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The Role of Telomere Biology in Cancer

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Abstract

Telomere biology plays a critical and complex role in the initiation and progression of cancer. Although telomere dysfunction resulting from replicative attrition constrains tumor growth by engaging DNA-damage signaling pathways, it can also promote tumorigenesis by causing oncogenic chromosomal rearrangements. Expression of the telomerase enzyme enables telomere-length homeostasis and allows tumor cells to escape the antiproliferative barrier posed by short telomeres. Telomeres and telomerase also function independently of one another. Recent work has suggested that telomerase promotes cell growth through pathways unrelated to telomere maintenance, and a subset of tumors elongate telomeres through telomerase-independent mechanisms. In an effort to exploit the integral link between telomere biology and cancer growth, investigators have developed several telomerase-based therapeutic strategies, which are currently in clinical trials. Here, we broadly review the state of the field with a particular focus on recent developments of interest.

INTRODUCTION

A defining characteristic of cancer cells is their ability to circumvent the multiple regulatory mechanisms that normally restrict cell proliferation. Replicative telomere attrition is one of these regulatory mechanisms, given that it provides a key barrier to indefinite cell proliferation. The path from normal cell to cancer cell must therefore include the engagement of a telomere-maintenance mechanism, which usually occurs through the upregulation of telomerase, the unique enzyme that maintains telomere length. As a result, the biology of telomeres and telomerase is intricately intertwined with the initiation and progression of cancer.

Here, we provide a broad review of the telomere biology of cancer, a vibrant research area that continues to evolve and expand. Given the breadth of this review and the sheer volume of work in this area, we are compelled to focus on particular topics and references and exclude others. We emphasize work in mammalian systems and highlight recent advances in the field. We regret the exclusion of so many great references and topics in the interest of space.

Shelterin: the six-subunit protein complex that binds and protects telomeric DNA repeats

Ataxia telangiectasia mutated protein (ATM): kinase that regulates the DNA-damage checkpoint response to critically short and TRF2-depleted telomeres

Telomere sister-chromatid exchange (T-SCE): homologous recombination between sister telomeres that is suppressed in normally functioning telomeres

TELOMERES AND TELOMERASE: PROTECTION AND MAINTENANCE OF THE CHROMOSOME ENDS

Telomeres solve two basic problems that are inherent in linear genomes. First, they distinguish chromosome ends from DNA double-strand breaks, thereby preventing unwanted DNA-damage signaling and genome instability. Second, they prevent loss of essential genetic information by providing a mechanism for telomere-length maintenance in proliferating cells. We begin by reviewing the mechanisms underlying these two critical functions.

Telomeric End Protection: Shelterins

Mammalian telomeres consist of hundreds to thousands of copies of tandem 5′-TTAGGG-3′ DNA repeats that are predominantly double stranded but terminate in a short 3′ single-stranded overhang (reviewed in Reference 1). The ability of this telomeric repeat sequence to protect chromosome ends rests largely on its ability to bind six-subunit protective protein complexes known as shelterins (**Figure 1*a***) (1). Three shelterin components bind in a sequence-specific manner to the TTAGGG repeats. TRF1 and TRF2 bind the duplex repeat regions (2), and POT1 binds the single-stranded overhangs (3). The other three shelterin components bind to the telomeres through protein-protein interactions. RAP1 binds TRF2; TPP1 binds POT1; and TIN2 binds TRF1, TRF2, and TPP1 simultaneously (4–10). Thus, TIN2 plays an essential role in stabilizing the shelterin complex and linking the single- and double-stranded binding components of shelterin.

Each of the shelterin components provides essential functions that ensure telomere integrity (1). Although the TRF homology domains (also known as dimerization domains) and myb DNA-binding domains of TRF1 and TRF2 share similar structural features (2), the functions of these proteins are quite different. Disruption of TRF2 activates an ataxia telangiectasia mutated protein (ATM)-mediated DNA-damage signal and causes end-to-end telomere fusions (11). TRF2 disruption also stimulates telomere homologous recombination, given that simultaneous loss of TRF2 and the DNA double-strand break repair factor Ku70 causes activation of telomere sister-chromatid exchange (T-SCE) (12). The association between Rap1 and TRF2 is important for repression of this homologous recombination because the increased T-SCE caused by knockout of TRF2 and Ku70 is suppressed by expression of wild-type TRF2 but not by expression of a mutant TRF2 that is defective in interacting with Rap1 (13). In contrast to TRF2, TRF1 does not play a primary role in preventing end-to-end fusions. Rather, TRF1 disruption compromises telomere replication and significantly increases the levels of fragile telomeres and sister-telomere associations (14).

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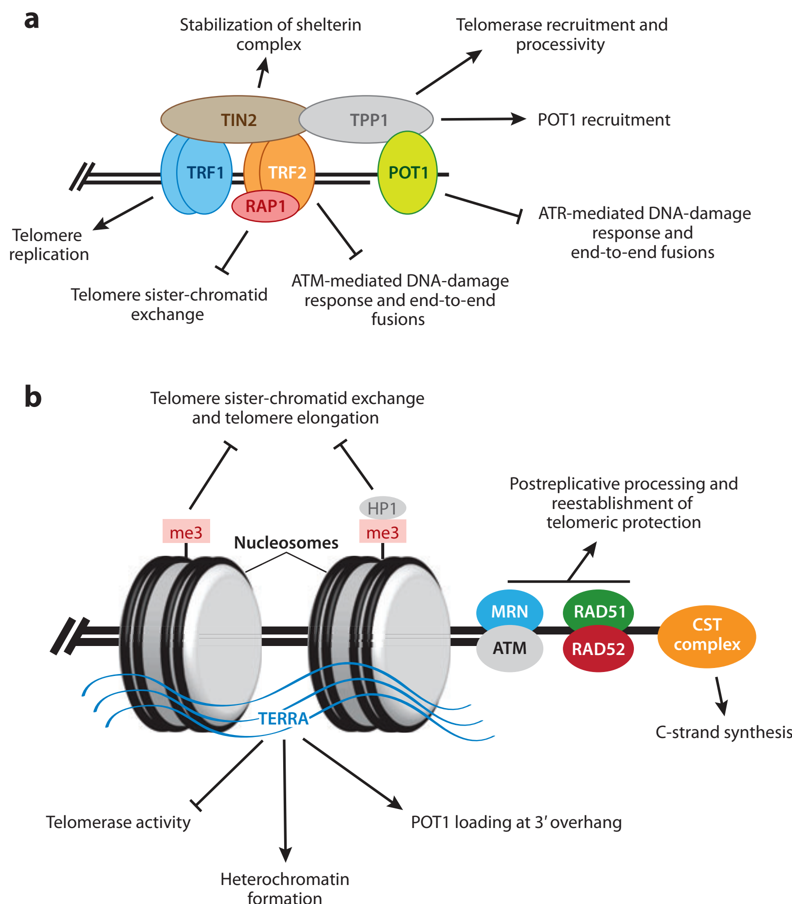

Figure 1

Telomere-protection mechanisms. (*a*) The shelterin complex, which binds in a sequence-specific manner to TTAGGG telomeric repeats. The basic function of each subunit is indicated. For simplicity, only a single shelterin complex is shown, although these complexes bind along the entire length of the telomere. (*b*) Nonshelterin telomere-protection mechanisms. For simplicity, these mechanisms are depicted in the absence of shelterin complexes, although shelterin components influence these other protective mechanisms. Indeed, understanding the dynamic interactions between all of these overlapping factors is a major challenge in the field. Histone trimethylation of H4K20 and H3K9 (me3), together with heterochromatic protein 1 (HP1) recruitment, reflects the heterochromatic state of the telomere, which restricts telomere elongation and sister-chromatid exchange. The MRE11/RAD50/NBS1 (MRN) complex, together with ataxia telangiectasia mutated protein (ATM), acts after S phase to coordinate telomere-end recognition and processing, and RAD51 and RAD52 may enable postreplicative t-loop formation by promoting homologous strand invasion of the 3′ overhang into the proximal duplex telomeric sequence. The proteins are not drawn to scale, and the temporal dynamics of protein binding are not illustrated. Abbreviations: ATR, ataxia telangiectasia and Rad3–related protein; TERRA, telomeric repeat–containing RNA; CST, CTC1/STN1/TEN1.

POT1 is recruited to telomeric overhangs through its sequence-specific single-stranded DNA (ssDNA)-binding activity and through its interaction with TPP1. POT1 is also recruited to duplex regions of the telomere through its interaction with TPP1. Because TPP1 interacts with both POT1 and TIN2, it bridges the single-stranded telomeric binding protein complex and the duplex telomeric binding protein complex. POT1 disruption leads to

Ataxia telangiectasia and Rad3–related protein (ATR): kinase that regulates the DNA-damage checkpoint response to exposed 3′ single-stranded telomeric overhangs

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an ataxia telangiectasia and Rad3–related (ATR)-dominated DNA-damage response and telomere fusions (15–17), and an equivalent phenotype is observed as a result of TPP1 disruption (17, 18). Although TPP1 does not bind to telomeric ssDNA by itself, it forms a complex with POT1 that binds to telomeric ssDNA with significantly increased affinity (19, 20). Furthermore, an in vitro assay showed that the TPP1/POT1 complex enables increased telomerase processivity (19). Recently, investigators reported that the telomeric localization of telomerase depends on TPP1 but not POT1 (21), which suggests that TPP1 might be directly involved in telomerase recruitment to telomeres.

Telomeric End Protection: Additional Components

Although shelterin complexes form the core telomeric protective structure, numerous other factors critically contribute to telomere integrity (**Figure 1*b***). We briefly review some of those factors here.

DNA-damage response and repair factors. Intriguingly, although a major role of telomeres is to prevent a genome-destabilizing DNA-damage response, the DNA-damage response machinery is nevertheless critical for proper telomere function. The MRN (MRE11/RAD50/NBS1) complex and ATM, along with numerous other DNA-repair proteins, are recruited to telomeres during the G_2 phase of the cell cycle, a point at which the telomeres transiently adopt a more open conformation (22–24). These DNA-damage response factors may, in cooperation with shelterin, promote the processing of telomere ends and the formation of t-loops, in which the single-stranded telomeric overhang loops back, invades, and base-pairs with the duplex region of the telomere (24, 25). t-Loops have been visualized by electron microscopy and are thought to sequester the telomeric ends, thereby bolstering end protection (26, 27). It remains to be determined whether telomeres alternate between the t-loop structure and a more accessible structure during the cell cycle and, if they do, what regulates the dynamic changes.

CST complex. The CST protein complex, which comprises CTC1, STN1, and TEN1, binds telomeres (28, 29). Unlike the shelterin complexes present at every telomere, CST complexes are detected at only a subset of telomeres. Depletion of CTC1 or STN1 leads to accumulation of telomeric 3′ overhangs, loss of telomere signals from chromosome ends, and formation of chromatin bridges. Because mammalian CTC1 and STN1 stimulate DNA polymerase α–primase activity (30), the CST complex may be involved in telomeric C-strand synthesis.

TERRA. Recent work has revealed that telomeric DNA is transcribed from several subtelomeric loci, generating telomere-associated telomeric repeat–containing RNA (TERRA) (31). Disruption of cellular TERRA equilibrium leads to multiple telomeric defects, including increased levels of telomere-free chromosome ends. Investigators recently demonstrated that heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) facilitates the displacement of replication protein A (RPA) by POT1 at telomeric overhangs and that TERRA regulates the RPA-displacing activity of hnRNPA1 (32). According to this model, in early and middle S phase, high levels of TERRA bind hnRNPA1 and prevent it from displacing RPA from telomere overhangs. In late S phase, TERRA levels decrease, releasing hnRNPA1 to displace RPA from telomeres. Telomere overhang–associated hnRNPA1 proteins are subsequently replaced by POT1. TERRA can also act as a direct telomerase inhibitor (33), and it has been implicated in the maintenance of the telomeric heterochromatic state (34).

Epigenetic state. In addition to TERRA, numerous other factors have been implicated in maintaining the appropriate epigenetic state of telomeres. Mammalian telomeric DNA is assembled into evenly spaced nucleosomes that

are enriched for repressive epigenetic marks that are characteristic of constitutive heterochromatin (35–37). The heterochromatic state of telomeres is important for proper telomere function. Disruption of histone methyltransferase (HMTase) Suv39h function leads to decreased H3K9 methylation and heterochromatic protein 1 (HP1) binding at telomeres, changes that are consistent with a more "open" chromatin conformation (38). In these Suv39h-deficient cells, T-SCE levels are elevated and telomere lengths are increased, perhaps because of the chromatin changes. Similarly, disruption of another HMTase, Suv4-20h, decreases H4K20 methylation, increases T-SCE, and lengthens telomeres (39). Abrogation of DNA methyltransferase (DNMT) function also causes increased T-SCE levels (40). Collectively, these results indicate that the epigenetic status of a telomere modulates its ability to undergo homologous recombination. We discuss this topic in more detail in the section titled Alternative Lengthening of Telomeres, below, because homologous recombination is particularly important for telomere-length maintenance in the subset of human tumors that lack telomerase.

Telomere-Length Maintenance

The second major function of telomeres is to prevent a loss of genetic information by providing a means for telomere-length maintenance in replicating cells. This maintenance role is usually filled by the enzyme telomerase, a reverse transcriptase complex that uses a short segment of its RNA subunit as a template to direct the addition of telomeric repeats onto chromosome ends (reviewed in Reference 41). Catalytically active telomerase complex purified from human cells is minimally composed of two molecules each of telomerase reverse transcriptase (TERT), template-containing telomerase RNA (TER), and dyskerin (DKC1) (42). In addition to the core telomerase components, the ATPases pontin and reptin are also required for telomerase holoenzyme assembly (43). Additional factors required for the appropriate function of telomerase holoenzyme have been identified through characterization of genetic mutations in a spectrum of human telomere diseases, including dyskeratosis congenita (DC), aplastic anemia, and pulmonary fibrosis (44–46). Cellular factors that are mutated in these disorders include (*a*) TERT and TER (47–50); (*b*) DKC1, which binds and stabilizes TER (51); (*c*) NHP2 and NOP10, which bind TER (52, 53); (*d*) TCAB1, which binds and directs TER to Cajal bodies (54); and (*e*) TIN2, the shelterin component (55, 56). Patients with mutations in these factors display characteristic premature telomere shortening and impaired stem cell function.

Telomerase is normally active in human stem/progenitor cells and germ-line cells, as well as in a subset of somatic cells (e.g., activated lymphocytes) (57). Most somatic cells lack telomerase activity entirely or have very low levels (58). Telomere length can also be maintained through telomerase-independent recombinational mechanisms termed alternative lengthening of telomeres (ALT). Both telomerase-dependent and telomerase-independent telomere-elongation mechanisms extend replicative life span and play an important role in cancer progression.

TERT: telomerase reverse transcriptase

TER: template-containing telomerase RNA

Alternative lengthening of telomeres (ALT): recombination-based telomere-elongation mechanisms that are independent of telomerase

THE DYSFUNCTIONAL TELOMERE AS A DNA DOUBLE-STRAND BREAK

Telomeres are critical for ensuring genome stability. In the absence of a protective mechanism, the ends of linear chromosomes would be indistinguishable from a one-sided DNA double-strand break and would engage the cellular DNA-damage response. Indeed, abrogation of telomeric protection, either through experimental disruption of shelterin components or through replicative attrition, initiates a canonical DNA-damage response (59, 60). This response has been extensively studied by monitoring the appearance of telomere dysfunction–induced foci at telomeres (which mimic the DNA-damage foci observed at other double-strand breaks) and by analyzing the activation of

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Dysfunctional telomere: a telomere that is not properly protected because of its critically short length and/or the loss of normal protective elements, such as shelterins

Replicative senescence: permanent cell-cycle arrest caused by telomere attrition–induced DNA-damage signaling in checkpoint-proficient cells

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downstream checkpoint pathways. Results indicate that the DNA-damage response emanating from a dysfunctional telomere is coordinated by ATM and/or ATR, depending on the nature of the telomeric lesion (reviewed in Reference 1). Replicative telomere attrition leads to activation of both ATM and ATR (59). TRF2 depletion leads predominantly to ATM activation, whereas deprotection of the 3′ single-stranded telomeric overhang, which occurs in response to POT1 loss, leads predominantly to ATR activation (11, 17). An ATR-coordinated DNA-damage response is also induced by replication stress at TRF1-depleted telomeres, analogous to what is observed at other difficult-to-replicate sites in the genome (14). Collectively, these studies suggest that there is no unique dysfunctional telomere signal, but rather that damaged telomeres activate the canonical pathways that operate at DNA double-strand breaks throughout the genome. However, as we highlight below, there are several unique features of dysfunctional telomeres—namely their persistence and their terminal chromosomal location—that distinguish them from DNA double-strand breaks elsewhere in the genome and modulate their influence on tumor biology.

THE DYSFUNCTIONAL TELOMERE: TUMOR SUPPRESSION VERSUS TUMOR PROMOTION

Cancer biology is rife with examples of double-edged swords: cellular mechanisms that alternately promote or inhibit tumorigenesis, depending on context. The dysfunctional telomere serves as a prototypical example of this phenomenon because its effect on cancer formation critically depends on the cellular milieu. In particular, the status of the cellular checkpoints is a key determinant of whether the DNA-damage signal emanating from a dysfunctional telomere protects or endangers genome stability (**Figure 2**). In this section, we discuss some of the factors that mediate this critical balance.

Tumor-Suppressive Effects of Telomere Dysfunction

Replicative senescence represents one mechanism by which telomere dysfunction can block cancer growth. The link between cell proliferative capacity in culture and telomere length was first established through studies of primary fibroblast proliferation in vitro. In 1961, Hayflick & Moorhead (61) discovered that primary human fibroblasts have limited replicative potential. This replicative potential was inversely related to the age of the fibroblast cell donor, which suggested that the cells have an intrinsic molecular counting mechanism that limits their replicative life span. After proliferating for approximately 40 to 60 population doublings, primary human fibroblasts become enlarged and enter a state now referred to as replicative senescence (reviewed in Reference 62). Because primary human fibroblasts contain very little to no telomerase, their telomeres progressively shorten with each cell division due to the inability of DNA polymerase α to replicate the extreme ends of human chromosomes (63). When one or more telomeres reach a critically short length, the deprotected chromosome ends are recognized by DNA-damage response factors, as described above. In checkpoint-competent cells, the result is typically senescence, in which the cells continue to live but are irreversibly blocked from further cell division. Consistent with this explanation is the finding that introduction of TERT into primary human fibroblasts allows them to bypass replicative senescence and become immortalized (64). Thus, telomere dysfunction resulting from attrition underlies the phenomenon of replicative senescence, and it was quickly recognized that this general mechanism might block the proliferation of incipient tumor clones.

The ability of dysfunctional telomeres to efficiently induce senescence is at least partly due to the persistence of the resulting DNA-damage signal. When replicative telomere dysfunction occurs in cells without active telomerase, there is no mechanism readily available to reverse the damage signal and reestablish

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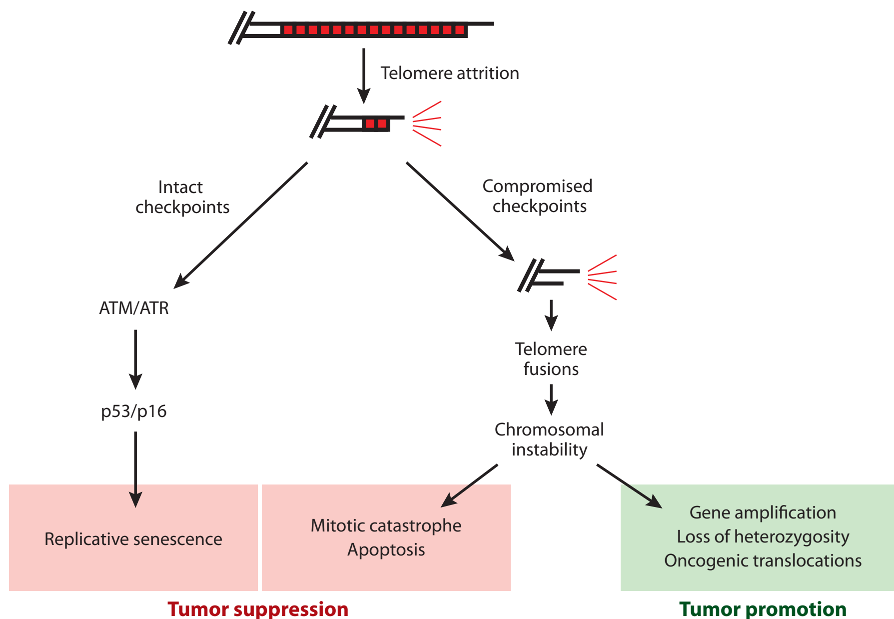

Figure 2

The impact of telomere attrition on tumor suppression and promotion. Replicative loss of telomeric repeats (*red squares*) causes deprotection of the chromosomal end and induction of a DNA-damage response (*red lines*). In checkpoint-competent cells, this response typically results in replicative senescence. In checkpoint-deficient cells, telomeres shorten further until they reach crisis, which is characterized by rampant telomere fusions and chromosomal instability. This instability blocks cell proliferation, but it can also promote oncogenic genomic changes. Abbreviations: ATM, ataxia telangiectasia mutated protein; ATR, ataxia telangiectasia and Rad3–related protein.

telomeric end protection without further destabilization of the genome. As a result, dysfunctional telomeres typically cause a persistent DNA-damage signal that induces senescence and the senescence-associated secretory phenotype (SASP) through activation of the ATM/CHK2 and p38 pathways in checkpoint-proficient cells (65–67). The SASP is characterized by the secretion of numerous proteins, including the cytokines interleukin (IL)-6 and IL-8, which enforce the senescent state in a cell-autonomous fashion. However, replicative senescence is itself a double-edged sword with respect to cancer. While senescent cells cease proliferating, factors secreted by these cells may promote inflammation and cancer development (recently reviewed in Reference 68).

The in vivo impact of replicative telomere shortening has been explored in TER- and TERT-null mice (69, 70). The mouse strain initially used for these knockouts, C57BL/6J, has very long telomeres ranging from 20 to 65 kb (71), and as a result, first-generation knockout mice appear essentially normal despite the complete absence of telomerase activity. However, continued breeding of these mice leads to progressive telomere shortening; clear loss of telomeric protection is evident from the fourth generation onward (69). These late-generation mice show cytogenetic evidence of telomere dysfunction, including chromosome ends lacking detectable telomere sequence, end-to-end chromosomal fusions, and aneuploidy. The cytogenetic defects are accompanied by shortened life span and multiorgan degeneration (72, 73). Defects include increased apoptosis and decreased cell proliferation in testis, which leads to infertility, as well

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as compromised bone marrow function. These degenerative phenotypes are linked to impaired proliferation of progenitor cells, particularly in highly proliferative tissues. The detrimental impact of telomere dysfunction on metabolic and mitochondrial function in diverse tissues has also been established (74). Although telomere attrition clearly compromises tissue function and genome stability, it also constitutes a critical barrier for cancer formation in various mouse tumor models (75–78). Importantly, several of these studies directly implicate p53-dependent senescence in mediating the tumor-suppressive effects of short telomeres (76, 77). Thus, the telomere dysfunction–induced senescence originally identified in vitro can protect against cancer development in vivo.

Tumor-Promoting Effects of Telomere Dysfunction

The impact of telomere dysfunction is dramatically altered when checkpoint pathways are disrupted, and this scenario has important implications for cancer biology. In particular, inactivation of the p53 and p16/Rb pathways allows bypass of replicative senescence and further telomere attrition. SV40 large T antigen, which disrupts both the p53 and p16/Rb pathways, can extend the proliferative life span of cells in vitro until they reach crisis, at which point further cell division is balanced by cell death (79–81). These concepts are summarized in a two-stage model of cell growth in the face of replicative telomere attrition (recently reviewed in Reference 82). Mortality stage 1 (M1) refers to replicative senescence that occurs in the presence of intact checkpoints, as described above. When M1 is bypassed, as in the presence of SV40 large T antigen, the cells proliferate further until the crisis stage (or M2) is reached. M2 is characterized by abundant telomere fusions, the mechanism of which is discussed in more detail below. Intriguingly, a recent study showed that persistent telomere dysfunction can also induce tetraploidy, a feature of some tumors, by causing bypass of mitosis and endoreduplication in p53-deficient cells (83). Crisis itself serves as another line of defense against the development of cancer because the resulting genome instability and DNA-damage signaling kill off the vast majority of cells. However, rare immortalized clones can emerge from crisis by reestablishing telomere maintenance through reactivation of telomerase or engagement of ALT mechanisms (82). Importantly, because the cells have passed through crisis, they may harbor secondary genetic changes that promote tumor progression. Indeed, there is a higher incidence of certain tumors in telomerase-null mice that also harbor p53 mutations; the tumors show significant genome rearrangements that are consistent with a period of crisis (84–86).

The dual role of dysfunctional telomeres in murine cancer was elegantly illustrated by two recent papers from the DePinho laboratory (87, 88) that used an inducible TERT expression system to model telomerase reactivation in the context of telomere dysfunction. In a prostate cancer model in p53/PTEN-null mice, telomere dysfunction drove cancer initiation, but progression of the resulting tumors was inhibited by the ongoing DNA-damage response caused by dysfunctional telomeres. Expression of TERT abrogated this DNA-damage signaling and enabled progression of aggressive, metastatic tumors. Notably, the resulting tumors showed marked genome rearrangement; many of the amplifications and deletions matched those observed in human prostate cancer. Similarly, in a T cell lymphoma model in ATM-null mice, the combination of initial telomere dysfunction followed by telomerase reactivation enabled the development of aggressive tumors with genome rearrangements that mirrored those observed in human T cell lymphomas. Together, these two studies provide strong support for the canonical view that telomere dysfunction and subsequent telomerase reactivation drive tumor initiation and progression.

Telomere Dysfunction in Human Cancers

Although tractable mouse models have provided remarkable insight into telomeres and

telomerase, the telomere biology of mice differs significantly from that of humans. Thus, it is important to consider evidence for the role of telomere dysfunction in human cancers. Multiple studies have looked directly at human tissue samples to glean evidence of telomere dysfunction during the progression to malignancy. In both colon and breast tissue, premalignant benign lesions typically show low levels of genomic instability and chromosomal aberrations, whereas in situ and invasive carcinomas show increased chromosomal rearrangements and anaphase bridges (89, 90). In the case of breast cancer, a combined analysis of tissue culture cells and patient samples was used to support the canonical model in which telomere attrition–induced crisis leads to outgrowth of telomerase-positive cancers harboring significant genome aberrations (89). A more recent analysis of chronic lymphocytic leukemia has similarly suggested that the fusion of chromosomes with short telomeres drives genome instability and disease progression (91). This study employed a single-molecule polymerase chain reaction–based approach that allowed more precise monitoring of telomere attrition and fusion in comparison to some prior studies, which relied on anaphase bridges as a proxy for telomere dysfunction–induced chromosome fusion.

The advent of technologies that enable whole-genome analysis of chromosomal instability has provided a new way to identify genomic rearrangements caused by telomere dysfunction in human tumors. A recent study that annotated chromosomal rearrangements in patients with pancreatic adenocarcinoma revealed a high number of distinctive fold-back inversions, which probably arise from sister-chromatid fusions (92). As discussed in more detail below, sister-chromatid fusions frequently result from telomere dysfunction or loss and are particularly interesting due to their association with gene amplification (**Figure 3**) (93). Importantly, the fold-back inversions were detected early in the development of pancreatic adenocarcinoma and were directly associated with the amplification of oncogenes, including *myc* and *cyclin E1* (92). This observation suggests that these mutations drive cancer progression. Intriguingly, the prevalence of fold-back rearrangements was much higher in pancreatic cancer (16%) than in breast cancer (2%), indicating very different patterns of instability depending on cancer type. These findings suggest that telomere dysfunction plays a significant role in the pathogenesis of pancreatic adenocarcinoma, although other mechanisms for the observed rearrangements cannot be excluded by such retrospective analyses (92).

Sister-chromatid fusion: covalent end joining of sister chromatids that can promote gene amplification

As outlined above, telomere attrition in mouse models can play both tumor-suppressive and tumor-promoting roles. Many studies have investigated whether telomere length similarly influences the risk of human cancers. These studies typically use leukocyte telomere length for analysis due to the ease of collecting blood samples. Whereas some studies find that short telomere length is associated with increased cancer risk (94–99), others find that long telomere length correlates with increased cancer risk (100–102). In addition, some studies have suggested that telomere length has no influence on cancer risk (103–105). Although these conflicting results may derive in part from different methodologies, they may simply mirror the diverse context-dependent roles of short telomeres in tumor suppression and promotion observed in the mouse.

THE DYSFUNCTIONAL TELOMERE AND GENOME INSTABILITY

As outlined above, dysfunctional telomeres mimic DNA double-strand breaks and influence cancer development and progression by engaging cellular checkpoints and destabilizing genomes. The ability of dysfunctional telomeres to promote genome instability derives in part from their terminal chromosomal location, which differentiates them from DNA double-strand breaks elsewhere in the genome. At internal double-strand breaks, the DNA-damage response coordinates the recognition and religation of the two broken

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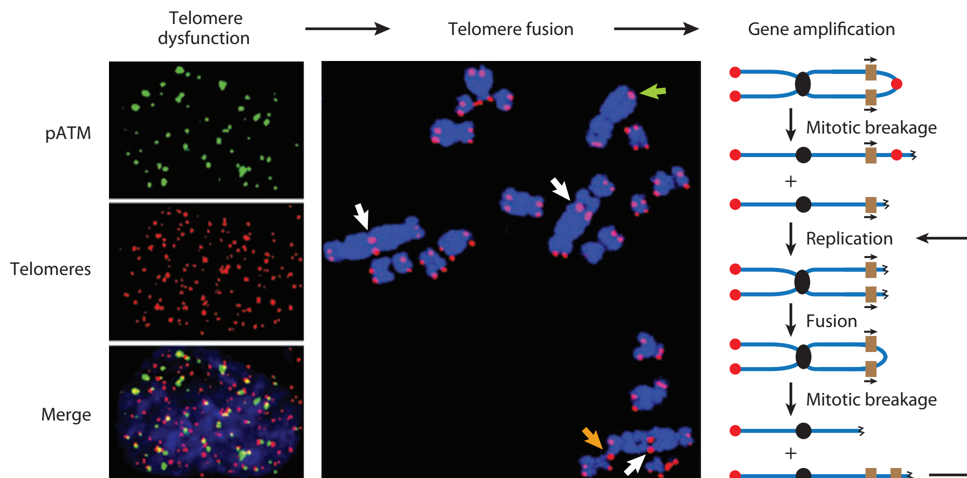

Figure 3

Consequences of telomere dysfunction. Telomeric protection was acutely disrupted in the human LOX melanoma cell line by the incorporation of mutant telomeric repeats that cannot properly bind shelterin complexes (109). Even though the telomeres remain long, end protection is abrogated and an ataxia telangiectasia mutated protein (ATM)-coordinated damage response ensues, as revealed by the presence of numerous telomere dysfunction–induced DNA-damage foci. Three types of resulting fusions can be seen in the partial metaphase spread (*center*). Dicentric chromosome-type fusions (*white arrows*) usually occur prior to S phase, and non-sister-chromatid fusions (*orange arrow*) and sister-chromatid fusions (*green arrow*) must occur after the chromosomes have replicated. Sister-chromatid fusions are particularly interesting because they can cause oncogene amplification (*right*). The brown rectangles represent a hypothetical oncogene, and the adjacent arrows indicate gene orientation. For simplicity, replication of only one of the broken chromatids is illustrated. As a result of sequential fusion and breakage, two copies of the oncogene end up in inverse orientation on one chromosome. Subsequent rounds of sister-chromatid fusion and breakage could continue amplification of the oncogene in a similar manner. In the fluorescence images, telomeres are highlighted with a fluorescently labeled peptide nucleic acid probe, activated ATM phosphorylated at serine 1981 (pATM) is detected with a phospho-specific antibody, and chromosomal DNA is stained with DAPI (4′,6-diamidino-2-phenylindole).

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ends, thereby maintaining chromosomal integrity. In contrast, a dysfunctional telomere cannot be repaired in this way, given that it is a double-stranded end without an appropriate repair partner. When dysfunctional telomeres engage the DNA-damage repair machinery, the result is genome-destabilizing telomere fusion. This distinction is clearly highlighted by experiments in cells lacking key components of the DNA-damage response, such as ATM and the E3 ligase RNF8. Whereas loss of these repair factors typically sensitizes cells to treatments that induce DNA double-strand breaks in the genome, their loss desensitizes cells to the acute effects of dysfunctional telomeres (106–109). Thus, the repair mechanisms that are so important for protecting chromosomal integrity can actually promote genome instability in the face of telomere dysfunction.

Given the proposed role of telomere fusion in destabilizing cancer genomes, significant research efforts have been devoted to understanding the mechanisms of dysfunctional telomere fusion. Initial experiments in which telomere dysfunction was induced by TRF2 disruption indicated that fusions were mediated by the classical nonhomologous end-joining (c-NHEJ) machinery, including Ku70/86 and ligase IV (reviewed in Reference 1). More recent work has highlighted the role of ligase

IV–independent alternative nonhomologous end-joining (a-NHEJ) pathways, particularly in situations wherein telomeres are critically shortened (110, 111). One of the factors that guides the choice of telomere fusion pathways is DNA sequence. When shelterins are acutely depleted, the presence of tandem 5′-TTAGGG-3′ repeats at the fusion site may minimize microhomology-based fusion mechanisms such as a-NHEJ while favoring c-NHEJ. In contrast, telomere dysfunction caused by replicative attrition exposes underlying subtelomeric sequences that provide regions of homology that may favor a-NHEJ. Indeed, recent sequence analysis of telomeric fusions caused by replicative attrition in human cells has revealed frequent subtelomeric microhomology at the fusion junctions (112).

Telomere dysfunction produces diverse chromosomal fusion types, which have various effects on genome stability (**Figure 3**). In the G_1 phase of the cell cycle, chromosome-type dicentric fusions typically predominate (113). These fusions occur when two different chromosomes fuse in an end-to-end fashion. In contrast, fusions that occur after S phase frequently involve chromatid-type fusions, either between chromatids of different chromosomes or between sister chromatids. Sister-chromatid fusions have received particular attention because of their long-recognized ability to induce gene amplification (recently reviewed in Reference 93). As discussed above, a recent analysis of genomic rearrangement patterns in pancreatic adenocarcinoma implicated sister-chromatid fusions in oncogene amplification early during tumor development (92). Given the critical role of telomere fusions in mediating chromosomal instability during cancer development, it will be important to better understand how telomeric and cellular factors conspire to modulate telomere fusion mechanisms and patterns.

ALTERNATIVE FUNCTIONS OF TELOMERASE

In the preceding sections, we focus on the central role of telomere attrition and dysfunction in cancer biology. Here, we examine other mechanisms by which the telomerase enzyme may influence cancer development and progression. Constitutive telomerase expression in mouse models results in increased incidence of spontaneous tumors (114–117). The canonical explanation for the tumor-promoting influence of telomerase is that it allows clonal neoplasms to bypass or escape the replicative barrier posed by telomere attrition. One of the most intriguing and controversial topics in the field is whether telomerase also supports the proliferation and survival of cells through mechanisms that are independent of its telomere-elongation function (**Figure 4**). Although many studies have hinted at such alternative functions, their importance in physiological settings remains unclear. If validated, these alternative functions could prove critical to the role of telomerase in supporting tumor progression. In this section, we review evidence for alternative telomerase functions and highlight the experimental challenges that have complicated this area of research.

Alternative function of telomerase: any cellular role for telomerase that does not involve its canonical telomere-elongation activity

A common strategy for exploring alternative telomerase functions is to acutely overexpress or deplete TERT and look for rapid changes in cell function. If these functional effects occur prior to bulk telomere-length changes or the appearance of an overt dysfunctional telomere response, then an alternative TERT function may be at play. Several TERT mutants are also valuable in evaluating putative alternative functions. A requirement for TERT catalytic activity can be demonstrated by showing that the putative alternative function is supported by wild-type TERT but not by catalytically dead TERT mutants such as TERT D868A (118). Also useful are TERT mutants such as TERT N-DAT 92, TERT-HA, and TERT-IA^- (119–121), which have robust catalytic activity in vitro but fail to maintain telomere length in cells. If a putative alternative function is supported by one of these TERT mutants but not by catalytically dead TERT D868A, then the catalytic activity may be required for a nontelomeric function. Finally, separation-of-function studies can be performed by

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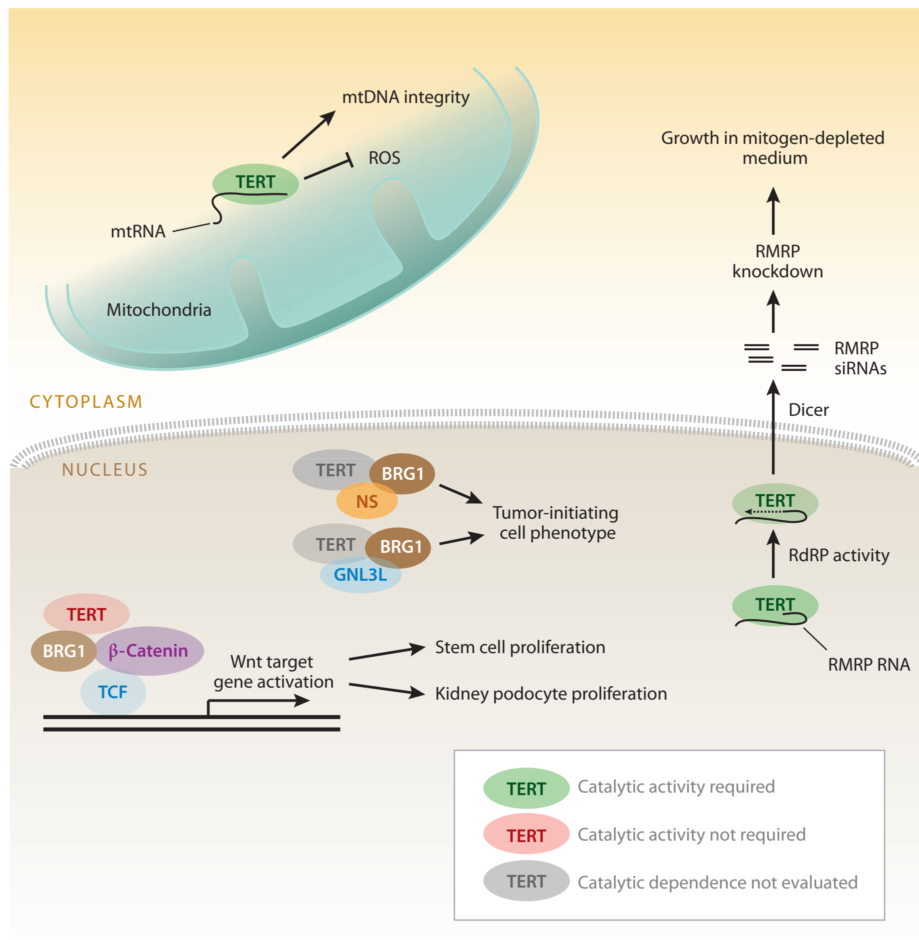

Figure 4

Four proposed alternative functions of telomerase reverse transcriptase (TERT). Abbreviations: GNL3L, guanine nucleotide binding protein–like 3–like protein; mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; NS, nucleostemin; RdRP, RNA-dependent RNA polymerase; RMRP, RNA component of mtRNA-processing endoribonuclease; ROS, reactive oxygen species; siRNA, small interfering RNA.

demonstrating TERT cellular effects in cells lacking TER expression (120, 122, 123).

Alternative Telomerase Functions Requiring Catalytic Activity

Using a combination of the above-described approaches, several groups have reported putative alternative functions of telomerase that depend on TERT catalytic activity. First, overexpression of wild-type TERT, TERT-HA, or TERT-IA^{-} promoted cell growth in mitogen-deficient medium by both inducing cell proliferation and limiting apoptosis (120, 124). Second, overexpression of either wild-type TERT or TERT-HA in immortalized ALT cells expressing oncogenic H-Ras promoted tumor formation in a mouse xenograft model, even though ALT cells do not require telomerase for telomere elongation (125). Third, acute small hairpin RNA (shRNA)-mediated TERT depletion in human

fibroblasts compromised the DNA-damage response, an effect that was rescued by overexpression of shRNA-resistant TERT N-DAT 92 (126). Finally, recent research showed that significant quantities of TERT are present in the mitochondria and that mitochondrial TERT may have positive and negative effects on mitochondrial function (122, 127, 128).

A key question posed by these studies is what TERT catalytic activity may do away from the telomeres that affects cell function. A possible answer was provided by several recent studies, which showed that the TERT protein forms complexes with RNAs other than TER. Most dramatically, a recent study reported that TERT binds RMRP, the RNA component of the mitochondrial RNA–processing endoribonuclease. TERT uses the RMRP RNA as a template for RNA-dependent RNA polymerization, thereby creating a double-stranded RNA that is processed in a Dicer-dependent manner to create small interfering RNAs (siRNAs) that regulate cellular RMRP levels (129). This report was notable not only for showing TERT acting with an RNA other than TER but also for revealing that TERT plays an RNA-dependent RNA polymerase role. Together with the recent finding that RMRP knockdown can promote cell proliferation in a mitogen-depleted medium (120), this observation suggests a possible mechanism through which catalytically active TERT influences cell proliferation and function in a telomere-independent manner, although additional work is needed to validate this model. A more recent investigation found that TERT interacts with other mitochondrial RNAs and drives reverse transcription of RNAs other than TER (122). Collectively, these results suggest that TERT polymerase activity, by use of templates other than TER, may underlie alternative TERT effects. However, the details of this putative mechanism remain to be determined and validated.

A key challenge in validating alternative telomerase functions that require TERT catalytic function is to show that the TERT catalytic activity serves a function other than its canonical telomere-elongation function. Several characteristics of mammalian telomere biology make this distinction particularly difficult. A primary problem is that mammalian telomeres widely differ in length from chromosome to chromosome and cell to cell, and cells frequently contain an ultrashort class of telomeres termed T-stumps (112, 130). Furthermore, even early-passage primary human cells whose average telomere lengths are long contain a low level of dysfunctional telomeres (131, 132). As a result, acute overexpression or depletion of TERT may rapidly influence cell behavior through its canonical telomere-elongation function, well before bulk telomere-length changes are evident. Although the use of the TERT separation-of-function mutants described above can provide additional evidence for noncanonical TERT activities, some TERT mutants that cannot maintain bulk telomere length, such as TERT+C and TERT-IA^{-}, may nevertheless act preferentially at very short telomeres, as evidenced by their ability to extend replicative life span while stabilizing telomeres at a very short bulk length (120, 133). Thus, these mutants do not necessarily provide a clean separation of function with which to distinguish canonical from noncanonical activity. Finally, many of the phenotypes ascribed to abrogation of noncanonical TERT activity—including impaired DNA repair, decreased cell proliferation, and disrupted mitochondrial function—can also be caused by dysfunctional telomeres (74, 131, 134), which makes it more difficult to identify the proximate cause. Together, these experimental challenges complicate efforts to pinpoint physiologically relevant noncanonical TERT functions.

Alternative Telomerase Functions Supported by Catalytically Dead TERT

Thus far, we have focused on noncanonical TERT functions that depend on its catalytic activity and the challenges in separating canonical from noncanonical effects. A second group of proposed alternative TERT

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functions, which do not require TERT to act catalytically, have lately received a great deal of attention, largely due to work in Steve Artandi's laboratory. Because these proposed alternative functions are supported by catalytically dead TERT mutants, there is little doubt that the phenotypes spring from a noncanonical TERT function. In 2005, the Artandi laboratory (123) showed that acute overexpression of TERT in mouse skin epithelium induces proliferation of the hair follicle bulge stem cells, resulting in hair growth. Remarkably, this effect occurred even in the absence of TER, which implicates a noncanonical function.

Several subsequent studies have defined the mechanism behind this unexpected phenotype (135–137). TERT stimulates the Wnt/β-catenin signaling pathway by serving as a transcriptional cofactor at Wnt target gene promoters. TERT associates with β-catenin/TCF complexes in a Brg1-dependent manner, thereby altering target gene expression. TERT overexpression also drives the proliferation of kidney podocytes in a Wnt-dependent manner, which causes glomerular pathology reminiscent of HIV-associated nephropathy (137). Intriguingly, a TERT/Brg1/nucleostemin complex was recently implicated in the maintenance of tumor-initiating cells, although the mechanism of this effect has not yet been explored in great detail (138). Together, these studies provide a plausible mechanism through which TERT, completely independently of its telomeric activities, may drive cell proliferation and modulate stem cell homeostasis.

Although the data described above clearly show that overexpressed TERT can drive Wnt signaling, there nevertheless remains significant disagreement regarding the importance of this noncanonical function in physiological settings. Initial studies with TERT knockout mice revealed that the first-generation animals are remarkably normal (139); more recent work found little evidence for Wnt signaling deficiency in first-generation knockout embryonic and adult tissues (140). Although a rib defect consistent with Wnt deficiency was reported in first-generation TERT knockout mice, the phenotype was relatively mild and was not observed in a separate cohort of mice (136, 140). These results suggest that, if TERT indeed plays a significant physiological role in Wnt signaling, the phenotype in knockout mice is largely masked by developmental compensation, as has been observed in other gene knockout models. Another issue to consider is that the most striking examples of TERT-induced Wnt activation in mice—including the proliferation of skin stem cells and podocytes—occur in the context of robust TERT overexpression (123, 136). It remains possible that these phenotypes do not reflect a role for TERT at its physiological levels, although conditional TERT knockout in murine embryonic stem cells in vitro significantly impaired Wnt target activation (136).

In summary, many interesting alternative functions for TERT have been proposed in recent years, and these functions could have profound implications for the role of telomerase in initiating and sustaining cancer. Although experimental challenges remain in terms of defining and validating these alternative functions, it seems increasingly likely that TERT may lead a double life that extends beyond the ends of the chromosomes.

ALTERNATIVE LENGTHENING OF TELOMERES

Whereas the previous section discusses telomerase independent of its role in telomere maintenance, here we examine the inverse: telomere maintenance independent of telomerase. The observation that mammalian telomeres can be maintained for many cell divisions in the absence of telomerase activity was first made in immortalized human cell lines (141–143). An initial survey of human tumors of different origins estimated that approximately 85% of human cancers have elevated telomerase activity (144). The telomerase-negative cancers maintain their telomeres through one or more telomerase-independent mechanisms collectively named ALT (**Figure 5**) (143). ALT

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is more common in certain types of tumors, including sarcomas, glioblastomas, and pancreatic neuroendocrine tumors (143, 145–148).

ALT Cell Characteristics

The telomeres of ALT cells are composed of canonical TTAGGG repeats bound by the usual shelterin complexes. Compared with normal primary human cells and telomerase-positive cancer cells, ALT cells possess several characteristic features. First, telomere lengths of ALT cells are very heterogeneous; they contain both extremely long (>50-kb) and short (<5-kb) telomeres (142). Second, a number of telomeres in some ALT cells cluster within a subset of promyelocytic leukemia (PML) nuclear bodies (149). These telomeric DNA-containing PML nuclear bodies have been observed only in ALT cells and are thus referred to as ALT-associated PML bodies. Third, the frequency of recombination is greatly increased, specifically at telomeres (150–152).

In normal primary human cells and telomerase-positive cancer cells, recombination between telomeres is almost undetectable. In contrast, T-SCE levels, measured by chromosome-orientation fluorescence in situ hybridization analysis (153), are significantly increased in ALT cells, whereas overall sister-chromatid exchange levels remain very low (150, 151). Interchromosome telomere-recombination rates are also significantly increased in ALT cells, as measured by the copying of a DNA tag engineered within the telomeric sequence of a specific chromosome to multiple telomeres on other chromosomes (152). The same DNA tag, if engineered within the subtelomeric sequence of a chromosome, fails to be copied into other chromosomes, which indicates that the recombination activity is specific to telomeres. Several proteins involved in recombination pathways are required for telomere maintenance in ALT cells. Knockdown of MRE11, RAD50, NBS1, or RAD51D by siRNA disrupts telomere maintenance and causes telomere shortening in ALT cells (154, 155). The recombination-based mechanisms that drive ALT remain to be fully characterized (see References 156 and 157 for recent reviews).

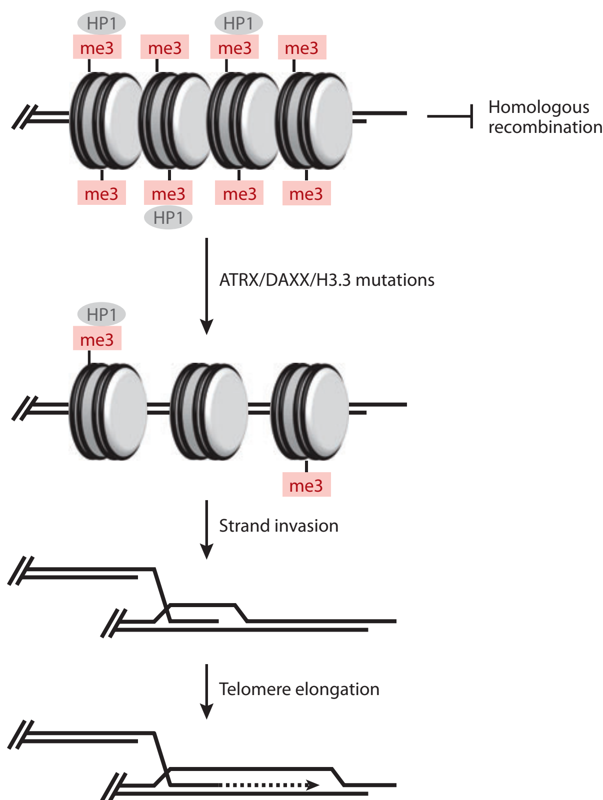

Figure 5

Proposed model for the induction of ALT (alternative lengthening of telomeres). The heterochromatic state of telomeres, which is reflected by H3K9 and H4K20 trimethylation (me3) and heterochromatic protein 1 (HP1) recruitment, inhibits telomeric homologous recombination. Because ATRX/DAXX/H3.3 mutations correlate with the ALT phenotype in human cancers, disruption of this chromatin-modifying pathway may promote loss of the heterochromatic state, thereby enabling homologous recombination–based telomere elongation. The precise mechanism of this transition, and the number of other cellular factors involved, is not yet clear. Only one pathway of strand invasion and recombination-based telomere elongation is illustrated; several recent reviews offer a more comprehensive view (156, 157). For simplicity, nucleosomes are omitted from the diagrams showing strand invasion and telomere elongation.

A Chromatin-ALT Connection in Human Cancer: ATRX, DAXX, and H3.3

With their long tracts of tandem TTAGGG repeats that terminate in a 3′ single-stranded

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overhang, telomeres might be expected to act as recombinational hot spots. The fact that telomere-recombination frequency is instead very low in primary cells and telomerase-positive cancer cells suggests that telomere recombination is actively repressed. As discussed above, two factors that mediate this recombinational repression are TRF2 (through its interactions with Rap1) and a heterochromatic epigenetic state (13, 37). Given that ALT cells display markedly increased telomere-recombination rates, one might expect ALT telomeres to display an altered epigenetic state. Indeed, exciting recent work has linked the ALT phenotype to the ATRX/DAXX/H3.3 chromatin-remodeling pathway, thereby validating this hypothesized link.

ATRX (α-thalassemia mental retardation X-linked) is a SWI/SNF chromatin remodeler that is enriched at pericentric heterochromatin, ribosomal DNA repeats, telomeric repeats and PML bodies (158–162). Constitutional mutations in the *ATRX* gene lead to ATR-X syndrome, which causes characteristic pleiotropic phenotypes in patients that include mental retardation, α-thalassemia, and facial and genital abnormalities (163). The ATRX protein contains an N-terminal ATRX/DNMT3/DNMT3L (ADD) domain, which includes a plant homeodomain (164), and a C-terminal ATPase/helicase domain (158). Most ATRX mutations in ATR-X patients are missense mutations that fall within either the ADD domain or the ATPase/helicase domain (165).

DAXX (death domain–associated protein) was initially identified as an apoptosis-promoting protein that binds to Fas (166). It was later found to associate with ATRX to form a chromatin-remodeling complex that deposits histone variant H3.3 at defined regions on chromatin (161, 167, 168). ATRX, DAXX, and H3.3 can be immunoprecipitated together from HeLa cell extracts (167). Although DAXX is required for ATRX interaction with H3.3, ATRX is not required for DAXX interaction with H3.3, which suggests that DAXX is a histone chaperone that bridges ATRX and H3.3 (167, 168). H3.3 is enriched around many transcriptionally active and repressed genes, at transcription regulatory sequences, and at telomeric repeats (167). ATRX is not required for H3.3 deposition around transcriptionally active and repressed genes or at many transcription regulatory sequences, but it is required for H3.3 localization at telomeric repeats and at a subset of transcription regulatory sequences. ChIP-Seq (chromatin immunoprecipitation sequencing) analysis revealed that ATRX is enriched at sites containing G/C-rich variable-number tandem repeats (VNTRs) and telomeres (162, 169). Both VNTRs and telomeres have a tendency to form G-quadruplex DNA structures, to which ATRX specifically bound in an in vitro gel-shift analysis (169). Thus, ATRX/DAXX chromatin complexes may be recruited to target sites and deposit H3.3 via ATRX's affinity for G-quadruplexes.

Two recent studies identified frequent somatic mutations in the ATRX/DAXX/H3.3 chromatin-remodeling pathway in human pancreatic neuroendocrine tumors and pediatric glioblastomas (147, 148). Strikingly, ATRX/DAXX/H3.3 mutations are highly correlated with the ALT phenotype, as defined by the presence of telomere aggregates/clustering by use of telomeric fluorescence in situ hybridization analysis. ATRX mutations are either (*a*) frameshift and nonsense mutations that lead to disruption of the ATPase/helicase domain or (*b*) missense mutations that fall within the ATPase/helicase domain. Most DAXX mutations are frameshift and nonsense mutations that are predicted to disrupt its association with ATRX and/or H3.3. Several DAXX mutations, however, are located toward the C-terminal end of DAXX, and their effects on the ATRX/DAXX/H3.3 pathway need to be further characterized. All mutations in histone H3.3, in contrast, are missense mutations at lysine 27 (K27M) or at glycine 34 (G34R or G34V). Notably, H3K27 often undergoes post-translational modifications, and trimethylation of H3K27 is associated with transcriptional repression. Glycine 34 of H3.3 is near another lysine residue (K36), methylation of which is

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correlated with transcriptional activation. Interestingly, some tumors harbor mutations in both ATRX and H3.3.

These findings offer new insight into the mechanism of ALT activation in tumors. It remains to be examined whether mutations in the ATRX/DAXX/H3.3 pathway alone are sufficient to activate ALT or whether additional mutations are required. Because of the direct involvement of ATRX and DAXX in modulating telomere heterochromatin (162, 167, 170), ATRX and DAXX mutations may cause a less repressed telomeric heterochromatin state that activates telomere recombination and engages ALT. However, because ATRX is required for deposition of H3.3 at many transcriptional regulatory sequences (167), it is also possible that ATRX mutations cause transcriptional alterations at genes that modulate telomere recombination, leading to activation of telomere recombination and ALT. Further work is necessary to distinguish between these possibilities.

TELOMERE-BASED THERAPEUTIC STRATEGIES

Because telomerase is upregulated in 85% of human cancers and is critical for ongoing cancer cell proliferation (144, 171), telomerase represents a compelling therapeutic target. Numerous telomerase-based therapeutic strategies have been tested in vitro and in vivo, and several approaches are now in early clinical trials (**Table 1**). Here, we review progress in these therapeutic efforts.

Telomerase Inhibition

The most straightforward telomere-related cancer therapeutic strategy is the direct inhibition of telomerase enzymatic activity. Given the shorter average telomere length of many tumors compared with that of adjacent normal tissue (172–175), systemic inhibition of telomerase could lead to telomere attrition–induced crisis and proliferative inhibition of cancer cells without severely affecting normal cells that also express telomerase. Although conceptually simple, this approach is complicated by several potential issues. First, systemic administration of a potent telomerase inhibitor could cause significant toxicity in telomerase-positive high-turnover cell populations, especially the hematopoietic system (176). The critical importance of telomerase activity for proper function of the hematopoietic compartment is dramatically underscored by haploinsufficiency of TERT or TER in DC patients, who frequently demonstrate aplastic anemia and other bone marrow deficiencies (177). Systemic telomerase inhibition may exhaust the telomere reserve and precipitate telomere and tissue dysfunction in the bone marrow and other stem and progenitor cell compartments. This problem could be avoided with targeted delivery of the telomerase inhibitor specifically to cancer cells, but such delivery for cancer therapeutic purposes remains a daunting challenge.

A second concern, with respect to systemic telomerase inhibition, is that cancer cells might escape treatment by engaging ALT pathways. In this case, the treatment-induced telomere dysfunction could cause genomic changes that further enhance tumor evolution and progression, analogous to what is proposed to occur at crisis during early tumor development. Indeed, when telomerase was experimentally removed from murine T cell lymphomas, tumor growth was ultimately restored in some cases by the emergence of ALT-enabled clones (88). Intriguingly, because of the cellular changes necessitated by the transition to ALT, the ALT-enabled outgrowth tumors were particularly susceptible to interventions targeting mitochondrial function and oxidative defense, which suggests that tumors that escape telomerase inhibition may have specific exploitable weaknesses as a result.

A third issue with systemic telomerase inhibition is that the resulting telomere dysfunction may have very different effects on different tumor types. For example, although telomere attrition in late-generation TER-null mice leads to a moderately increased incidence of spontaneous tumor in highly proliferative cell types (e.g., lymphomas and teratocarcinomas),

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Table 1 Cancer therapeutic strategies involving telomere biology

Strategy	Proof of principle	Clinical trials
Telomerase inhibition	Telomerase knockdown (shRNA, siRNA)	GRN163L (imetelstat)
Telomere disruption	G-quadruplex ligands,[a] mutant TER	—
Telomerase vaccination	—	GV1001, GRNVAC1
Telomerase-targeting virus	TERT promoter–driven suicide gene	TERT promoter–driven oncolytic virus

[a]G-quadruplex ligands have entered early clinical trials, but the particular ligands used do not appear to act through telomere disruption. For this reason, we keep the G-quadruplex telomere disruption strategy in the proof-of-principle column. Abbreviations: shRNA, small hairpin RNA; siRNA, small interfering RNA; TER, template-containing telomerase RNA; TERT, telomerase reverse transcriptase.

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such mice are resistant to the development of skin tumors (78). Finally, in tumors with long telomeres, there may be a significant delay from treatment onset to the induction of tumor proliferative inhibition.

GRN163L. Currently, there is only one telomerase inhibitor in clinic trials: GRN163L (also known as imetelstat) from Geron. GRN163L is a short-chain lipidated oligonucleotide (5′-TAGGGTTAGACAA-3′) that is complementary to the TER template region (178–180). When GRN163L binds telomerase, it blocks the catalytic function of telomerase and causes progressive telomere attrition, ultimately inducing telomere crisis and proliferative inhibition in cancer cells both in vitro and in vivo. Extensive Phase I and Phase I/II trials with GRN163L alone in breast cancer, lung cancer, myeloma, and chronic leukemia have been completed, and randomized Phase II trials are ongoing. A trial of GRN163L in combination with the microtubule inhibitor paclitaxel and the angiogenesis inhibitor bevacizumab is also under way (181, 182). Although outcomes have been promising thus far, side effects including cytopenias, impaired coagulation, and neuropathy have been observed (181, 182). The mechanisms that underlie these side effects, and the extent to which they will hamper treatment efforts, are not yet clear.

GRN163L (imetelstat): a telomerase catalytic inhibitor currently in clinical trials

Recent results have suggested an important role for cancer stem cells in tumor progression and resistance to chemotherapy. Intriguingly, GRN163L depletes cancer stem cells in breast and pancreatic cancer cell lines (183), which indicates that telomerase inhibition may be particularly effective at targeting this important subset of cells.

Knockdown of telomerase by RNA interference. Another approach to limiting telomerase activity is through the depletion of TER and/or TERT by siRNAs or shRNAs. This approach can efficiently and rapidly inhibit cancer cell growth both in vitro and in vivo (184, 185). The speed of the response suggests that depletion of the telomerase ribonucleoprotein may abrogate a telomere-independent function of telomerase, but this hypothesis has not been definitively proven. Although this approach is promising, the specific and efficient delivery of inhibitory RNAs to tumors in vivo remains a difficult challenge.

Telomere Disruption

Another general cancer therapeutic approach is to directly target telomere integrity, thereby precipitating rapid telomere dysfunction and cancer growth inhibition. This approach can induce a more rapid growth response than telomerase catalytic inhibition can, given that it is not necessary to wait for critical telomere shortening. However, this strategy is complicated by the fact that the telomere-disrupting agents

may dramatically affect survival and genome stability in normal cells as well. Thus, these approaches will probably require the development of better targeting strategies in order to limit unwanted toxicity.

G-quadruplex ligands. Under physiological conditions, G-rich sequences can generate four-stranded helical structures termed G-quadruplexes (186). Given the G-rich nature of the telomeric sequence, it is not surprising that G-quadruplexes can be detected at telomeres in vivo. These telomeric structures may affect telomere-telomere associations, sister-chromatid alignment, and telomere capping (recently reviewed in References 187 and 188). Stabilization of G-quadruplexes at the single-stranded G-rich telomere overhang by G-quadruplex ligands can displace telomere-binding proteins, including POT1, and can inhibit telomere elongation by telomerase, thereby causing rapid tumor growth inhibition both in vitro and in vivo. However, a major concern is the lack of specificity of G-quadruplex ligands, given that G-quadruplex structures are also present at many other genomic loci (including the promoters of c-MYC, vascular endothelial growth factor, hypoxia-inducible factor 1α, and Bcl2). Stabilization of G-quadruplexes in such promoter regions can cause disrupted gene expression and potential toxicity in normal cells. A G-quadruplex ligand has moved into early clinical trials, although it is unlikely that this agent acts through telomere disruption (188).

Mutant template telomerase RNA. Another way to disrupt telomere capping relies on mutant versions of TER to induce rapid telomere dysfunction. The catalytic subunit TERT uses the 11-bp template region of TER to add new TTAGGG telomeric repeats to the telomere ends (189, 190). When mutations are introduced into the template region of TER, the resulting mutant telomerase adds mutant telomeric repeats that cannot properly bind the protective shelterin proteins, thereby causing rapid telomere dysfunction (191). The outcome is rapid cancer cell death and inhibition of proliferation in vitro and in vivo (185, 191, 192). There are several significant hurdles facing this therapeutic strategy. First, although overexpression of mutant TER blocks the growth of only telomerase-positive cells (193), this approach would probably lead to senescence and/or genome instability in telomerase-positive stem and progenitor cells. Second, because this technique represents a gene therapy approach to cancer, it will not be feasible until better in vivo delivery methods are developed. Finally, as with telomerase catalytic inhibition, cells may be able to circumvent treatment by enabling ALT telomere maintenance.

GV1001: an injectable telomerase peptide vaccine that is currently in clinical trials

GRNVAC1: a telomerase-targeting autologous dendritic cell vaccine

Telomerase-Targeted Immunotherapy

Tumor-associated antigens presented at the cell surface by major histocompatibility complex class I molecules can trigger the activation and expansion of $CD8^+$ cytotoxic T lymphocytes (CTLs) (194, 195). These CTLs then specifically recognize and kill the tumor-associated antigen–presenting cells. A major obstacle to such cancer immunotherapy is the heterogeneous expression of tumor antigen within a tumor and lack of antigens that can be widely used to target various tumors. Because telomerase is expressed in most human tumors and its presence is critical for tumor proliferation and progression, it is a compelling target for tumor vaccine development. Previous studies in human and murine models have demonstrated that CTLs can specifically and effectively kill cancer cells that have TERT epitopes presented on the cell surface (196). Several TERT-based immunotherapies that rely on two major strategies for CTL activation have been developed: (*a*) direct injection of tumor antigen and (*b*) ex vivo treatment of antigen-presenting cells.

The most advanced TERT-directed vaccines are GemVax's GV1001 and Geron's GRNVAC1. GV1001 contains a 16-mer peptide derived from TERT (amino acids 611–626: EARPALLTSRLRFIPK) (197, 198). Results from Phase I and II clinical trials

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suggest that GV1001 is well tolerated and that it induces a TERT-specific T cell response that prolongs patient survival (197–200). GRN-VAC1 relies on ex vivo treatment of autologous antigen-presenting dendritic cells isolated from cancer patients (201). The dendritic cells are transduced with messenger RNA encoding full-length TERT (202), which may enable a robust T cell response against multiple TERT peptides. Results from Phase I and II clinical trials indicate that GRNVAC1 is well tolerated and that TERT-specific T cell activation can be detected in the majority of patients. As with telomerase inhibition, a significant concern with these immunotherapeutic approaches is that the treatment might endanger telomerase-positive stem and progenitor cells.

Telomerase-Targeted Suicide Genes and Oncolytic Viruses

Because telomerase expression is high in most cancer cells and low to absent in most adult somatic cells, the TERT promoter has been exploited to drive the expression of suicide genes or the replication of oncolytic viruses in cancer cells. The suicide genes that have been used include *TRAIL* (tumor necrosis factor–related apoptosis-inducing ligand) and several prodrug-activating enzymes, including *E. coli* cytosine deaminase, herpes simplex virus thymidine kinase, *E. coli* purine nucleoside phosphorylase/6-methylpurine deoxyribose, and carboxypeptidase G2 (203–207). In the case of oncolytic viruses, the TERT promoter has been used to drive expression of adenovirus E1A and E1B proteins, thereby restricting viral replication to TERT-positive cells. One such virus, telomelysin, was tested in a Phase I clinical trial through intratumoral injection in patients with various solid tumors (208). Initial results suggested specific targeting of tumor cells (208). As with most other TERT-targeting strategies, the impact on telomerase-positive stem and progenitor cells will be an important safety issue to consider.

LOOKING AHEAD

Recent research with mouse models and patient samples has provided compelling evidence in support of the critical interplay of telomere dysfunction and telomerase expression in driving cancer initiation and progression. Beyond this canonical model, new research has suggested that telomerase may promote cell proliferation through telomere-independent mechanisms, although it remains to be determined whether and under what circumstances these mechanisms drive cancer growth. Furthermore, recent studies have identified key epigenetic changes that may engage telomerase-independent ALT telomere-elongation mechanisms. Collectively, these studies highlight the incredible complexity of telomerase and telomeres, working together and separately, in supporting cancer growth. A more complete understanding of these intricate pathways will undoubtedly suggest new avenues for cancer therapeutic approaches. In the meantime, with a telomerase catalytic inhibitor and telomerase vaccines in clinical trials, we will soon have new results with which to evaluate the benefits and challenges of telomerase-targeted cancer therapy.

SUMMARY POINTS

1. Telomeres protect chromosomal ends from recognition by the DNA-damage response machinery, thereby maintaining genome stability.
2. Dysfunctional telomeres can suppress cancer development by engaging replicative senescence or apoptotic pathways, but they can also promote tumor initiation and progression by causing oncogenic chromosomal rearrangements.

3. Although the primary function of telomerase is to elongate telomeres and extend the cellular replicative life span, recent evidence suggests that telomerase may also support cell growth through telomere-independent functions.
4. A subset of human tumors elongate telomeres through telomerase-independent ALT recombinational mechanisms, and recent work in human tumors has revealed that mutations in the ATRX/DAXX/H3.3 chromatin-remodeling pathway strongly correlate with the ALT phenotype.
5. Several cancer therapeutic strategies have been developed to exploit the intricate connection between telomere biology and cancer, and ongoing clinical trials are putting some of these approaches to the test in various human malignancies.

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DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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