutations in the protein, (56) which is required for both ribosomal RNA (rRNA) pseudouridine modification and cellular accumulation of hTR. Important insight into the etiology of X-linked DC came from experiments in which hTR was added back to cells derived from DC patients (194). With this approach, the Collins laboratory demonstrated that overexpression of hTR can rescue the telomere maintenance phenotype. Further, they showed that rRNA biogenesis occurred at the normal rate. These results demonstrate that X-linked DC is truly a disease caused by aberrant telomerase biogenesis.

9.2. Diseases associated with telomere processing

Several syndromes result from genetic abnormalities in genes with telomere functions that overlap with their other cellular roles. Common symptoms associated with these diseases include premature aging, susceptibility to cancer, sensitivity to DNA damage, and critically short telomeres. Mutations in the Mre11 complex members Mre11 and NBS1 give rise to Ataxia-telangiectasia-like disorder (ATLD) (195) and Nijmegen breakage syndrome (NBS) (196), respectively. Blood cells from NBS patients have shortened telomeres and introduction of hTERT and NBS1 were required to regain telomere lengths, consistent with an active role for the Mre11 complex in telomere extension (196). Even though both Mre11 and Nbs1 are part of the same Mre11 complex, deficiency in either protein in humans does not lead to the same clinical symptoms, suggesting that the components of the complex may also act separately (110). Mutations in ataxia-telangiectasia mutated (ATM) protein, a DNA damage response kinase, cause ataxia-telangiectasia. ATM functions in mobilizing the cellular response to double strand breaks in the DNA and its loss results in accelerated telomere shortening (197). Mutations in the telomere-related flap endonuclease ERCC1/XPF cause xeroderma pigmentosum (85). Fanconi anemia (FA) is a rare genetic disease characterized by chromosome instability, progressive bone marrow failure and cancer susceptibility. Accelerated shortening of telomeres, a high frequency of extra-chromosomal TTAGGG signals, and chromosome ends with undetectable TTAGGG repeats have been observed in FA cells. The observation of extra-chromosomal telomeric DNA is suggestive of intensive breakage at telomeric sequences (198). Interestingly, telomerase was found to be 4.8-fold more active in some FA patients than in age-matched healthy controls (199). Since FA cells have a defect in oxygen metabolism leading to an increased burden of reactive oxygen species and attending 8-OxodG accumulation it has been speculated that oxidative damage at the G-rich telomere gives rise to the telomere-shortening phenotype inherent in FA cells (110, 200).

Bloom's and Werner's syndromes are caused by defects in related RecQ type helicases BLM and WRN, respectively (201). Patients with Bloom's syndrome are predisposed to early onset of cancer, growth retardation, and immunodeficiency. Werner's syndrome is more complex and patients present premature aging with several complex aging related phenotypes, including graying and thinning of the hair, bilateral cataract formation, type II diabetes mellitus, osteoporosis and atherosclerosis. Because both BLM and WRN have demonstrable G-quadruplex resolvase activity and are required for proper telomere maintenance, it is tempting to speculate that they are involved in resolving G-quadruplex structures that arise during DNA replication at the telomere to allow complete replication (135). Perhaps, the absence of WRN and BLM results in an increased incidence of stalled replication forks within telomeric regions of the chromosomes owing to G-quadruplex formation, which is known to stop DNA polymerization when present in the template strand. Since the G-rich telomeric DNA is the template for lagging strand DNA synthesis, WRN and BLM should only affect lagging strand telomeres if the effect is related to G-quadruplex resolution within the template, as was observed by the Karlseder lab for WRN (112). Alternatively, replication of the repetitive telomeric DNA could lead to stalled replication forks that undergo regression leading to a four-way junction characterized as a chicken foot DNA structure.

10. TELOMERE MAINTENANCE AS A DRUG TARGET

Because telomere maintenance is an absolute requirement for the continued proliferation of cancer cells, several aspects of telomere maintenance have been examined as anticancer drug targets. The most advanced of these is inhibition of telomerase (202). Several strategies to inhibit telomerase activity through direct and indirect means have been investigated (203-206). These can be classified as targeting production of the telomerase complex using antisense approaches, targeting telomerase activity with specific inhibitors, or targeting the telomere with G-quadruplex stabilizers. Targeted disruption of telomerase production has been achieved through antisense–mediated disruption of hTERT transcription and translation (207, 208) and by using siRNA (153, 154). Recently, antisense mediated redirection of hTERT splicing has been demonstrated to generate, in situ, a dominant negative telomerase complex (209, 210). One of the more active and promising areas of research directed at inhibiting telomere-maintenance has been direct inhibition of telomerase activity. These inhibitors fall into two general categories: oligonucleotide inhibitors that target the template portion of hTR (204) and small-molecule inhibitors including reverse transcriptase inhibitors (211), nucleotide analogs (212, 213), and other small molecules (Figure 9) (214-217). Each of these strategies to inhibit telomerase has been met with some success, and in general, telomerase inhibition by these methods leads to erosion of telomeric DNA. As telomere length decreases, the functional telomere structure becomes increasing difficult for cells to maintain and eventually the dysfunctional telomeres initiate senescence or apoptosis (77). Importantly, the use of telomerase inhibitors has been validated in cultured tumor cells and human tumor

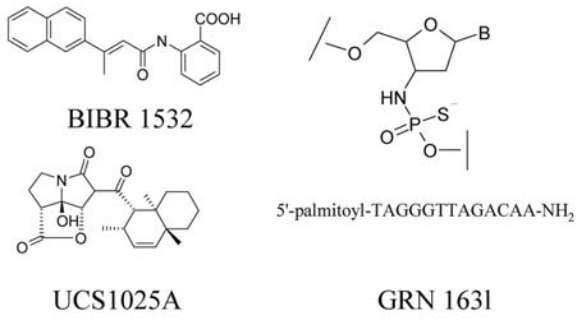

Figure 9. Representative telomerase-targeted inhibitors. BIBR 1532 was identified by high throughput screening followed by optimization of lead compounds. UCS1025A is a natural product isolated from the fungus *Acremonium* sp. GRN 163L is a telomerase template antagonist that is a short oligonucleotide that is complementary to the telomerase template. GRN163L contains a unique N3'→P5' *thio*-phosphoramidate backbone.

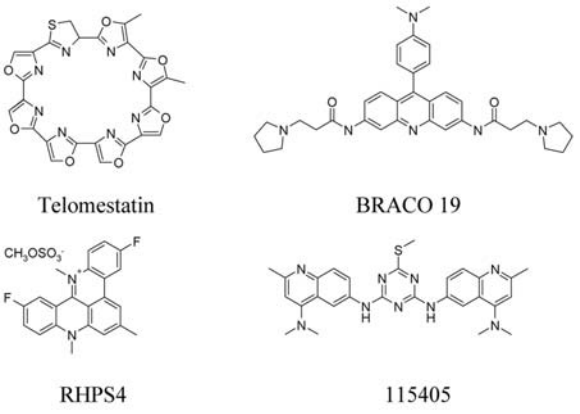

Figure 10. G-quadruplex binding ligands. Telomestatin is natural product isolated from *Streptomyces amulatus*. BRACO 19 is a 3,6,9 trisubstituted acridine derivative. RHPS4 is a pentacyclic acridine derivative. 115405 is a trisubstituted triazine derivative.

xenografts in nude mouse models (216, 218-220). Telomerase assemblage has also been targeted by affecting the chaperones involved in assemblage or by targeting the protein-RNA interactions involved in establishing telomerase activity (65, 221-223). Telomerase requires the proper association of hTERT and hTR to afford an active complex, and telomerase assemblage appears to require several accessory proteins including Hsp90 and p23, which function as chaperones (59). Thus, assemblage of the telomerase complex has been blocked by using Hsp90 inhibitors geldanamycin (221) and novobiocin (155). However, it was recently demonstrated that the role of hsp90 is more complex than mediating telomerase assemblage (60), and inhibition of cellular hsp90 results in degradation of hTERT (61), precluding the formation of a dominant negative telomerase complex. A dominant negative complex has been generated by transfecting cells with a mutant of hTR with an altered template sequence, though this approach required concomitant knock down of hTR level with RNAi (224, 225). The era of telomerase-based therapeutics is close at hand as the biotechnology company Geron has recently led one of its lead compounds into the phase I/II clinical trials, trial NCT00124189 starting July 2005, for chronic lymphocytic leukemia. Furthermore, several telomerase-based vaccines are also in phase I/II clinical trials (226).

Another area that has shown promise is the use of G-quadruplex stabilizing molecules (227). This field owes its existence to the observation that telomerase cannot extend the G-quadruplex structures that telomeric DNA can form. Based on this, it is hypothesized that G-quadruplex stabilizing ligands can bind telomeric DNA, induce the formation of the unique, four-stranded G-quadruplexes, and inhibit telomerase; or they can bind the DNA in the nascent product-telomerase complex. In reality, G-quadruplex stabilizing compounds can affect telomerase and telomere maintenance by a variety of mechanisms. Focusing on telomerase, these ligands can prevent telomerase from interacting with the telomere, inhibit telomerase during primer extension after four telomeric repeats are available,(228) suppress activation of hTERT transcription by binding to the c-MYC promoter (229), and affect splicing of the hTERT transcript (230). Aside from their effects on telomerase specifically, G-quadruplex ligands can affect telomeres more directly by blocking the interaction of telomere-binding proteins with the telomere.

The combined efforts of the Hurley and Neidle labs provided the first study of G-quadruplex-binding ligands, 2,6-diamidoanthraquinones, as telomerase inhibitors (231). Since that seminal work, a wide variety of pharmacophores have been explored as G-quadruplex-based telomerase inhibitors (Figure 10). From a pharmacological perspective, two of the best studied G-quadruplex-binding ligands are BRACO-19, a 3,6,9-trisubstituted acridine (232), and telomestatin, a natural product with a unique circular structure (233). Two reports demonstrate that the association of POT1 with the telomere is inhibited by telomestatin, but does not affect the telomere localization of the double-stranded telomere-binding protein TRF2 (234, 235). Treatment of HT1080 human tumor cells by telomestatin induces a rapid decrease of the telomeric G-overhang and of the double-stranded telomeric repeats, provokes a strong decrease of POT1 and TRF2 from their telomere sites, uncaps telomere ends, and causes an increase in telomeric □-H2AX foci (235). These results indicate a DNA damage response at the telomere. Other foci were also present, indicating the presence of additional DNA targets for telomestatin. POT1 overexpression allowed resistance to telomestatin treatment characterized by protection against telomestatin-induced growth inhibition and G-overhang shortening. This protection is rather related to the initial G-overhang length than to its degradation rate and is overcome by increased telomestatin concentration. In a

separate report, telomestatin was shown to dissociate TRF2 from telomeres in cancer cells within a week, when given at a concentration that does not cause normal cells to die (236). In this study, the G-tails were dramatically reduced in cancer cell lines, but not in normal fibroblasts and epithelial cells. In addition, telomestatin also induced anaphase bridge formation in cancer cell lines. These results indicate that telomestatin exerts its anticancer effect not only through inhibiting telomere elongation, but also by rapidly disrupting the capping function at the very ends of telomeres.

Based on the observation that the telomere-associated RecQ helicases BLM and WRN efficiently disrupt G-quadruplex DNA, it was predicted that G-quadruplex ligands could inhibit the G-quadruplex resolvase activity. It was then demonstrated that trisubstituted acridine ligands are potent inhibitors of the helicase activity of the BLM and WRN proteins on both G-quadruplex and B-form DNA substrates (237). Inhibition was associated with both a reduction in the level of binding to G-quadruplex DNA and a reduction of the DNA-dependent ATPase activity.

11. TELOMERE MAINTENANCE AND ADVANCES IN TISSUE ENGINEERING

Because of its relationship with cellular aging, telomerase has received substantial interest by groups looking to take advantage of its immortalizing ability to enhance tissue engineering campaigns. In this setting, the ectopic expression of telomerase is currently being investigated as a tool to increase the potential of cultured cells for uses in tissue engineering (238). Because telomerase activity in cells is limited by the levels of the catalytic subunit hTERT, introduction of hTERT is generally sufficient to establish telomerase activity in telomerase negative cells. Importantly, ectopic expression of hTERT is not sufficient for the acquisition of cancerous phenotypes such as chromosomal abnormalities, anchorage-independent growth, and tumorigenicity in immunodeficient mice. Thus, ectopic expression of hTERT and the attending telomerase activity allows the production of cells that can be grown for extended periods of time in culture. In the absence of hTERT, of course, cells have a limited proliferation capacity owing to telomere-length dependent replicative senescence.

Immortalization of cells by telomerase has been employed in two general scenarios. One is to directly immortalize cells that will latter be used for tissue engineering. The second is to use telomerase immortalized-differentiated cells to drive the differentiation of undifferentiated stem cells. The direct immortalization of cells with hTERT has been successful with several cell types. The first example of xenotransplantation using hTERT-expressing cells was reported by Thomas et al., who successfully rescued adrenal gland activity in a mouse model after adrenalectomy by implanting hTERT-immortalized bovine adrenocortical cells (239). hTERT-mediated lifespan expansion has also been investigated for the treatment of spinal cord injury by spinal neuroepithelial cells transduced with hTERT. The cells were propagated for over 168 population doublings and successfully matured into neurons upon xenograft to rat brain and injured adult spinal cord (240). Several other cell types have been immortalized with hTERT including muscle satellite cells, retinal pigment epithelia, dermal fibroblasts, vascular endothelium, osteoblasts, hepatic satellite cells, and mesenchymal stem cells (238). The differentiation of embryonic cells by using coculture with hTERT-transduced, differentiated cells was recently reported to allow the production of dopaminergic neurons (241). These were successfully transplanted in parkinsonian rats in an exciting example of the use of hTERT-immortalized cells.

Using hTERT to overcome replicative senescence for uses in tissue engineering has several potential drawbacks. For example, long term culturing of telomerase-induced cells led to neoplastic transformation of the mesenchymal stem cells though differentiated vascular smooth muscle cells did not suffer similar transformation (242). The problem of telomerase-associated neoplastic growth could theoretically be overcome by conditional or reversible hTERT expression. Towards this reality, a reversibly immortal human β-cell line was recently established and used to successfully establish normal blood glucose control in a diabetes mouse model (243). Another possible drawback is the expansion of undifferentiated cells post grafting. This is not a problem associated with telomerase per se, but is an inherent problem of tissue engineering.

12. PERSPECTIVES

The past ten years have witnessed an explosion of accumulated knowledge about human telomeres, but have left us with more questions then when telomeres were first characterized. In the next ten years, we can anticipate a clearer picture of telomere structure to emerge. Details regarding the telomere structure, how and when it forms, and how it is resolved during DNA replication will certainly provide unique insights into human disease and provide new pharmacological targets for the manipulation of cellular physiology towards the benefit of human health. Other avenues of advancement certainly include understanding the role of telomerase and telomere maintenance in cancer biology. Both the process of transformation during tumorigenesis and the maintenance of cancers cells are affected by telomere biology, yet the details are nebulous. Lastly, one area that seems particularly under explored is the use of chemical biology to probe the salient features of telomere-associated proteins. As the structures of the telomere-binding proteins and telomerase become available, we can anticipate an increased interest in targeting them with small molecules. Such efforts will undoubtedly produce a useful set of tools and lead compounds to probe telomere biology and test in the clinic.

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Send correspondence to: Dr. Michael B. Jarstfer, Division of Medicinal Chemistry and Natural Products, CB # 7360, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599-7360, Tel: 919-966-6422, Fax: 919-966-0204, E-mail: jarstfer@unc.edu

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