

# Telomeres, Telomerase, and DNA Damage Response in Cancer Therapy

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## 1 Overview

Faithful genome maintenance is essential to an organism's growth and survival. To preserve genome fidelity, the DNA Damage Response (DDR) pathway has evolved to coordinate the surveillance and repair of genomic DNA, damaged by normal metabolic or environmental insults [1]. DDR surveillance mechanisms scan for discontinuities and structural changes in the DNA double helix. Upon detection of any damage to the DNA molecule, these surveillance sensors activate signal transduction cascades to amplify the damage signal, and coordinate the arrest of proliferation for proper DNA repair [1–4]. Alternatively, apoptosis may be initiated if repair is not possible. The abrupt termini of linear eukaryotic chromosomes pose specific challenges to DDR surveillance, as these natural ends are indistinguishable from damaged double-stranded DNA. In most eukaryotic organisms with linear chromosomes, phylogenetically conserved nucleoprotein structures, known as telomeres, differentiate chromosome ends from nonspecific DNA breaks [5–7]. Telomeres mask the ends of chromosomes from DDR surveillance sensors and protect the chromosome ends from inappropriate repair by DDR mechanisms [8].

Over the past two decades, we have learned a great deal about the structure of telomeres, their homeostatic maintenance, and the cellular consequences of their dysfunction. We know that while telomeres suppress the erroneous activation of the DDR pathways by chromosome ends, the structural and functional integrity of these structures are dependent on the activities of the same DDR pathways. In this chapter, we describe the protein and nucleic acid components of telomeres, both

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stable and transient. We then describe the physiological mechanisms of telomere maintenance by the enzyme telomerase, its biogenesis and regulation, and how this reverse transcriptase might be utilized in anticancer chemotherapy.

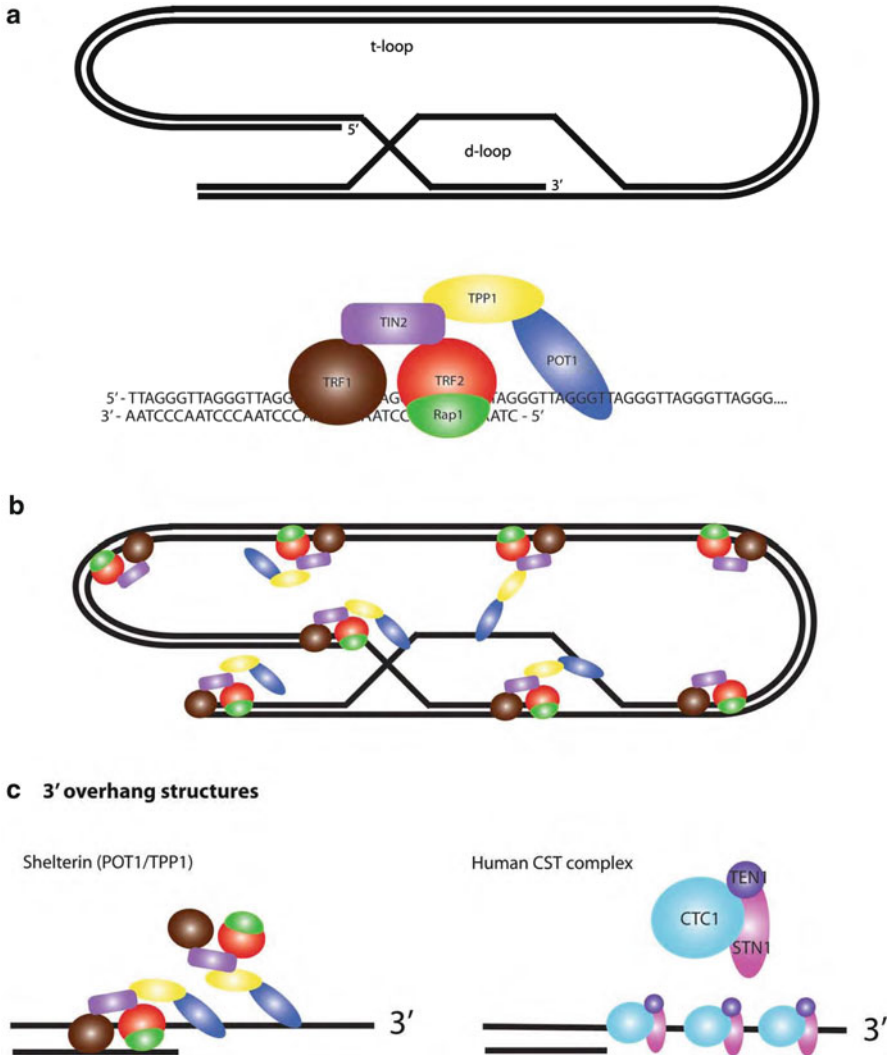
## 2 Telomeres

### 2.1 *Telomere Structure*

At the ends of most eukaryotic chromosomes are highly conserved, tandem DNA repeats. These highly repetitive sequences are associated with their specific binding proteins, and together, these chromosome-end structures are known as telomeres. Telomeres cap chromosome ends and protect them from nonspecific nuclease digestion, as well as preventing them from being recognized as double-stranded DNA breaks. In the absence of telomeres, erroneous DNA repair can lead to chromosomal end-to-end fusions and genetic recombination [5]. The length of telomeric DNA repeats vary between species, ranging from ~300 to 600 bp in yeast [9], to ~150 kb in mice [10]. Human telomeres measure ~5–15 kb in length [11, 12]. In all vertebrate chromosomes, telomeres are made up of a G-rich hexanucleotide sequence (TTAGGG)<sub>n</sub> [13]. Telomere repeats run 5'-3', terminating in a single-stranded 3' overhang of the G-rich strand [14, 15]. The length of this overhang is also species-specific, measuring ~50–100 nucleotides in length in mouse and human telomeres [16].

Mammalian telomeres were previously thought to be linear. However, electron microscopy analysis of psoralene cross-linked telomeric DNA from human and mouse were visualized to end as large duplex loops [17]. At the molecular level, double-stranded telomeric DNA folds back onto itself to form a lariat structure termed the telomeric loop (Fig. 1a). This allows for the G-rich 3' overhang to invade the duplex section of telomeric repeats, thereby forcing the formation of a single-stranded DNA displacement loop [18]. The resulting higher order chromatin structure is distinct from damaged DNA and thus serves to differentiate the normal chromosomal termini, preventing them from being recognized as double strand breaks. This differentiation mechanism is crucial in preventing the initiation of DNA damage checkpoint responses [5, 6, 16].

A six-member protein complex, termed shelterin, associates with telomeric DNA in a sequence specific manner (Fig. 1b, Table 1). This complex facilitates formation of the telomeric loop to protect chromosome ends from DNA damage surveillance mechanisms, as well as to functionally maintain telomere length. The shelterin complex is composed of six distinct proteins: telomere repeat binding factors 1 and 2 (TRF1 and TRF2), protection of telomeres 1 (POT1), TRF1- and TRF2-interacting nuclear protein 2 (TIN2), repressor/activator protein 1 (Rap1), and TPP1 (formally known as PTOP, PIP1, or TINT1) [7, 19]. TRF1 and 2 are sequence specific telomeric DNA binding proteins that recruit the other four proteins to the telomeres



**Fig. 1** Human telomeres. (a) Telomere repeats at chromosome ends fold back to form a lariat structure (t-loop). The 3' telomeric DNA overhang invades the double-stranded DNA region of telomeric repeats to form a displacement-loop (d-loop). (b) Shelterin protein complex aids in t-loop formation and stabilization: TRF1 and TRF2 interact with double-stranded telomeric repeats, recruiting the other four shelterin proteins, POT1, TIN2, TPP1, and Rap1, to the telomere end. TIN2 links TRF1 to TRF2, contributing to the stabilization of these proteins on the telomere. POT1, which has strong binding specificity for single-stranded telomeric repeats, together with its heterodimeric partner TPP1 associates with TRF1 and TRF2 through a bridge formed by TIN2. Rap1 is recruited by TRF2, forming a TRF2-Rap complex. (c) The human telomere 3' overhangs exist in two structural forms. Shelterin components POT1-TPP1 bind single-stranded telomeric DNA with high sequence specificity. Recently, the human homologs of yeast CST complex have been identified to associate with single-stranded telomeric DNA structure, with low sequence specificity and in the absence of shelterin

**Table 1** Telomere Binding Proteins

	Protein	Gene name	Major functions at telomere	Telomere binding	Other biological functions
Shelterin complex	TRF 1	<i>TERF 1</i>	<ul style="list-style-type: none"> <li>- Negative regulator of telomere length</li> <li>- Translate double-stranded telomere length to POT1 recruitment at the telomeric terminus, indirectly regulate telomerase access</li> </ul>	<ul style="list-style-type: none"> <li>- Bind to double-stranded telomeric DNA</li> <li>- Poly (ADP) ribosylase tankyrase 1 and 2 regulate TRF 1 binding to telomeres</li> </ul>	<ul style="list-style-type: none"> <li>DDR protein interactions: ATM, BLM, PML, PINX1, DNA-PK</li> </ul>
	TRF2	<i>TERF2</i>	<ul style="list-style-type: none"> <li>- Telomere end protection</li> <li>- Formation of T-loop</li> <li>- Suppression of ATM activation</li> <li>- Suppression of erroneous DNA repair at the telomere by HR or NHEJ</li> </ul>	<ul style="list-style-type: none"> <li>- Bind to double-stranded as well as double/single-stranded junction of telomeric DNA</li> <li>- Phosphorylation and arginine methylation of TRF2 regulate its binding to telomeres</li> </ul>	<ul style="list-style-type: none"> <li>DDR protein interactions: ATM, DNA-PK, Apollo, MRN, WRN, BLM, Ku70, ERC 1/ XPF, PARP1, FEN1, MUS81</li> </ul>
	POT1	<i>POT1</i>	<ul style="list-style-type: none"> <li>- Telomere protection and length regulation</li> <li>- Suppression of the activation of ATR</li> <li>- Determine the structure of the extreme ends of telomeres</li> <li>- Decrease telomerase recruitment at the telomere but increase telomerase processivity together with TPP1</li> </ul>	<ul style="list-style-type: none"> <li>- Bind to single-stranded G-rich-telomeric DNA</li> </ul>	<ul style="list-style-type: none"> <li>DDR protein interactions: WRN, BLM</li> </ul>
	TPP1	<i>ACD</i>	<ul style="list-style-type: none"> <li>- Telomere proteion</li> <li>- Together with TIN2, mediate the formation of the shelterin complex by the bridging of TRF1 and TRF2</li> <li>- Bind to and increase the processivity of telomerase together with POT1</li> </ul>	<ul style="list-style-type: none"> <li>- Do not bind telomeric DNA directly</li> <li>- Protein-protein interaction with TIN2 and TRF1 mediates the recruitment of itself and of POT1 to the telomere</li> </ul>	<ul style="list-style-type: none"> <li>Adrenocortical dysplasia is a spontaneous autosomal recessive mouse disorder with a splice donor mutation in the ACD gene</li> </ul>

TIN2	<i>TINF2</i>	<ul style="list-style-type: none"> <li>- Telomere length regulation</li> <li>- Modulate the ribosylation of TRF1 by inhibiting TRF1 association with tankyrase</li> <li>- Together with TPP1, mediate the formation of the shelterin complex by the bridging of TRF1 and TRF2</li> </ul>	<ul style="list-style-type: none"> <li>- Do not bind telomeric DNA directly</li> <li>- Protein-protein interaction between TRF1/TRF2 to TPP1/POT1 complex</li> <li>- Long isoforms (TIN2L) may anchor telomers to the nuclear matrix</li> </ul>	Genetic mutations were found in dyskeratosis congenita and Revesz syndrome patients
RAP1	<i>TERF2IP</i>	<ul style="list-style-type: none"> <li>- Telomere length regulation</li> <li>- Suppression of homology-directed repair at the telomere</li> <li>- Subtelomeric gene silencing</li> </ul>	<ul style="list-style-type: none"> <li>- Do not bind telomeric DNA directly</li> <li>- Protein-protein interaction with TRF2 mediates its telomere localization</li> </ul>	Global regulation of gene expression in yeast and mouse
Human CST complex	<i>CTC1</i>	<ul style="list-style-type: none"> <li>- Homolog of mouse CTC1</li> </ul>	<ul style="list-style-type: none"> <li>- As a complex, these three proteins bind to telomeric G-rich single-stranded DNA in a sequence non-specific manner, analogous to the binding of ssDNA by replication protein A (RPA)</li> </ul>	Also identified as subunits of DNA polymerase alpha:primase complex
	<i>STN1</i>	<ul style="list-style-type: none"> <li>- Homolog of <i>S. pombe</i> STN1</li> <li>- Bridge the association between CTC1 and TEN1</li> </ul>	<ul style="list-style-type: none"> <li>- As a complex, these three proteins bind to telomeric G-rich single-stranded DNA in a sequence non-specific manner, analogous to the binding of ssDNA by replication protein A (RPA)</li> </ul>	Also identified as subunits of DNA polymerase alpha:primase complex
	<i>TEN1</i>	<ul style="list-style-type: none"> <li>- Homolog of <i>S. pombe</i> TEN1</li> </ul>	<ul style="list-style-type: none"> <li>- Bind to telomeric single-stranded DNA in the absence of TPP1-POT1</li> </ul>	

[19]. Both TRF1 and TRF2 contain a C-terminal SANT/Myb-type DNA binding domain that binds to the 5'-TTAGGG-3' sequence in duplex DNA, making the entire shelterin complex highly specific for telomeric repeats [20, 21].

TRF1 is a homodimeric protein that aids in telomeric loop formation and stabilization [22]. Its binding to arrays of telomeric repeats induces shallow bends and results in the formation of DNA loops, demonstrating the protein's architectural role on telomeres [20]. This protein has also been shown to affect telomere length. Over-expression of TRF1 results in telomere shortening while expression of a dominant negative TRF1 mutant, lacking the Myb type domain, causes telomere lengthening. This suggests a negative correlation between TRF1 function and telomere length [23, 24]. On the other hand, accumulations of TRF1 and TRF2 at telomere ends were shown to positively correlate with telomere length [23, 24]. This led to a protein counting theory of telomere length regulation, which proposed that a feedback mechanism mediated by protein interactions with TRF1 is responsible for steady-state telomere length maintenance [23].

Like TRF1, TRF2 also binds to double-stranded telomeric DNA as a homodimer [25] and plays a role in telomeric loop assembly. In contrast to TRF1, TRF2 is believed to bind near the loop-tail junction where it stabilizes the G-rich single-stranded telomeric overhang at the displacement loop by facilitating strand invasion and preventing the single-stranded sequence from being recognized as a DNA break [17, 26]. In corroboration to this model, electron microscopy of a telomere DNA track containing ~2 kb of telomeric repeats at the end of a linearized DNA plasmid and terminating in a 3' single-stranded overhang, revealed the specific binding location of TRF2 at the telomeric loop junction [27]. Like TRF1, TRF2 also serves as a negative regulator of telomere length. TRF2 over-expression results in shortened telomeres and induces senescence in telomerase negative cells [28].

POT1 is the most highly conserved component of shelterin, and has a strong specificity for single-stranded 5'-(T)TAGGGTTAG-3' sites [29, 30]. Following DNA replication, single-stranded telomeric overhangs initially associate with replication protein A (RPA). Heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) binding displaces RPA binding, while the increase in TERRA expression levels (see below) following S phase removes hnRNPA1 from telomeric DNA, allowing for sequence-specific binding by POT1 [31]. POT1 accumulation at chromosome ends is believed to regulate telomerase activity by relaying telomere length information from the double-stranded region of the telomeric loop to the single-stranded region through its interaction with TRF1 [32]. Studies have also demonstrated that POT1 plays a positive role in telomere length maintenance, as ectopic expression of POT1 results in an increase in telomeric DNA [33, 34].

TPP1, the heterodimeric partner of POT1 [35, 36], enhances POT1 affinity for single-stranded telomeric DNA [36]. Most of the POT1-TPP1 complexes are associated with TRF1 and TRF2 through a bridge formed by TIN2, which functions to stabilize the interactions between these proteins [37]. In addition to the protection of telomere ends, the TPP1-POT1 complex also serves as a regulator of telomere length maintenance. Through its oligonucleotide- and oligosaccharide-binding

fold, TPP1 has been suggested to regulate telomerase activity and the enzyme's access to single-stranded telomeric DNA, both negatively and positively in a context dependent manner [36].

TIN2 co-localizes with TRF1 on metaphase chromosomes [38]. TIN2 forms bridges that join POT1 to TRF1 and TRF2 and also TRF1 to TRF2, contributing to the stabilization of these proteins at telomeres [39, 40]. The binding of TRF1 to TIN2 leads to the compaction of telomeric DNA and telomeric loop stabilization. Both events limit the accessibility of telomerase to telomere ends and thereby functions as a negative regulator of telomere length [38]. Genetic lesions of TIN2 underlie a subpopulation of autosomal dominant form of dyskeratosis congenita, representing the only shelterin protein associated with this genetic disease of telomere dysfunction [41].

Rap1 is recruited to the telomere by protein interactions with TRF2, forming a TRF2-Rap1 complex [42]. Rap1 affects telomere length homeostasis through its interactions with telomere length regulator proteins Rif1 and Rif2. Like the other shelterin proteins, Rap1 is a negative regulator of telomere length. Over-expression of Rap1 leads to telomere shortening, while expression of dominant negative mutants results in the gain of telomere length [43]. In addition, RAP1 binds to non-telomeric sequences and is implicated in diverse cellular activities including gene silencing and the transcriptional regulation of gene targets involved in adhesion, metabolism, and cancer [44].

In *Saccharomyces cerevisiae*, the *cdc13-stn1-ten1* (CST) complex binds single-stranded telomeric DNA in place of POT1-TPP1 [45, 46]. Recently, the human version of this protein complex has been identified. The human *ctc1-stn1-ten1* (CST) complex contains two human homologs of the ScCST complex (Stn1 and Ten1), and a third component, the conserved telomere maintenance component 1 (*ctc1*) [47]. Similar to the ScCST complex, human CST binds single-stranded G-rich telomeric DNA, in the absence of POT1-TPP1. Unlike ScCST, human CST does not exhibit sequence specificity for telomeric repeats, and likely associates with other single-stranded DNA in a manner analogous to the binding of single-stranded DNA by replication protein A (Fig. 1c, Table 1). A significant increase in the G-strand overhang was observed in Stn1 depleted human cells, indicating a role of the CST complex in single-strand telomeric DNA regulation [47]. Whether this newly identified CST complex functionally interacts with the shelterin complex is currently under investigation.

In addition to the binding of telomere-specific shelterin and the single-stranded DNA-specific CST complexes, heterochromatin formation via the epigenetic regulation of telomeric chromatin is also observed. DNA methylation of subtelomeric regions [48], together with histone methylation of the telomeric chromatin [49], are postulated to negatively regulate gene transcription, suppress homologous recombination and prevent telomerase access for telomere elongation [50]. Telomere-repeat containing RNAs (TERRA) are long UUAGGG-repeat containing noncoding RNA transcripts that have been recently identified [51, 52]. TERRAs are transcribed starting from the subtelomeric region, using the C-rich strand of the telomere as a template. TERRAs are found to be stably associated with telomeric

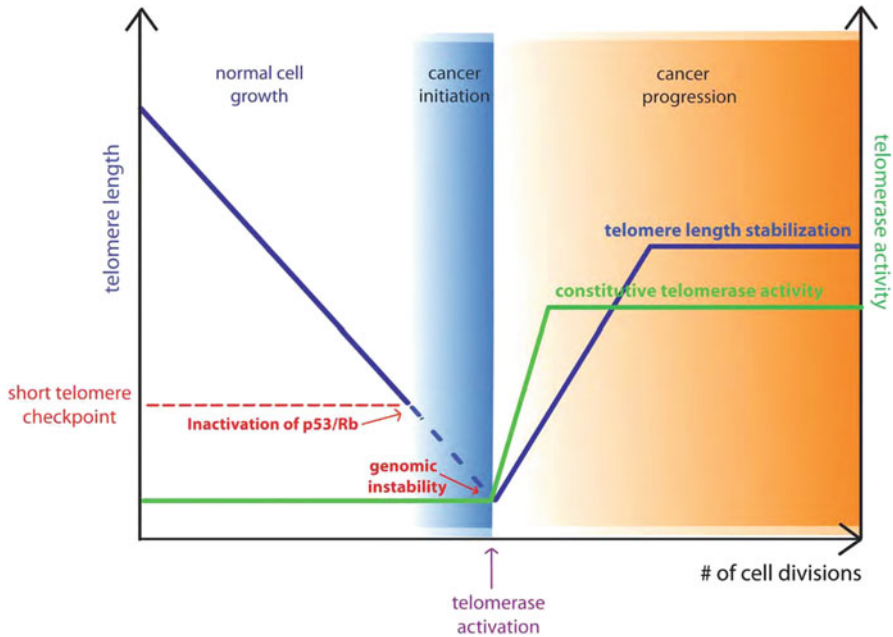
chromatin and cellular machinery responsible for the nonsense mediated decay of dysfunctional RNA transcripts. Current models of these noncoding RNA functions prescribe a role in the induction of heterochromatin formation, serving as a negative regulator of telomerase access to telomeres [53]. Cell-cycle phase-specific changes in TERRA expression levels are also postulated to mediate the switch from RPA to POT1 binding at single-stranded telomeric DNA termini [31].

## ***2.2 Telomere Function: End Replication Problem and the Hayflick Limit***

Besides structurally protecting the ends of chromosomes, telomeres also serve as a solution to the end-replication problem. Because DNA polymerases fail to completely copy chromosomes to the very end, the placement of telomeres at the extreme ends of chromosomes allows them to buffer gene coding sequence from being eroded [11, 54]. Instead, telomeric DNA is lost after every round of DNA replication. Telomeric DNA loss is cumulative and with continual proliferation; telomeres will eventually reach a critical short length. At this point, genome surveillance mechanisms will trigger replicative senescence, an irreversible cellular growth arrest state where cells can no longer divide into daughter progeny, but remain metabolically active (Fig. 2) [5, 55–57]. This short telomere checkpoint serves as a “mitotic clock” which counts down the number of cell divisions in each cell lineage. Leonard Hayflick first described this relationship by observing the replicative potential of human primary fibroblasts in culture in 1965 [58]. Termed the Hayflick Limit, the number of times a cell lineage could divide before short telomere-induced proliferative arrest was determined by the structural integrity of telomeres and the activities of biological pathways responsible for maintaining the length of these specialized DNA tracts [59, 60]. Incidentally, this process can be viewed as a tumor suppressive mechanism: by limiting the number of cell divisions that can occur in a particular cell lineage, one can reduce the accumulation of deleterious mutations that precede cellular transformation [61].

In rare cases, some somatic cells are able to bypass this short telomere checkpoint by inactivating the genome surveillance mechanisms mediated by the tumor suppressor genes p53 and retinoblastoma protein (Rb) (Fig. 2). Further cell divisions in p53/Rb-inactivated cells continue to deplete telomeric DNA, leading to the disruption of the telomere structure [62]. Uncapped telomeres are recognized by cellular repair mechanisms as damaged DNA, resulting in cells attempting to repair these damages. Erroneous repair leads to chromosome end fusions and rampant genomic instability. When this happens, a second checkpoint termed “crisis” [57] is activated and cells are triggered to undergo apoptosis. Under this extreme selective pressure, most cells will die. In extremely rare cases (~1 in 10 million cells), genomic instability can lead to the reactivation of a specialized cellular reverse transcriptase, termed telomerase, which is capable of adding telomeric repeats to chromosome ends [63]. Telomerase expression





**Fig. 2** Telomere dynamics and cancer development. With each cell division, approximately 50–100 bp of telomeric DNA is lost from chromosome ends. With continual proliferation, telomeres will eventually reach a critical short length and are triggering replicative senescence. Inactivation of genome surveillance mechanisms mediated by the tumor suppressor genes p53 and Rb allow continual cell divisions, further depleting telomeric DNA leading to rampant genomic instability and the induction of apoptosis. A rare cell (~1 in 10 million) can be forced to reactivate telomerase, allowing the cell to replace lost telomeric repeats, prevent further genomic instability and confer the unlimited proliferative capacity required for the formation of a malignant tumor cell

allows cells to replace lost telomeric repeats and prevent further chromosome instability. In these cases of forced reactivation of telomerase enzyme expression, constitutive telomerase activity confers the unlimited proliferative capacity required for the formation of a malignant tumor cell (Fig. 2).

### 2.3 *Dysfunctional Telomere Capping Is Recognized as DNA Damage*

Dysfunctional telomeres are created when telomeric sequences are shortened beyond a critical length allowing for the formation of a higher order chromatin structure [5], or by the genetic deletion or protein dysfunction of key shelterin components [28, 56]. Uncapped telomeres expose the ends of chromosomes, thereby inducing the DDR and resulting in the cascade of genomic instability through erroneous chromosome end repair. Genetic deletion of different shelterin components in

mice resulted in overlapping yet distinct phenotypes, underscoring the complexity and the distinct roles of shelterin components in chromosome end capping [64–67]. In parallel, cellular biological experiments using human cell models have demonstrated that TRF2 and POT1 have independent roles in the normal suppression of distinct DDR pathways, and that their dysfunctions cause severe molecular cytogenetic phenotypes [19, 56, 68].

Expression of dominant negative TRF2 that cannot bind DNA leads to the induction of a potent DDR mediated by the Mre11-Rad50-NBS (MRN) complex and the ataxia telangiectasia mutated (ATM) protein [26, 69]. As a protein kinase, ATM activates the DNA repair machinery through a cascade of phosphorylation activity, including targets such as the histone variant H2AX and the p53 binding protein (p53BP) [70]. Using immunofluorescent labeling, the MRN complex, ATM,  $\gamma$ H2AX, and p53BP can all be seen to form DNA damage foci at uncapped telomeres resulting from the loss of TRF2 binding [56]. Known as telomere dysfunction induced foci (TIF), these structures contain DDR factors similar to those found in double-stranded DNA damage foci. Thus, part of the normal function of TRF2 binding to the telomere is the suppression of the ATM DDR pathway [19, 69].

Reducing the expression of POT1, or its binding partner TPP1, activates the ataxia telangiectasia and Rad3-related (ATR) kinases [69, 71]. Together with its obligate subunit, ATR interacting protein (ATRIP), ATR phosphorylates DDR effectors responsible for diverse DNA damages, such as those induced by UV exposure, exposure to nucleophilic crosslinking agents or resulting from collapsed replication forks [72]. *In vitro* biochemical experiments have shown that ATR is activated by replication protein A (RPA)-coated single-stranded DNA. This is reminiscent of the single-stranded telomeric G-rich overhang left vacant by the removal of POT1 binding. Thus, part of the normal function of POT1 binding to the telomeric terminal is the suppression of the ATR DDR pathway [19, 64, 69].

## **2.4 Key DDR Players Are Required for Normal Telomere Maintenance**

The relationship between telomeric chromatin and the DDR machinery extends beyond simple antagonism. DDR components are known to play positive roles in the normal homeostatic maintenance of telomeres, in the absence or presence of telomerase activity. Evidence that key DDR components have important roles in normal telomere homeostasis comes from studies of inherited human diseases and from animal models of these diseases. Genetic disorders such as Ataxia telangiectasia, Nijmegen break syndrome, Bloom syndrome and Werner syndrome all exhibit molecular phenotypes of accelerated telomere shortening [70, 73]. Initially believed to be the function of an increased rate of telomere attrition due to the higher cellular turnover, animal models and cellular biology studies later revealed the normal functions of these proteins in telomere homeostasis.

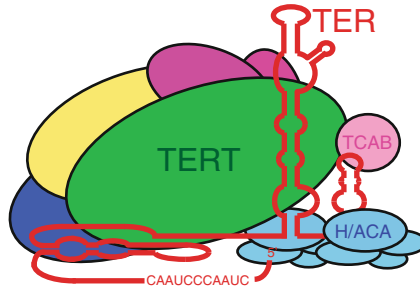
DDR mediators are activated each time telomeric DNA undergoes replication [78, 79]. Normal replication through telomere ends requires the resolution of the telomere loop, followed by leading and lagging strand synthesis through the ends. This requires the action of RecQ helicases, such as Bloom and Werner, the elective de novo synthesis of G-rich telomeric DNA when telomerase is active, nuclease trimming by Apollo, XPF or the MRN complex to create the correct telomeric DNA terminus and overhangs, and the reformation of the telomeric loop structures through the actions of Rad51D, RPA and other homologous recombination pathway effectors. In addition, the steps necessary to “open up” a telomere for DNA replication machinery access predicts that the transient recognition of these “open” telomere structures by DDR sensors. Indeed, both ATM-MRN and ATR-ATRIP complexes are found at functional telomeres during the DNA replication phase of the cell cycle [78, 79]. Despite the data supporting these models, detailed mechanisms of how telomeric binding proteins coordinate with transient DDR signals to direct telomere formation, instead of promoting erroneous DNA repair at these sites, still need to be elucidated.

### 3 Telomerase

#### 3.1 *Telomerase Structure and Biogenesis*

The human telomere terminal transferase enzyme, more commonly referred to as telomerase, is a ribonucleoprotein (RNP) responsible for the de novo synthesis of telomere repeats. This unique reverse transcriptase extends chromosome ends by utilizing an integral RNA subunit as a template to synthesize the TTAGGG telomeric DNA repeats. The core components of this enzyme complex consist of the telomerase reverse transcriptase catalytic subunit (TERT) and the telomerase RNA (TER), which contains the template sequence for telomere synthesis. In the human enzyme, RNA-binding proteins such as the H/ACA proteins dyskerin, Nop10, and Nhp2 are also found to associate with the core enzyme complex (Fig. 3, Table 2). Other proteins transiently associate with the core enzyme complex and play important roles in the regulation of the catalytic activity, enzyme stability, cellular localization and intracellular trafficking of the enzyme (Table 3) [80–82].

TER and TERT were identified as the catalytic core of this complex by virtue of their ability to form a complex and elongate telomeres *in vitro*, in the absence of other protein factors [83]. However, *in vivo*, telomerase employs an intricate biogenesis pathway involving specific factors for enzyme assembly, trafficking and the subcellular localization of the holoenzyme complex. TER transcription is ubiquitous in all human cells. The stability of TER is dependent on biogenesis protein factors Shq1 and NAF-1 mediated complex formation with the H/ACA proteins (dyskerin, Nhp2, and Nop10). TER association with the H/ACA complex results in the formation of a stable but inactive telomerase RNP. Assembly of this



**Fig. 3** Human Telomerase. Schematic depiction of the human telomerase enzyme. Telomerase is a specialized reverse transcriptase carrying its own RNA template (TER). Telomerase RNA serves multiple functions. The template domain allows sequence specific alignment of the linear chromosome ends into the catalytic site and provides the 6nt template sequence for RT. Other domains of the RNA serve structural and catalytic functions, RT activity is provided by the protein subunit, telomerase reverse transcriptase (TERT). Together, TER and TERT comprise the minimal functional unit that can be reconstituted *in vitro* for telomerase activity. The *in vivo* accumulation and stability of TER requires the association of RNA with two sets of H/ACA proteins. Other protein factors involved in the regulation of enzyme functions through cellular localization (TCAB1), assembly (Pontin and Reptin) and other mechanisms associate with the holoenzyme complex in a transient manner

inactive telomerase RNP with TERT is required for catalytic activity [84]. Telomerase enzyme assembly is cell cycle and subcellular localization dependent [85]. Numerous biogenesis factors, including staufen, L22, SmB/SmD3, PinX1, 14-3-3, nucleolin, pontin, reptin, Hsp90, p23, and telomerase Cajal body protein 1 (TCAB1) have all been demonstrated to play important roles in TER/TERT subcellular localization and enzyme assembly. Finally, following the formation of a functional telomerase enzyme, additional trafficking factors, such as TCAB, hEST1A, and hnRNPs, are required for proper transport of the active enzyme to chromosome ends.

### 3.2 Telomerase RNA

TER is a noncoding RNA that serves as a template for TERT-dependent addition of telomeric repeats. Ubiquitously expressed, human TER is synthesized by RNA polymerase II (pol II) and processed into a mature 451-nucleotide (nt) product with a 5' trimethyl guanosine cap that lacks a polyadenosine tail at its 3' end [86, 87]. TER contains a 341-nt pol II-type promoter region upstream of the transcription start site [88]. Nuclear factor-Y (NF-Y), Sp1 and Sp3 are essential regulators of TER promoter function.

Primary and secondary structural elements of TER contain many motifs that are essential for telomerase activity as well as cellular accumulation of mature TER. The 11-nt telomeric repeat template sequence is contained within the 5' portion of TER in the pseudoknot domain (nt 1–209). The H/ACA motif (nt 275–451)

**Table 2** Core Telomerase Holoenzyme Subunits

Holoenzyme component	Role in telomerase	Other suggested biological functions	Effects of functional/genetic changes to telomere maintenance
TERT	<ul style="list-style-type: none"> <li>- Reverse transcriptase</li> <li>- Expression levels is transcriptionally regulated</li> <li>- Expression levels control temporal pattern of telomerase activity in human cells</li> </ul>	<ul style="list-style-type: none"> <li>- Promotes double-stranded DNA damage repair</li> <li>- Suggested to act as a transcription factor in mouse, in the absence of TERT</li> </ul>	<ul style="list-style-type: none"> <li>- Knock down results in the lost of telomerase activity</li> <li>- Haploinsufficient in humans</li> <li>- Genetic mutations are associated with autosomal dominant dyskeratosis congenita</li> </ul>
TER	<ul style="list-style-type: none"> <li>- RNA template</li> <li>- Ubiquitously expressed</li> </ul>	<ul style="list-style-type: none"> <li>- Inhibit ATR activation, in the absence of TERT</li> </ul>	<ul style="list-style-type: none"> <li>- Knockdown results in the lost of telomerase activity</li> <li>- Haploinsufficient in humans</li> <li>- Genetic mutations are associated with autosomal dominant dyskeratosis congenita</li> </ul>
DKC1	<ul style="list-style-type: none"> <li>- Member of H/ACA protein complex</li> <li>- Binds to TERT and direct correct processing, localization and promotes in vivo stability of TERT</li> </ul>	<ul style="list-style-type: none"> <li>- Pseudouridine synthase</li> <li>- Use H/ACA RNA as a guide to modify rRNA and snoRNA</li> </ul>	<ul style="list-style-type: none"> <li>- Knockdown results in decreased TERT accumulation</li> <li>- Genetic mutations in DKC1 are associated with X-linked dyskeratosis congenita</li> </ul>
Nop10	<ul style="list-style-type: none"> <li>- H/ACA protein</li> <li>- Necessary for the correct processing, localization and in vivo stability of TERT</li> </ul>	<ul style="list-style-type: none"> <li>- Together with DKC1 and Nhp2, forms the RNA binning surface for association with H/ACA RNAs</li> </ul>	<ul style="list-style-type: none"> <li>- Genetic mutations in NOP10 are associated with autosomal recessive dyskeratosis congenita</li> </ul>
Nhp2	<ul style="list-style-type: none"> <li>- H/ACA protein</li> <li>- Necessary for correct processing, localization and in vivo stability of TERT</li> </ul>	<ul style="list-style-type: none"> <li>- Together with DKC1 and NOP10, forms the RNA binning surface for association with H/ACA RNAs</li> </ul>	<ul style="list-style-type: none"> <li>- Genetic mutations in Nhp2 are associated with autosomal recessive dyskeratosis congenita</li> </ul>

**Table 3** Telomerase Biogenesis and Regulation Factors

TER biogenesis and regulation	Protein components	Roles in telomerase pathways	Other biological functions	Effects of changes in expression levels in telomere maintenance
	NAFI Shq1	Early assembly factors for H/ACA RNA (including TER)	H/ACA RNPs biogenesis	ND Knockdown of SHQ1 causes a decrease in TER accumulation
	hSaufen L22	TER associating complex	Proper RNA localization	ND
	SmB and SmD3	TER associating complex, binding to the CAB box of the CR7 region	Component of 60S ribosomal unit SMN complex	ND
TERT biogenesis and regulation	Hsp90 and p23 14-3-3	TERT chaperones Nuclear localization of TERT by masking of the TERT nuclear export signal	Chaperone functions in nuclear and ligand activated receptors Controlling intracellular signaling, molecular chaperone functions and intracellular localization	Inhibition of Hsp90 or p23 block the assembly of telomerase Mutant 14-3-3 leads to the decrease of TERT nuclear accumulation but does not reduce telomerase activity <i>in vitro</i>
	Nucleolin	TERT nucleolar localization	Synthesis and maturation of ribosome RNAs	Nucleolin mutants prevent TERT nucleolar localization but have no effects on telomerase activity
	PimX1	TERT nucleolar localization	rRNA and snoRNA maturation	Over-expression of PimX1 inhibits telomerase activity and leads to telomere shortening
	c-Abl	c-Abl phosphorylates TERT and inhibits its activity	Cell differentiation, cell division, cell adhesion and stress response	c-Abl <sup>-/-</sup> causes increase in telomere length

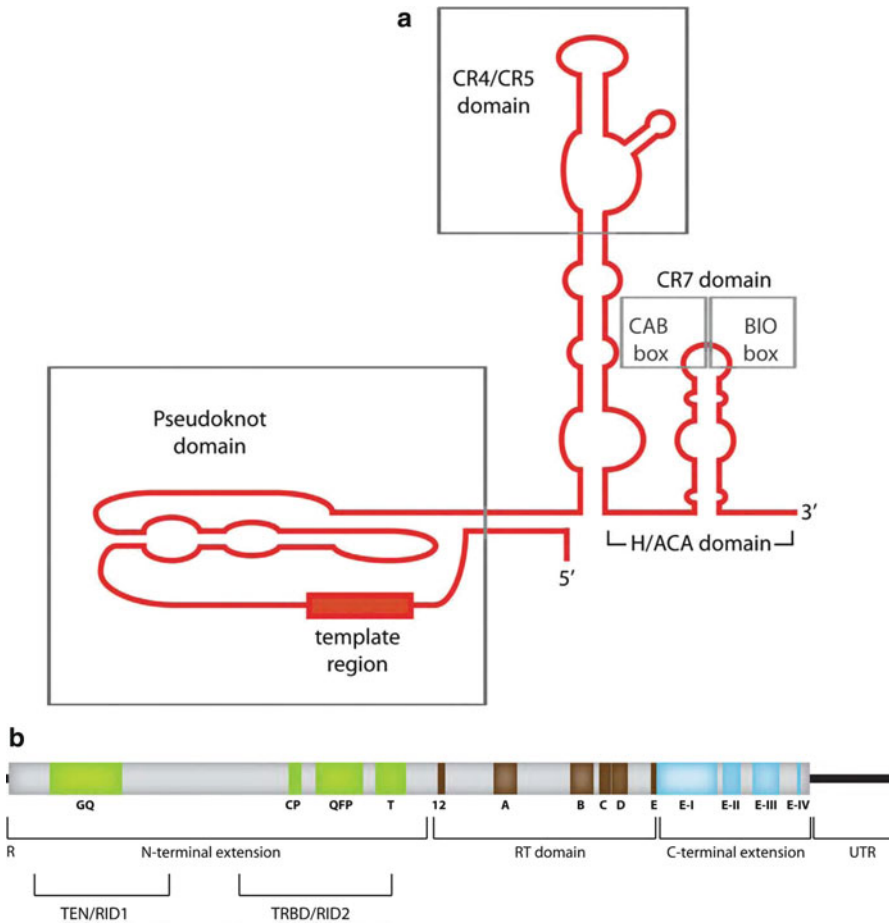
Akt	Akt phosphorylates TERT and inhibits its activity	Angiogenesis, cell survival and cell division	Over-expression of Akt decreases telomere length in HTC cells
MKRN	Regulates ubiquitination of TERT	Lysine ubiquitination, targeting proteins for proteasome degradation	Over-expression of MKRN1 decreases telomerase activity and telomere length
SMURF	SMURF2 activated in presence of shortened telomeres	Lysine ubiquitination, targeting proteins for proteasome degradation	SMURF2 is increased by telomere shortening
Telomerase assembly and localization factors	TER associating protein, binding to the CAB box of the CR7 region	Small Cajal body RNAs biogenesis and localization	Mutant TCAB is associated with AR Dyskeratosis congenita TCAB knockdown leads to telomere shortening
pontin and reptin	Assembly of TER, dyskerin and TERT	AAA + ATPase chromatin remodeling, transcription co-regulation, DNA damage responses	Pontin knockdown decreases dyskerin steady state levels and telomerase activity by 10–20%
NAT10	Telomerase assembly, disassembly and localization	Nucleolar assembly, cytokinesis and microtubule stabilization	NAT10 over-expression causes a decrease in telomere length but does not affect telomerase activity
GNL3L	Telomerase assembly, disassembly and localization	Nucleolar GTPase	GNL3L over-expression causes a decrease in telomere length but does not affect telomerase activity
Telomere tethering/ binding	Recruitment of TERT to telomeric terminus. Bind TERT independent of TER.	Involved in nonsense mediated mRNA decay. Implicated in TERRA-mediated telomere chromatin formation.	Recombinant expression of EST1A in the presence of TERT increases telomere length when compared to cells receiving TERT alone. EST1A expression alone causes a loss of telomere repeats.

(continued)

**Table 3** (continued)

EST1B	Associate with telomerase activity. Bind TERT independent of TER.	Involved in nonsense mediated mRNA decay. Implicated in TERRA-mediated telomere chromatin formation.	Recombinant expression of EST1B did not significantly change telomere length
hnRNPs	Telomere telomerase interaction	Regulation of alternative splicing and mRNA transport and packaging	Over-expression of hnRNP C1 and U leads to a decrease in telomere length
Pif1	DNA helicase negatively regulate telomerase by unwinding DNA/RNA hybrid	In yeast, the mitochondrial form of Pif1 is involved in mitochondrial DNA repair	Expression of wildtype Pif1 in human fibrosarcoma cells resulted in telomere shortening





**Fig. 4** Human TER and TERT organization. **(a)** Secondary structure of TER. The 451-nt RNA includes the 11-nt template region in addition to conserved regions: pseudoknot domain (nt 1–209), CR4/CR5 (nt 214–330), CR7 3' terminal hairpin domain, which contains the CAB box and BIO box, and H/ACA domain (275–441). **(b)** Functional organization of TERT protein. The reverse transcriptase (RT) domain is flanked by an N-terminal domain which is subdivided into an RNA binding domain (TRBD/RID2) and a TERT essential N-terminal (TEN/RID1) domain. The seven universally conserved RT motifs are illustrated as *purple boxes*

is essential for TER association with the chaperone H/ACA protein complex. Association with the H/ACA proteins is crucial for cellular accumulation and 3' end processing of TER. The 3' terminal hairpin domain (CR7; nt 408–422) contains a Cajal body specific localization signal (CAB box) necessary for the accumulation of TER to the Cajal bodies (CBs), as well as a biogenesis box (BIO box), which is necessary for *in vivo* accumulation of TER (Fig. 4a) [89, 90].

### 3.3 *The Hinge/ACA Proteins (H/ACA)*

The H/ACA proteins dyskerin, Nop10, and Nhp2 form the core trimer that acts as a chaperone to promote the *in vivo* accumulation of TER. The binding of these proteins with TER immediately following transcription is essential for its cellular accumulation, processing and stability [93]. In contrast to other protein factors described in the later sections, H/ACA proteins associate with TER throughout the enzyme's life span and are considered stable components of the telomerase holoenzyme, as illustrated by affinity purification experiments [92, 93].

Two sets of H/ACA proteins bind to the distal and proximal stem loops of the TER H/ACA motif (nt 275–441) [80]. Mutations in the H/ACA motif in TER, as well as in the members of the H/ACA core trimer complex (dyskerin, Nhp2, and Nop10), are associated with genetic diseases with the common etiology of telomerase deficiencies and overlapping clinical presentations of premature tissue aging phenotypes [94–98].

### 3.4 *Other TER-Associating Factors*

RNA binding proteins, staufen and L22, have been shown to independently associate with TER *in vivo* and are involved in TER processing, localization and telomerase assembly [99]. The Sm-fold proteins, SmB and SmD3, have also been shown to associate with TER and are involved in its subcellular localization to Cajal bodies. SmB and SmD3 both interact with the CAB box sequence on TER, located in the CR7 domain, through an extended C-terminal tail modified with symmetric dimethyl-arginine. Deletion of this modified C-terminal sequence disrupts their association with TER [94]. However, it is not known whether this association is mediated through direct interactions between Sm proteins and TER or through the interactions with a tether protein. Mutations in TER's CAB box result in a significant decrease in SmB and SmD3 association and a loss of CB localization [100, 101]. Notably, the novel RNA binding protein TCAB1 was also shown to bind TER at the CAB box [103]. It is currently unknown whether SmB/SmD3 and TCAB1 proteins coexist on the same telomerase molecule, or if associations with these specific protein factors occur at different stages of the telomerase enzyme's maturity.

### 3.5 *Telomerase Reverse Transcriptase*

Catalytic activation of the telomerase complex requires the transcriptional activation of TERT. The TERT gene, located on chromosome 5p15.33, is composed of 16 exons and encompasses more than 37 kb [103, 104]. The GC-rich promoter region is located 1,100 bp upstream from the ATG start codon [104, 105]. This region lacks both TATA and CAAT boxes [103] and was found to be hypermethylated in somatic

cells which correlates with its transcriptional inactive state. The TERT promoter contains numerous c-myc, as well as other oncogenic transcription factors, such as c-Jun and c-fos binding sites, which have been demonstrated to mediate TERT transcriptional activation in transformed cells [105]. Transcription activation of the TERT locus produces a full length TERT-mRNA, as well as a variety of alternative spliced forms. TERT alternative splicing is believed to regulate the levels of functional telomerase in a development stage specific manner [107]. Following protein translation of the full length 125 kDa polypeptide [108, 109], TERT associates with chaperones Hsp90 and p23, and is transported to the nucleus via its nuclear localization signal where it is assembled with the TER-H/ACA complex to form the fully functional telomerase enzyme [110].

TERT contains a central reverse transcriptase (RT) domain that is flanked by a N-terminal region and a C-terminal domain. The TERT N-terminal region is further subdivided into two domains: an RNA binding domain (TRBD) and a TERT essential N-terminal (TEN) domain. A large non-conserved linker region separates the two N-terminal domains (Fig. 4b) [112].

The RT domain contains the seven universally conserved RT motifs (1, 2, A, B', C, D, and E) [113]. An invariant trio of aspartic acids (found in motifs A and C) is directly involved in catalysis, as mutations of these residues results in abolished catalytic activity *in vitro* and *in vivo* [84, 114–117]. Mutations of other amino acid residues in any of the conserved RT motifs were also found to reduce or eliminate telomerase reverse transcriptase activity (Fig. 4b) [84, 115, 117].

The high affinity RNA binding domain (TRBD), also known as the RNA interacting domain 2 (RID 2), contains telomerase specific motifs CP, QFP, and T, also referred to as domains II, III, and IV, respectively [118–120]. These motifs mediate TER recognition and have a relatively high binding affinity to structured RNA stem loops, interacting with the CR4/CR5 domain of TER [121]. This domain plays a role in promoting stable enzyme assembly, as mutations in these motifs result in severe defects in TER–TERT association (Fig. 4b) [122].

The TERT essential N-terminal (TEN) domain or RNA interacting domain 1 (RID 1), contains the non-conserved extreme N-terminus motif [123] and moderately conserved GQ motif (also referred to as domain I) [112, 122]. The GQ motif is further divided into domains IA and IB, separated by a DAT (dissociates activities of telomerase) domain [124]. The TEN domain interacts with the TER pseudoknot-template domain [121], but is not considered a major TER binding surface as mutations in this region only result in modest reductions of TER–TERT association [122]. This region also displays high single-stranded telomeric DNA binding affinity, suggesting an important role in substrate recognition and primer binding (Fig. 4b) [121, 124–126].

The smaller, less-conserved C-terminal domain (TEC or CDAT) plays several roles in telomerase function: it contributes to telomerase catalytic activity [121, 127], regulates the cellular localization of the enzyme, and plays a role in polymerase processivity [128, 129]. However, this domain is not essential for RNA binding, as mutations in this region were not found to impair TER–TERT association (Fig. 4b) [129].

### 3.6 *TERT Chaperones and Localization Factors*

Molecular chaperone proteins p23 and Hsp90 were identified as key factors in the assembly and functionality of the telomerase holoenzyme. Both were found to associate with TERT and aid in its nuclear import and localization. They were also demonstrated to be required for the assembly of active telomerase enzyme both *in vitro* and *in vivo*, as inhibition of either chaperone protein disrupts telomerase assembly leading to a reduction in enzyme activity ([110]; see geldanamycin mechanism below).

The nuclear retention of TERT is dependent on its association with the 14-3-3 proteins, a protein family involved in intracellular trafficking/targeting, cell cycle regulation, cytoskeleton structure, and transcription [129]. TERT and 14-3-3 interact via their respective C-termini. This interaction is required for the nuclear accumulation of TERT, as 14-3-3 proteins promote the nuclear retention of TERT by masking the nuclear export signal (NES)-like motif in the C-terminal region of TERT. Binding of 14-3-3 inhibits the binding of CRM1/exportin 1 to TERT NES, resulting in the nuclear accumulation of the reverse transcriptase.

Nucleolin is a phosphorprotein that binds to TERT through its RNA binding domain 4 and the carboxyl terminal RGG domain. RNA binding domain 1 may also be involved in the nucleolar localization of telomerase holoenzyme through its interactions with TER. Biochemical experiments had shown that the binding of TERT with the nucleolin-4R fragment, which lacks a nucleolar localization signal, resulted in the mislocalization of TERT in the cytoplasm, thereby implicating this protein in the subnuclear localization of TERT [130].

PINX1, a PIN2/TRF1 interacting protein, is involved in TERT nucleolar localization and has also been characterized as an inhibitor of telomerase activity and a negative regulator of telomere length. Inhibition of endogenous PINX1 resulted in an increase in telomerase activity, whereas over-expression of PINX1 decreases telomerase activity and shortens telomeres [131]. PINX1 was found to bind directly with TERT at its RNA binding domain and indirectly associate with TER through TERT [132].

### 3.7 *TERT Post-translational Modifications*

Telomerase activity is regulated via post-translational modifications of TERT. Several studies have demonstrated that the phosphorylation of TERT is required for the catalytic activity of the enzyme [133–136]. Protein kinase B (Akt) and protein kinase C $\alpha$  have both been shown to interact with and phosphorylate TERT *in vitro* and *in vivo* [133–135], resulting in the increase in telomerase activity. Conversely, protein phosphatase 2A inhibits telomerase activity via the dephosphorylation of TERT directly [133, 137] or indirectly, through the dephosphorylation and inhibition of Akt. c-Abl protein tyrosine kinase associates with TERT and mediates TERT phosphorylation *in vitro* and *in vivo*. In contrast to the activation models above, c-Abl phosphorylation of TERT resulted in the inhibition of telomerase activity, making this kinase a negative regulator of TERT [138].

The E3 ubiquitin ligase MKRN1 was shown to have a negative role on telomere length homeostasis. MKRN1 is responsible for the ubiquitination of TERT, targeting TERT for protease degradation. Over-expression of MKRN1 results in the decrease of telomerase activity and subsequently in the shortening of telomere length [139].

### **3.8 *TER–TERT Biogenesis/Assembly Factors***

Pontin and reptin, members of the AAA+ family of DNA helicases [140], play pivotal roles in telomerase assembly. These helicases are found to bind to dyskerin and play a role in the formation of the TER-dyskerin complex. Subsequently, these helicases bind to endogenous TERT and mediate the assembly with TER-dyskerin complex to form the catalytically active telomerase enzyme [141]. The formation of the TERT-pontin-reptin complex is regulated by cell cycle stages, with the highest level of complex formation occurring during S-phase, providing evidence for another level of cell cycle dependent regulation of TERT.

Nucleoplasmic Cajal bodies (CBs) have been suggested as one of the sites for telomerase assembly. The novel RNA binding protein TCAB1 was shown to be required for telomerase localization to these sites. Knockdown of TCAB using retroviral shRNA and RNA interference resulted in a significant reduction in the percentage of cells with TER staining in CBs by microscopic analysis [102], indicating its role in CBs localization of telomerase. TCAB1 was found to associate with TER by binding specifically to the CAB-box sequence (CR7 motif). Inhibition of TCAB1 by shRNA also reduced the amount of TER at telomeres during S phase of the cell cycle, resulting in telomere shortening. This data suggested that TCAB1 plays a role in controlling the access of telomerase complex to telomeres, representing an additional level of enzyme activity regulation [102] and see below).

### **3.9 *Targeting Telomerase Holoenzyme to Telomere***

Newly assembled, catalytically active telomerase enzyme must travel to and associate with the limited number of telomere ends for its proper function. As illustrated with the earlier discussion on TCAB, the Cajal Bodies were suggested as sites where the delivery of the active enzyme to the telomeres occurs [142–144]. TER is found localized at CBs in cancer cells throughout the cell cycle [101, 142]. Mutations in the CAB box motif decrease the accumulation of TER in CBs as well as the frequency of TER association with telomeres, resulting in shorter telomere length [101, 144]. Recent analysis of genetic lesions responsible for the rare autosomal recessive isoforms of dyskeratosis congenita (AR-DC) identified TCAB1 compound heterozygous mutations in a small subpopulation of AR-DC. While TER accumulations were within the normal range, telomerase RNA was found to

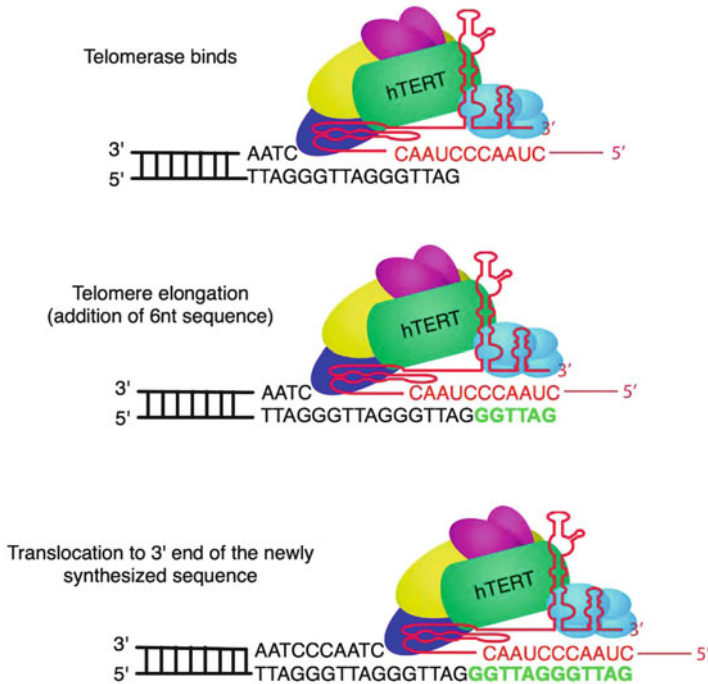
accumulate at nucleoli instead of Cajal bodies. Mislocalization of the telomerase holoenzyme prevented telomere access leading to a loss of telomere length maintenance. This data identified TCAB1 as a critical telomerase regulation factor, which recruits the holoenzyme complex to Cajal bodies for proper telomere access and synthesis [145].

The presence of TERT was also found to be necessary for the localization and accumulation of TER in CBs as well as trafficking of telomerase to telomeres during S phase of the cell cycle [142, 146, 147]. However, outside of S phase, TERT resides in subnuclear foci, termed TERT foci [143], indicating that these two components are not transported to CBs as an assembled complex. Inhibition of TERT resulted in a decrease of TER colocalized with CBs and telomeres without affecting the levels of TER in cells. Additionally, expression of TERT in telomerase negative cells resulted in the accumulation of TER at both sites [146]. These observations again suggest that CB localization of telomerase is connected to enzyme biogenesis and catalytic activity in transformed cells.

hEst1A has also been suggested to play an important role in telomere maintenance in a manner similar to its yeast homologue Est1p. Yeast Est1p interacts with TLC (yeast TER) and the yeast telomere binding protein Cdc13, thereby recruiting telomerase to the proximity of the telomeres [148, 149]. Using *in silico* methods, three human homologs of yeast telomerase telomere-recruitment factor Est1p were identified. Of these three proteins, Est1A shows the highest sequence homology with ScEst1p [150]. Over-expression of Est1A reduced the steady state telomere length, but co-expression of TERT and Est1A increases telomere length substantially, suggesting that Est1A's role in telomere length regulation is completely telomerase dependent. The human Est1p homologs have recently been implicated in TERRA and telomeric chromatin regulation [52, 151].

hnRNPs are also implicated in the localization of telomerase to telomeric ends for the *de novo* synthesis of telomeric repeats. *In vitro* studies demonstrated that hnRNPs A1/UP1, A2, A3, C1/C2, and D bind to TER and single-stranded telomeric DNA [153–156], suggesting possible roles in the bridging and recruitment of telomerase holoenzyme to the telomeres. In parallel, hnRNP A1/UP1 was found at telomere ends *in vivo* and was suggested to stimulate telomerase activity through the disruption of G-quadruplex structures formed during telomere synthesis by telomerase [156].

The human homolog of yeast DNA helicase Pif1 negatively influences the regulation of telomere length, by modulating telomerase activity [157]. hPif1 reduces telomerase processivity at the telomere by binding to and unwinding the DNA substrate and RNA template hybrid, resulting in the removal of telomerase from chromosome ends. hPif1 expression is regulated by cell cycle progression, peaking at late S/G2 [158]. Over-expression of hPIF1 induces telomere shortening in human HT1080 cells through telomerase activity modulation [157].



**Fig. 5** Telomere repeat synthesis. Due to its short RNA template sequence, telomerase relies on two movement behaviors to add multiple 6-nucleotide (nt) telomeric sequences to chromosome ends. Addition of each 6-nt repeat to the 3' end of the template is followed by telomerase translocation. This mediates realignment of the chromosome end from the 5' end to the 3' end of the template to enable subsequent rounds of repeat addition. Telomerase's ability to carry out these two movement behaviors is termed nucleotide addition processivity and repeat addition processivity, respectively

## 4 Telomerase Catalytic Cycle

TERT directs the addition of deoxynucleotide triphosphates (dNTPs) to the ends of the G-rich strand of the chromosome by copying the last six nucleotide of the 11-nt telomere repeat template sequence of TER [159, 160]. This activity results in the *de novo* synthesis of a single, 6 nt repeat. Because the TER RNA template region is quite short, to generate multiple repeats within a single catalytic event, telomerase holoenzyme undergoes multiple rounds of transient dissociation from the DNA substrate, to reposition the enzyme-substrate complex. Telomerase relies on its unique ability to transiently move away from the active site after the addition of a single 6-nt repeat, translocate towards the 3' end of the newly synthesized chromosome and mediate the realignment of the new chromosome end with the TER RNA template, in order to continue subsequent rounds of multiple telomeric repeat addition (Fig. 5). Following the addition of each telomeric repeat, the enzyme may either

disassociate from the chromosome end, stay bound without continuing elongation, or translocate and continue additional cycles of repeat addition [124, 161]. Translocation of the enzyme requires the DNA substrate to remain bound to telomerase. This interaction is mediated through an “anchor site” within the N-terminal domain of TERT (Fig. 4b) [125, 162].

## 5 Alternative Lengthening of Telomeres

Telomere maintenance can also be achieved by a process named alternative lengthening of telomeres (ALT) [163, 164]. ALT was discovered in 1995 when telomere elongation was observed in immortal human cells without detectable telomerase activity [165]. In yeast, this process involves either a rolling circle recombination mechanism or a strand exchange recombination mechanism. ALT is believed to occur by similar processes in humans [166], as it requires the activity of many homologous recombination protein factors including Rad50, MRE11, and NBS1 [167, 168].

One of the defining characteristics of ALT cells is the presence of a special class of promyelocytic leukemia (PML) bodies, known as the ALT-PML bodies [169]. ALT-PML bodies are microscopically defined, multi-protein domains in the nucleus that associate with telomeres in a cell cycle-specific manner [170, 171]. Observation of the ALT cell line U2OS revealed that TRF1 and FANCD2, a member of the Fanconi anemia protein family, colocalized with ALT-PML bodies at the same stages of the cell cycle. Monoubiquitination of FANCD2 is essential for this association, as depletion of FANCA, a member of the ubiquitination complex, or FANCL, the E3 ubiquitin-protein ligase, resulted in the loss of FANCD2 signals in ALT-PML bodies [172]. Depletion of FANCA or FANCD2 also resulted in an increase in telomere-signal-free chromosome ends in ALT cells. Due to the heterogeneity of telomere length in ALT cells, there were no significant changes in the average telomere length corresponding to these events. However, examination of newly synthesized telomere ends revealed that in the absence of FANCA or FANCD2 there is a significant decrease in sister chromatid exchange, supporting a role for monoubiquitination of FANCD2 in the ALT-mechanism of telomere maintenance through homologous recombination. Mutations in the chromatin remodeling proteins, the alpha thalassemia/mental retardation syndrome X-linked protein (ATRX) and death-associated protein 6 (DAXX) were found to associate with the ALT phenotype in a panel of pancreatic neuroendocrine tumors. Loss of ATRX-DAXX function is postulated to compromise heterochromatin states at telomeres, leading to the development of ALT by providing a permissive environment for nonreciprocal homologous recombination [173].

Although detected in human cells, ALT is not considered to be the normal physiological process for the maintenance of telomeres in humans. It has only been observed in a small number of human tumors (carcinoma and osteocarcinoma) and some transformed cell types in culture (mainly fibroblasts) [165, 174]. Long-term



telomerase inhibition could potentially select tumor cells for ALT activation, as recently described in a mouse model of inducible TERT expression [175]. The under-representation of ALT-positive tumors was puzzling, until a 2002 study proved that the ALT mechanism cannot fully substitute telomerase in tumorigenesis: expression of the oncogenic H-Ras allele in the immortal human fibroblast ALT cell line GM00847 did not result in malignant transformation when injected into nude mice. In contrast, the co-expression of TERT in these cells imparted a tumorigenic phenotype [176]. This tumorigenic phenotype was again observed with the introduction of a mutant TERT, TERT-HA, which retains its enzymatic activity *in vitro* but is incapable of maintaining telomere length *in vivo*. Additionally, recombinant telomerase expression in ALT models accelerates cell growth and promotes anchorage-independent growth. Telomerase-positive ALT cells pass through cell-division phases of the cell cycle more quickly, implying that the observed cell-growth advantage is cumulative over cycles of proliferation [177]. The ALT recombination mechanism was not able to completely replace telomerase in the process of cellular transformation, implicating an additional, tumor growth-promoting role of TERT, independent of its role in telomere length maintenance.

## 6 Telomerase and Cancer

Most normal human somatic cells do not express detectable levels of telomerase activity as TERT expression is rapidly down-regulated following embryonic development [178]. In some human cell types, such as germline cells and stem cells, where there is a high demand for proliferation, TERT transcription is periodically activated to allow for transient expression of the enzyme. In contrast, more than 85% of human tumors surveyed harbor robust telomerase activity [179]. In almost all cases, the transcriptional up-regulation of TERT is responsible for the increase in ectopic telomerase activity in tumor cells [180]. Proof-of-concept experiments showed that the inhibition of telomerase in human cancer cells resulted in telomere-induced crisis and apoptosis in cell culture models [181, 182].

Telomerase expression is not considered to be oncogenic, as it alone does not lead to the development of cancer [183]. Additionally, it has been shown that TERT expression alone is not sufficient for the immortalization of human mammary epithelial cells, keratinocytes [184], prostate epithelial cells [185], or airway epithelial cells [186]. Cooperation between TERT and other oncogenic factors are essential for the transformed phenotype [187].

Paradoxically, early neoplastic lesions typically have undetectable or low telomerase activity, when compared to advanced malignant lesions that over-express the enzyme [188, 189]. This suggests that initiation of tumor development may require the absence of telomerase activity. Indeed, data from tumor cytogenetic studies have demonstrated that telomere length from precancerous lesions are much shorter than in normal tissues [12, 190, 191]. Several studies have reported critically short telomeres as a common early feature of many human cancers, such as colon [189],

lung [192], breast [193], pancreatic [194], and prostate [195]. The telomere dysfunction model of carcinogenesis suggested that rampant chromosome instability following the uncapping of dysfunctional, short telomeres contributes to the eventual development of aneuploidy, a genetic signature of cellular transformation and carcinogenesis [57, 196]. Telomere dysfunction is thus recognized as a late event in the process of cancer initiation. After which, telomerase activity has to be induced to prevent further chromosome instability that hinder cancer growth, and provide a mechanism for the indefinite proliferation and immortality phenotype in malignant tumors [197] (Fig. 2).

## 7 NON-telomere Maintenance Roles of Telomerase

Besides its role in telomere maintenance, there is growing evidence pointing to telomerase's additional role in the cancer biology. Higher mRNA levels of several DNA repair and chromatin modifying genes, as well as better double-stranded break repair kinetics, were observed in human foreskin fibroblast cells expressing TERT as compared to cells lacking ectopic expression of the enzyme [198]. Importantly, these effects occurred rapidly before any significant telomere lengthening was observed. A transcriptome study done by Smith and colleagues [199] demonstrated that the ectopic expression of telomerase in human mammary epithelial cells reduced the need for exogenous mitogens for cellular proliferation, correlating with the telomerase dependent induction of gene expression that promotes cell growth and survival. This latter study provided evidence for a role of telomerase in cellular proliferation by affecting the expression profiles of growth and survival-related genes. In corroboration of this model, TERT is shown to act as a transcriptional co-activator of the beta-catenin transcriptional complex in mice [200], a function that is independent of its reverse transcriptase activity and its association with the telomerase RNA [200, 201]. These data have been recently corroborated by Masutomi and Hahn's model implicating human TERT in the promotion of TWIST expression and the resultant epithelial–mesenchymal transition. In conformity with the mouse models, TERT was found to complex with the BRG1, a SWI/SNF-related chromatin-remodeling factor, in transformed human cells. Distinct from mouse models, human BRG1-TERT complexes with additional nucleolar proteins nucleostemin (NS) and/or GNL3L [202].

Aside from transcriptional regulation, TERT activity is also implicated in optimal mitochondrial function, independently of TER [203, 204]. Although direct molecular proof of TERT's functionality in the mitochondrion has not yet been established, TERT has intriguingly been shown to exhibit a RNA-dependent RNA polymerase activity when partnered with a mitochondrial RNA, RMRP [203]. DePinho's group showed that a switch to the ALT mechanism of telomere maintenance in mouse T-cell lymphoma, through the inhibition of TERT expression, was accompanied by a specific induction of mitochondrial enzymes that reduce oxidative damage. This supports the hypothesis that TERT harbors mitochondrial functions, independent of TER [175].

In addition to transcription co-activator functions, constitutive TERT expression is also involved in enhanced DNA repair. Normal, diploid human fibroblasts over-expressing TERT were found to be more resistant to apoptosis and necrosis induced by DNA damages, but equally susceptible to the cytotoxic effects of oxidative agents as normal fibroblasts without TERT expression [205]. This suggested that telomerase is involved in enhancing cellular survival following genotoxic stress. Direct evidence implicating telomerase's role as a regulator of the DNA damage response pathway was provided by a cell biology study [206]. By suppressing endogenous TERT expression in diploid human fibroblasts using either a TERT-coding sequence specific shRNA or an TERT 3' untranslated region-specific shRNA (TERT 3' UTR shRNA), it was shown that TERT participates in DNA damage responses and chromatin maintenance in a manner that is separate from its role in telomere length maintenance. Following ionizing radiation (IR), irinotecan, or etoposide treatment, phosphorylation of H2AX and the ataxia telangiectasia mutated (ATM) protein was greatly impaired in telomerase knock-down cells as compared to control cells expressing normal levels of TERT. As a direct consequence, the phosphorylation of BRCA1 tumor suppressor proteins was not observed and protein levels of p53 were not up-regulated. These results indicate impaired DNA damage responses in cells lacking TERT. Telomerase knock-down cells also exhibited increased sensitivity to IR as shown by the decreased relative survival in clonogenic growth assays. When wildtype recombinant TERT was introduced into cells expressing the TERT 3' UTR shRNA, which does not target these recombinant copies of TERT, the cells ability to respond to DNA damage was restored. The molecular mechanism of how TERT perform this role to promote DNA damage survival remains unclear, but is suggested to be associated with TERT's chromatin remodeling activities. In agreement with these data, our laboratory showed that transient telomerase inhibition synergistically increased the cytotoxicity of double-stranded DNA-damaging agents, in a cell-cycle phase-specific manner. This short-term telomerase inhibition was not predicted to significantly reduce telomere length, and the synergistic cellular toxicity may be ascribed to the inhibition of a non-telomere-related telomerase function in tumor cell growth [207].

## **8 Targeting Telomeres and Telomerase in Anticancer Chemotherapy**

Uncapped telomeres induce a dramatic DDR response culminating in cell cycle arrest and programmed cell death [19, 56, 69]. While targeted disruptions of the telomere structure could have been a viable strategy in anticancer therapy, the therapeutic index would be extremely low, considering that the same DDR activation will be induced in cancer and normal cells alike. Conversely, the apparent lack of TERT expression in normal somatic cells, and the growing evidence for TERT's additional roles in cancer biology, makes telomerase an ideal target for anticancer therapies. Telomerase is constitutively over-expressed in over 85% of all human cancers [179]. Early proof-of-principle experiments demonstrated that the expres-

sion of a dominant negative form of TERT completely inhibited telomerase activity and substantially reduced telomere length in several cancer models [180]. The resulting telomere dysfunctions led to the formation of dicentric chromosomes and other types of chromosome fusions, resulting in the loss of cellular viability and apoptosis. This inhibition of TERT was demonstrated to limit tumorigenicity of mouse xenograft models of cancer [181].

Following these proof-of-principle experiments, numerous strategies targeting the telomerase holoenzyme components are described. In the following sections, we discuss some of the more notable strategies of telomerase inhibition in targeted therapy against cancers.

## 9 Telomerase Catalytic Activity Inhibitors

### 9.1 BIBR1532

BIBR1532 is a small molecule, non-nucleoside inhibitor that interferes with telomeric DNA repeat addition by telomerase through the targeting of the catalytic component TERT [208]. Treatment of cancer cells with low doses BIBR1532 reduces their growth capacity and sensitizes them to other chemotherapeutic drugs, in a telomere length-dependent manner [209]. At high doses of BIBR1532, cells exhibited off target cytotoxic effects independent of telomerase's catalytic function [210, 211]. Leukemia cells, but not normal hematopoietic stem cells, treated with 30–80  $\mu$ M BIBR1532 displayed an immediate reduction in proliferative capacity. In particular, telomere dysfunctions are manifested as increases in telomere signal free ends, formation of chromosome end-to-end fusions, and an increase in phosphorylation of p53 and a loss of TRF2 signals at the telomere. However, BIBR1532 induced cytotoxic effects may not be confined to the formation of dysfunctional telomeres and this off-target effect hampers its further development as an anticancer therapeutic agent.

### 9.2 3'-Azido-2', 3'-Dideoxythymidine (AZT)

AZT is a reverse transcriptase inhibitor used in the highly active antiretroviral therapy (HAART) against HIV infection and in the treatment of virus-associated cancers. As a thymidine analog, AZT has been shown to inhibit telomerase *in vitro* and *in vivo*. Upon its activation through phosphorylation by thymidine kinase, this nucleoside analog is incorporated into telomeric DNA as a chain terminator, blocking further reverse transcription and telomere elongation [212, 213]. Prolonged treatment of adult T-cell leukemic cells with AZT results in telomere attrition, accompanied by increased expression of p14<sup>ARF</sup> and activation of the p53-dependent apoptotic pathway [214]. This leads to an increase in the p53 target p21<sup>WAF</sup> expression and the accumulation of p27<sup>KIP</sup>, to induce cell cycle arrest

or program cell death of the tumor cells. In combination with chemotherapy agents such as 5-fluorouracil, AZT has been shown to increase treatment toxicity in colorectal cancer cell model, most likely in a synergistic manner.

### ***9.3 Oligonucleotide-Based Specific Inhibitors of Telomerase***

Oligonucleotide-based inhibitors of telomerase designed to target the TER template may provide a highly specific, telomerase-based antitumor therapy [86, 215, 216]. GRN163L is a 13-base, lipid modified N3'-P5' thiophosphoramidate oligomer, complementary to the template region of TER. GRN163L binds with high affinity to telomerase [217, 218] and has been demonstrated to effectively inhibit the enzyme, resulting in telomere length shortening and subsequent growth arrest. The 5'-lipid palmitoyl domain facilitates cellular and tissue penetration, as well as makes this agent more acid resistant than other anti-telomerase oligonucleotides, thereby increasing the cellular uptake and bioavailability of the drug [220]. GRN163L shows antitumor effects in several cancers, including breast, liver, lung, and multiple myeloma, both *in vitro* and *in vivo* [219–223]. This drug is currently undergoing clinical trials in patients with chronic lymphocytic leukemia, multiple myeloma, solid tumor malignancies, locally recurrent or metastatic breast cancer and advanced or metastatic non-small cell lung cancer [224].

## **10 Telomerase Expression, Biogenesis and Assembly Inhibitors**

### ***10.1 Costunolide***

Costunolide is a sesquiterpene lactone isolated from *Magnolia sieboldii*. Reported to harbor anti-inflammatory, antifungal, and antiviral properties [225–228], it was also shown to suppress cell proliferation and induce apoptosis in several tumor cell lines, including breast cancer and leukemia cells [229, 230]. Costunolide exerts its anticancer properties through transcription regulation of TERT. A decrease in c-Myc or Sp1 binding to their cognate DNA binding sites on the TERT promoter was observed after costunolide treatment, in a dose-dependent manner [229]. Corresponding to the decrease in TERT mRNA levels, there is a reduction in telomerase activity resulting in an inhibition of cell growth and an increase in apoptosis.

### ***10.2 Geldanamycin***

Geldanamycin is a benzoquinone ansamycin antibiotic and inhibits the binding of cofactor ATP and partner p23 to the molecular chaperone Hsp90 [231, 232]. The Hsp90-p23 complex is a molecular chaperone that binds to and stabilizes

cytoplasmic TERT at intermediate stages for folding, assembly and movement across nuclear membranes. Geldanamycin blocks the assembly of active telomerase both *in vitro* and *in vivo* [110] by disrupting the Hsp90-p23-telomerase interaction. Geldanamycin actions result in the ubiquitination and proteasome degradation of TERT and the reduction of telomerase activity [139]. However, since Hsp90 and p23 form chaperone complexes that have integral roles in numerous biological processes, geldanamycin mediated inhibition of Hsp90 function lacks specificity for the telomerase pathway. Given that many of the Hsp90-p23 binding partners are key players in cancer progression, such as v-Src, Bcr-Abl, Raf-1, and ErbB2 [233–236], geldanamycin promiscuous activities might be beneficial in anticancer chemotherapy. The utility of geldanamycin disruption of Hsp90-p23 formation should be revisited in specific cancer types, based on the molecular etiology of the disease.

## 11 Telomerase Immunotherapy

Telomerase is tested as a novel target for cancer immunotherapy. In telomerase-positive cancers, TERT peptides are presented as epitopes on the tumor cell surface by the major histocompatibility complex (MHC) class I pathway. TERT antigen presentation was demonstrated to produce cytotoxic T lymphocyte responses [237–239]. Two first-generation vaccines have been developed: GRNVAC1 and GV1001. Telomerase cancer vaccine, GRNVAC1, uses an *ex vivo* process where mature dendritic cells are isolated from the patient's blood and transfected with TERT mRNA. These cells are then returned to the body where they stimulate the production of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells specific for TERT [240]. GV1001 is a peptide vaccine derived from the active functional domain of telomerase. GV1001 binds multiple human leukocyte antigen (HLA) class II molecules and harbors putative HLA class I epitopes, and also illicit CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses specific for TERT [240]. Both vaccines were test successful in phase I/II clinical trials for efficacy in producing telomerase specific CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes [240, 241]. GV1001 is currently in two phase III clinical trials for the treatments of pancreatic cancer while GRNVAC1 is being investigated in a phase II clinical trial in patients with acute myeloid leukemia [242].

## 12 Telomerase-Telomere Recruitment Inhibitors

### 12.1 Tankyrase1 Inhibitors

Poly(ADP-ribose) polymerase (PARPs) is a large family of enzymes that use NAD<sup>+</sup> as a substrate to generate ADP-ribose polymers onto glutamic acid residues on protein acceptors [243–245]. Tankyrase 1 and 2 are PARP family members

specifically known for their telomeric poly (ADP-ribosyl) polymerase activities. Tankyrase 1 and 2 ribosylates TRF1, preventing TRF1 from binding to telomeric DNA, and leading to TRF1's proteolytic degradation [246]. Over-expression of tankyrase 1 reduces TRF1 binding to the telomere, enables telomerase access at the telomere ends and the corresponding telomere elongation. Conversely, inhibition of tankyrase 1 induces telomere shortening and cell death through a telomere length independent mechanism: in the absence of tankyrase 1, cells undergoing mitosis are unable to resolve sister telomeres cohesion and were arrested at the mitotic phase [248, 249].

Small molecule inhibitors of tankyrase 1's PARP activities have been shown to complement telomerase inhibition to enhance the rate of telomere attrition [249]. However, given PARPs are known to mediate the ribosylation of multiple protein acceptors, the likelihood of off-target effects by these small molecules PARP inhibitors is high.

## 12.2 G-Quadruplex Stabilizers

G-quadruplexes are stable 4-stranded DNA structures made up of G-rich sequences where the guanine residues form square arrangements. The 3' telomeric DNA overhang is guanine rich and can form these higher order molecular structures, in addition to the normal telomeric DNA structures. Small molecule, non-nucleoside compounds such as telomestatin, BRACO-19, TMPyP4, and carbocyanine dyes, are predicted to bind within the grooves [250] or intercalate [251] G-quadruplex DNA, to stabilize these structures. Compounds that intercalate into the DNA to stabilize the G-quadruplex tend to have large, flat aromatic surfaces and are cationically charged to allow for  $\pi$ -stacking interactions. Examples of such molecules are porphyrins and cisplatin [252]. These older platinum containing complexes are shown to potently inhibit telomerase, leading to telomere shortening, arrested cell growth and subsequent cell death. Newer platinum (II) containing structures are also reported to inhibit telomerase *in vitro*, with distinct covalent linkage that could lock the G-quadruplex structure irreversibly [253].

Several different G-quadruplex inhibitors have been shown to disrupt the binding of telomere-associated proteins, inhibit telomerase activity and induce apoptosis *in vitro* [254–258]. However, as G-quadruplex binding agents, these compounds are predicted to bind elsewhere in the genome and disrupt their local structure, leading to altered functions. For example, telomestatin can bind to non-telomeric G-rich DNA found in the promoter region of the c-myc oncogene [259, 260]. Expression of myc is reduced by telomestatin binding, which stabilizing the G-quartet structure in its promoter and prevent transcription factor access. In addition to these off-target effects, another major problem with G-quadruplex stabilizer is their inability to penetrate the cell membrane. Optimal delivery protocol for these types of drugs has yet to be developed.

## 13 Genetic Therapy Against Telomerase

### 13.1 *TER with Mutant Template*

The expression of mutant-template human telomerase RNA (MT-TER), in telomerase positive cells, has been tested as an anticancer gene therapy. MT-TER assemble with endogenous TERT and the recombinant enzyme then erroneously adds DNA repeats with mutant sequence to chromosome ends. A few copies of mutant DNA repeats are enough to disrupt the binding of telomeric proteins. The resulting compromised telomere structure leads to a loss in cellular viability by inducing apoptosis [260–262]. Even though mutant TER is dominant over endogenously expressed wild-type TER, it can only be expressed at low levels, thereby limiting its cytotoxic efficiency in cancer cells. To overcome this deficiency, co-expression of siRNA against endogenous TER, as well as lentiviral expression of mutant TER, has proven to increase the therapeutic efficacy of MT-TER [262].

### 13.2 *TERT-Promoter Driven Suicidal Gene Therapy*

Based on the selective activation of the TERT promoter in cancer cells, several groups reported the use of recombinant DNA vectors, with TERT promoter driving the expression of cytotoxic transgenes, including the herpes simplex virus thymidine kinase, Bcl2-associated X protein, caspase 8 and bacterial nitro-reductase, delivering suicidal enzymatic activities in a cancer cell specific manner [263–271]. While these proof-of-principle experiments provided the framework for a cancer specific targeting strategy, more work is still needed for the development, delivery, and clinical validity of these cancer gene therapies.

### 13.3 *Hammerhead Ribozyme*

Hammerhead ribozymes targeting either the RNA component or reverse transcriptase component of telomerase are shown to be effective strategies in several cancer models. Colon and gastric carcinoma cells treated with retrovirus delivered ribozyme targeted TERT displayed a significant decrease in telomerase activity and rapid induction of apoptosis [272]. In endometrial and hepatocellular carcinoma cells, ribozyme targeting of TER resulted in a dose dependent decrease in telomerase activity [273, 274]. Up to 90% inhibition of telomerase activity could be achieved at relatively low concentrations of the ribozyme. As with other genetic means of telomerase activity inhibition, the current lack of efficient delivery protocols hamper their use in clinical settings.



### 13.4 Zinc Finger Proteins

Zinc Finger Proteins are synthetic peptides designed to target specific chromosomal loci and alter their functionality or sequence identity. Transcription activation of TERT in tumor cells relies on the activation at multiple transcription factor binding sites on TERT's promoter, including that for SP1, c-MYC, ER, E2F-1, WT-1 and MZF-2 [105, 275, 276]. Conceivably, ZFP designed to target these chromosomal loci will interfere with TERT transcription activities. Recently, a ZFP that recognizes a 12 bp sequence within the core TERT promoter fused to a KRAB repressor domain has been described [277]. *In vitro* expression of this ZFP resulted in >80% reduction of TERT expression. Cancer cell lines engineered to express this ZFP are shown to have significantly lower endogenous TERT mRNA levels, a decrease in telomerase activity and inhibition of cell proliferation within 8–12 days. Longer-term repression of endogenous TERT transcription in human cancer cell lines expressing this ZFP in a stable fashion mirrored these results and displayed shortened telomeres. Despite the positive laboratory data, several issues such as ZFP's treatment efficacy, target efficiency and specificity, as well as the availability of appropriate delivery protocols will need to be addressed before the adoption of these novel therapeutic options into clinical applications.

## 14 Therapeutic Considerations

### 14.1 Combination Chemotherapies

Despite the demonstrations of several successful strategies targeting telomeres and telomerase in cancer cells, their usefulness in the clinics has been marred by several deficiencies. The timeline of inducing cytotoxicity by telomerase inhibition relies completely on the kinetics of telomere shortening to a critically short length. As telomere length decreases at a rate of 50–100 bp per cell division, this process can be quite long, and tumor specific. This time lag can range from weeks to months of continual telomerase inhibition therapy. However, prolonged inhibition of the telomerase enzyme could affect normal human cells that are also dependent on transient telomerase activity for their functionality [61]. In these cases, telomere erosion in off-target cells from telomerase inhibition therapy could precipitate adverse treatment effects in these normal cell types. Premature telomere shortening translate to the accelerated rate of tissue aging. If these cells were allowed to divide beyond the short telomere check point, due to the inactivation of tumor suppressive mechanism, new rounds of chromosome instability cycles could trigger the development of secondary tumors. This paradox, in addition to the lack of proper delivery methods for genetic-based inhibition of TERT function, argues that telomerase inhibition on its own is not efficacious as an anticancer therapy.

On the other hand, telomerase inhibition has been demonstrated to increase the sensitivity to chemotherapeutic agents by overwhelming the DNA repair mechanism, with the creation of unprotected chromosome ends. For example, telomere dysfunction in late generation *TERC*<sup>-/-</sup> mice, lacking the mouse telomerase RNA gene, resulted in decreased cellular survival after exposure to IR [278]. At the cellular level, the rate of apoptosis in gastrointestinal crypt cells and primary thymocytes was higher in telomerase deficient mice as compared to control. These *TER*<sup>-/-</sup> cells also displayed delayed DNA break repair kinetics, as well as persistent chromosomal breaks, complex chromosomal aberrations and massive fragmentation.

Reduction of telomerase activity also resulted in increased cell sensitivity to topoisomerase inhibitors. The MCF-7 breast cancer cell line and HBL-100 immortal breast cell line expressing an anti-TERT ribozyme, which cleaves human telomerase mRNA, resulted in inhibition of telomerase activity, decreased telomere length and induced apoptosis. Additionally, an increased sensitivity to the topoisomerase II inhibitor doxorubicin was also observed in these cell lines. In parallel, when exogenous TERT was introduced into telomerase-negative human fibroblasts, there was a decrease in the sensitivity of these cell lines to doxorubicin, as well as two other topoisomerase inhibitors: mitoxantrone and etoposide [279].

Telomerase inhibition via the ectopic expression of dominant negative-TERT (DN-TERT) in human cancer cells resulted in telomere shortening, growth arrest and apoptosis [181, 182]. Expression of recombinant DN-TERT in BCR-ABL positive leukemia cells completely inhibited endogenous telomerase activity and resulted in an increase in apoptosis following treatment with the tyrosine kinase inhibitor imatinib [280].

Telomerase inhibition was also demonstrated to increase telomerase positive pharynx FaDu tumor cell's sensitivity to paclitaxel [281]. Telomerase inhibition was achieved using either antisense TER, which blocks the template for telomere synthesis, or 3'-azido-3'-deoxythymidine (AZT), a nucleoside analog reverse transcriptase inhibitor. The combination of AZT and paclitaxel resulted in decreased tumor size, increased apoptosis, and prolonged survival in FaDu xenograft tumor mice models. This effect was not observed in telomerase negative human osteocarcinoma Saos-2 cells, indicating that the increase in sensitivity to paclitaxel was due to telomerase inhibition [282].

Knockdown of telomerase activity in human cells can also be achieved via retroviral transfer of siRNA targeting TERT. These telomerase knockdown cells displayed increased sensitivity to IR and chemotherapeutic agents etoposide, bleomycin, and doxorubicin [283]. In addition, the combination therapy using the TERT siRNA increased the apoptotic effect of cisplatin, a platinum-based chemotherapeutic agent, on the hepatocellular cell line SMMC7721 *in vitro* and also greatly reduced SMMC7721 and HepG2 tumor growth in the mouse xenograft model as compared to cisplatin monotherapy [284].

In 2005, Ward and Autexier reported the effects of telomerase inhibition on drug resistant leukemia and breast cancer cells by the non-nucleosidic small molecule

inhibitor BIBR1532, a proprietary formulation from Boehringer Ingelheim [285]. This drug impairs telomere elongation by affecting telomerase translocation or promoting the disassociation of the enzyme from the telomere end [208]. They observed an increase in chemotherapy sensitivity when drug resistant leukemia and breast cancer cells were concurrently treated with BIBR1532. Continuous BIBR1532 treatment was found to decrease the proliferative capacity of these cells. As the number of population doublings with BIBR1532 increased these cells are progressively sensitized cells to the chemotherapeutic agents. This observation suggested that the effects of BIBR1532 treatment were telomere length dependent [211].

Combination chemotherapy studies demonstrated synergistic effects of GRN163L in combination with ionizing radiation [286]. Enhanced radiation sensitivity by GRN163L application was observed following long-term (42 days) drug treatment, with no significant differences in short-term (2 and 9 days) and intermediate inhibition (20 days) [286]. Accordingly, this synergistic effect was attributed to the generation of critically short telomeres following long-term telomerase inhibition. With breast cancer models, previous studies have also demonstrated that GRN163L in combination with the microtubule stabilizing agent paclitaxel [287], and trastuzumab, a monoclonal antibody against the HER-2 receptor [288], has synergistic treatment effects, in a telomere-length dependent manner.

Combination studies have provided genetic and biological evidence linking telomere dysfunction and increased sensitivity to chemotherapeutic agents, making telomerase inhibition an effective therapeutic option for many different types of cancers. Many of these studies concluded that the observed increase in sensitivity of cancer cells to cytotoxic agents was telomere length dependent [209, 222, 286]. However, telomere shortening caused by the continuous inhibition of telomerase may affect normal human cell types that also require telomerase activity for growth and proliferation [61]. Recent data from our laboratory demonstrated that a transient inhibition of telomerase activity, at the time of the induction of DNA damage, also elicit a synergistic cytotoxicity response in breast and colon cancer cells. This potentiation of cytotoxicity is dependent on the timing and mode of action of the genotoxic agents, as only S/G2 specific DNA damage inducers are observed with increased cytotoxicity in combination with telomerase inhibition [207]. Even though the exact mechanism by which telomerase inhibition increases cellular toxicity in this manner, independent of telomere length, is not known, our work also demonstrated that inhibiting the ATM kinase, in conjunction with telomerase inhibition, synergistically increases the cytotoxicity of these S/G2 specific double-stranded DNA-damaging agents, suggesting a role for telomerase in DNA repair [207].

## ***14.2 Transient Telomerase Activation as a Genome Maintenance Mechanism***

Higher telomere attrition rates are often seen with chronic inflammation [289]. Accelerated telomere shortening in these conditions is associated with increased

cellular turnover, leading to premature loss of tissue renewal capacity, and an increased risk of genomic instability. Proof-of-principle experiments showed that telomerase activation extends the replication lifespan of tissues with high turnover [290, 291]. Spurred by these early reports, telomerase activation strategies using small-molecule transcription activators are being heavily pursued. One of these agents, TAT2 (cycloastragenol) is extracted from the root of *Astragalus membranaceus*, a flowering plant used extensively in traditional Chinese herbal medicine [292]. TAT2 has been shown to activate telomerase in cell culture models, through the induction of TERT transcription. Short-term TAT2 treatment (12–18 days) improves the proliferative capacity of CD8+ T-lymphocytes from HIV-infected individuals by moderately increasing telomerase activity. By delaying the onset of immunosenescence of these T-cell models, TAT2 treatment increases the cytokine/chemokine production and antiviral (HIV) activity of T-cells in vitro. This effect is blocked by the addition of a specific telomerase inhibitor, GRN163L, confirming that the improvement of immune function by TAT2 is mediated by telomerase activation [291].

There is a strong negative correlation between mean telomere length and chronological age in humans [293, 294]. Conceivably, strategies to stimulate telomerase-dependent telomere maintenance in later life not only will contribute to boost tissue renewal capacity but will also help preserve the stability and integrity of the genome. This protection against genomic alterations which are frequently associated with cancers could be invaluable to older individuals, as age is one of the biggest risk factors for cancer [197]. Several studies have been initiated to test this. In one such study, TA-65, a compound related to TAT2, also isolated from *Astragalus membranaceus*, is being given as one of the active components of a dietary supplement. Other active ingredients include standard vitamins and trace minerals. An interim (1-year) report of this study revealed moderate improvement in participants' immune system profiles with a continuous regimen of TA-65 in low doses [295]. The long-term utility of these strategies in health promotion including an improved tissue renewal capacity and cancer prevention, as well as the off-target/untoward effects of such therapies require further investigation.

### 14.3 Concluding Remarks

The integrity of telomere function has a paramount role in promoting chromosome stability. Loss of telomere function is implicated in the replicative aging of human tissues, and also has a major effect on cellular transformation related to carcinogenesis [1, 197]. The relationship between telomere structural maintenance and DDR pathways is an illustration of functional adaptation. Telomeres exist to protect the ends of chromosomes from being recognized by DDR sensors and undergoing erroneous repair by DDR mechanisms [7, 19, 69]. Yet, normal homeostatic maintenance of this nucleoprotein structure relies on many of the same DDR factors that need to be kept in check [78, 79]. How do these simple 6nt DNA

repeats and their protein-binding partners accomplish these conflicting tasks? What are the regulatory/signal transduction events that allow the same DDR machinery to adapt its functions for the specific requirement of maintaining telomeres?

Interindividual variations in telomere maintenance capacity are an understudied area of telomere biology [289]. TERT A1062T non-synonymous single nucleotide polymorphism (SNP) has recently been discovered to associate, with a high prevalence, to acute myeloid leukemia (AML). Patients diagnosed with AML had three times higher prevalence of the 1062T-TERT isoforms compared to controls. 1062T-TERT was found to exhibit decreased telomerase activity compared to wild type (1062A) [296]. Conceivably, a decreased telomere maintenance capacity could accelerate the rate of telomeric DNA loss leading to the premature exhaustion of replicative cell pools and the precipitation of genomic instability. With increased availability of data from large-scale epidemiology studies, it is expected that genetic variations in telomerase and other telomere pathway components could also be named as risk factors in other types of malignancies, as well as idiosyncratic and orphaned tissue failure syndromes [289].

To maintain an immortal phenotype, cancer cells need to replenish lost telomere repeats. Accordingly, high telomerase activity is observed in more than 85% of all human cancers [179]. Even though telomerase inhibition alone has limited clinical efficacy as an anticancer treatment, chemotherapy regimens targeting telomerase, when combined with other cytotoxic stress, are reported to be effective against multiple types of malignancies. Several Phase I and II clinical studies with non-small cell lung cancer and breast cancer patients are currently underway [297, 298]. The results of these trials may provide new clinical anticancer strategies.

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