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Invited Review

Cytological and functional aspects of telomere maintenance

A.T. Dandjinou, I. Dionne, S. Gravel, C. LeBel, J. Parenteau and R.J. Wellinger

Department of Microbiology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Québec, Canada

Summary. The fact that eukaryotic chromosomes are linear poses a special problem for their maintenance: the natural ends of chromosomes must be distinguished from ends generated by chromosomal breakage and somehow, the chromosome ends must also be fully replicated to maintain their integrity. Telomeres, the complex structures at the ends of chromosomes are thought to be instrumental for both of these functions. However, recent insights in telomere biology suggest that these terminal structures do much more than just fulfill these two basic functions. Cytological data demonstrate that telomeres may play leading roles in chromatin organization and nuclear architecture during mitosis and meiosis. Moreover, non-functional telomeres may lead to genetic instability, a common prelude to cancer. Here, we review the basic functions of telomeres during chromosome replication and discuss the cytological aspects of telomere function during mitosis and meiosis.

Key words: Telomeres, Chromosome organization, DNA replication

Introduction

The concept of telomeres was defined by the geneticist Hermann J. Muller, working on *Drosophila melanogaster*. The word telomere derives from Greek roots: *telos* meaning, "end", and *meros* meaning, "part". After irradiation with X-rays, Muller noted that terminal deletions and terminal inversions were unusually hard to find as compared to other rearrangements on the chromosomes. Thus, Muller inferred that chromosome stability requires a specialized terminal structure, the telomere (Muller, 1938). His observations on Drosophila chromosomes were complemented by the studies of Barbara McClintock, working on *Zea mays*. In her studies, McClintock noticed that broken chromosomes were reactive and often fused with each other whereas natural chromosome ends were stable (McClintock, 1939).

It is now known that telomeres are essential nucleoprotein structures at the ends of eukaryotic chromosomes. They serve several functions, two of which are essential for genome integrity, namely: protecting the ends from degradation, recombination and random fusion events; and enabling complete DNA replication (for recent reviews, see Blackburn and Greider, 1995; Zakian, 1995; Greider, 1996). Cytologically, telomeres in some organisms are located in a non-random manner near the nuclear periphery and in others, there is evidence that they associate with the nuclear matrix (Cockell et al., 1995; Luderus et al., 1996). Thus, it has been postulated that telomeres may be involved in organizing the nuclear architecture (Gilson et al., 1993). They may interact with the nuclear envelope and other telomeres, either directly or mediated by various telomere-binding proteins. Moreover telomeric regions are thought to be organized into a heterochromatin-like structure that influences transcription, replication or both of genes located near the telomeres (Blackburn and Greider, 1995). Here, we will briefly review the main functions of telomeres and then focus on the cytological aspects of telomere biology.

Telomeric DNA

In the vast majority of eukaryotes, telomeric DNA consists of tandem repeats of short (6-26 bp) simple sequences (Wellinger and Sen, 1997; Table 1). For all organisms analyzed so far, the strand running 5' → 3' towards the end contains clusters of 3 to 4 guanines and is commonly referred to as the G-rich strand. The actual number of repeats, and hence, the length of the telomeric repeat tracts is highly variable between organisms, between the telomeres within the same species and even within the same cell. Thus, the regulatory mechanisms determining repeat length in general do not lead to a fixed number of repeats, but rather a distribution of lengths around a certain species-specific median length.

Offprint requests to: Dr. Raymund J. Wellinger, Department of Microbiology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Québec, J1H 5N4 Canada. Fax: +819 564 5392. e-mail: rwelli01@courrier.usherb.ca

However, it appears that a certain minimal number of these terminal repeats is essential for most, if not all functions ascribed to telomeres.

Telomere proximal, a variety of more complex repeated DNA is found. These sequences are called TAS (for Telomere Associated Sequences) or subtelomeric region. The actual repeats found in this area range from rather complex, middle repetitive elements to relatively short, direct repeats that are organized in a mosaic fashion (Pryde et al., 1997). The functions of the subtelomeric regions remain unclear. There is good evidence that this area of the genome is very dynamic, being involved in frequent exchanges of sequences. Thus, it has been proposed that the variable subtelomeric areas would constitute an ideal place for the amplification and variation of useful genes and hence play an important role for adaptation and evolution of the genome (Biessmann and Mason, 1992; Pryde et al., 1997).

Table 1. Telomeric repeats (5→3').

ORGANISMS	SEQUENCE	LENGTH
Vertebrates	TTAGGG	5-100kb (human:5-15kb)
Plants	T2-4AGGG	3 kb
Brewer yeasts	(TG)1-6 TG2-3	350pb
Ciliates (protozoa)	TTGGGG or TTTTGGGG	20-300pb

Telomere replication: the importance of telomerase

Until recently, models of telomere replication predicted that due to the properties of the polymerases implicated in semiconservative replication of linear DNA, the two daughter molecules generated after S-phase have one end that is blunt and one end with a short 3' overhang (Lingner et al., 1995; Wellinger et al., 1996). The most important component ensuring the complete telomere replication is a ribonucleoprotein enzyme called telomerase. Telomerase can elongate the short single-stranded G strand in a DNA template independent manner to create long G-tails (Greider and Blackburn, 1985; for review, see Greider, 1996). Support for this initial model came from studies in yeast, in which such long, single-stranded tails are present on telomeres in late S-phase when conventional replication has essentially been completed (Wellinger et al., 1993a,b). The fill-in synthesis on the G-tails would complete the replication of telomeres, preventing the loss of sequences and reestablishing short 3' overhangs. The telomeres on which the newly synthesized strand is made by leading-strand synthesis were thought to become blunt-ended after conventional replication is complete. However, if the telomeric end-structure needs to be uniform on all telomeres, this blunt end must be converted into an end with a short 3' overhang. We have recently shown that transient, long G-tails also occur on ends replicated by leading strand synthesis and that these overhangs can be generated in the absence of telomerase (Dionne and Wellinger, 1996; Wellinger et al., 1996). These observations have prompted us to postulate a new step in telomere replication, namely the involvement of a strand specific 5'-3' exonuclease or a helicase combined with a single strand specific endonuclease (Fig. 1; Dionne and Wellinger, 1996). Such activities would thus allow the reformation of the proper end-structure on all telomeres and could therefore be essential activities for cell cycle progression.

It is clear that an active elongation process for telomeric repeats must be functional for the chromosome to be entirely propagated to the daughter cells in a stable manner over many generations. However, in most human somatic cells, telomeres shorten with each cell division and telomerase can not be detected. There is now a direct evidence for a causal relationship between telomere shortening and cellular senescence (Bodnar et al., 1998; de Lange, 1998; Greider, 1998; Vaziri and Benchimol, 1998) giving importance to the fact that telomere shortening may be part of the molecular clock

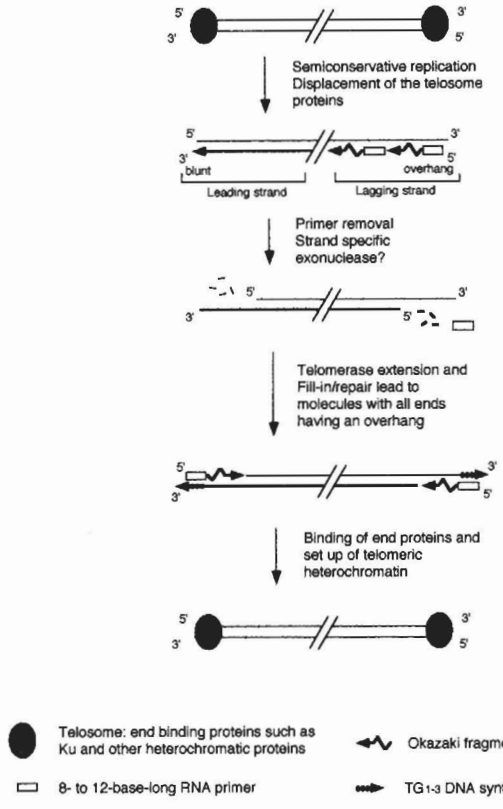

Fig. 1. Model for telomere replication and maintenance in Yeast. Replication at both ends of the chromosome for one of the daughter molecules is shown. Conventional replication at the leading strand presumably yields a blunt end on one side of the molecule. However, the discontinuous replication of the lagging strand leaves at least an 8- to 12-base gap after the removal of the RNA primer on the other end. After replication, a strand-specific exonuclease exposes tails of the G-rich strand on both telomeres of individual molecules. Telomerase elongation and DNA polymerase fill in/repair generates 3' overhangs on all telomeres after the removal of the RNA primer.

that triggers senescence and that a threshold level of telomerase activity is required for life-span extension (Bodnar et al., 1998; Vaziri and Benchimol, 1998). Interestingly, telomerase activity can be detected in immortalized cell lines and in about 85% of human tumors (Kim et al., 1994). Thus, it has been postulated that human cancerous cells would be subject to telomere shortening and senescence in the absence of telomeric repeat reforming activities. In most cases, reestablishment of telomeric repeat synthesis would thus be acquired by a reactivation of telomerase, but there is also evidence for alternative modes for telomere repeat maintenance in the absence of telomerase (Murnane et al., 1994; Bryan et al., 1995).

Telomere Capping: Terminus-binding proteins

A double strand break in chromosomal DNA can lead to chromosomal fusion and genomic instability (breakage-fusion-bridge cycles) if not adequately repaired (McClintock, 1939). In yeasts, a double stranded break in DNA is recognized by the DNA damage checkpoint that is dependent on the *Rad9p* gene product (Weinert and Hartwell, 1988), the cell cycle is arrested and the break is usually repaired by homologous recombination or can be repaired directly by the addition of telomeric repeats by telomerase (Sandell and Zakian, 1993). If there is no rescue events, the double stranded break will expose the DNA to degradation and chromosome loss will ultimately lead to inviability of the cells. Thus, one of the functions of natural chromosome ends must be to provide the chromosome with a "Cap" that will prevent aberrant degradation and also prevent telomeres from being recognized as a chromosomal break. It is thought that factors associating with the telomeres will be implicated in these functions and thus factors binding to the extreme ends of the chromosomes will be crucial for these functions.

The physical structure of the very ends of the chromosomes is known for some protozoans and there consists in a short 10-16 bases overhang of the G-rich strand (Klobutcher et al., 1981; Pluta et al., 1982; Henderson and Blackburn, 1989). Proteins that bind specifically to this terminal structure have been identified in Oxytricha and related ciliates (Gottschling and Zakian, 1986; Price, 1990; Sheng et al., 1995). These proteins are intimately associated with the chromosomal termini and there is evidence that they protect them from exonucleolytic degradation.

The Ku protein complex, a heterodimer composed of a 70-kDa and a 80-kDa subunit, is known to bind directly to double-stranded DNA ends and to be critical for non-homologous DNA double-strand break repair (Weaver, 1995; Lieber et al., 1997). We have recently shown that Ku also plays a direct role in telomere maintenance, at least in yeast (Gravel et al., 1998). In cells lacking a functional Ku complex, unusually long overhangs of the G-rich strand are present throughout the cell cycle. In addition, by using an in vivo cross-linking protocol, we demonstrated that Ku is bound to telomeric DNA. The transcriptional repression of genes located near telomeres is dramatically altered in Ku-cells, reinforcing the conclusion that Ku is present in normal telomeric chromatin (Boulton and Jackson, 1998; Gravel et al., 1998). These data suggest that Ku establishes a normal DNA end-structure on yeast chromosomes by functioning as a terminus-binding factor (Fig. 1).

Little is known about terminus binding factors in vertebrates. Recently, it has been demonstrated that the termini of mammalians telomeres carry long (~150 nt) protrusions of the G-rich strands (Makarov et al., 1997; McElligott and Wellinger, 1997; Wright et al., 1997). Heterogeneous nuclear ribonucleoproteins (hnRNPs) have been shown to bind to single-stranded repeats of the G-rich strand in vitro (McKay and Cooke, 1992; Ishikawa et al., 1993). More direct evidence that hnRNP A1 (hereafter called A1) functions at telomeres in vivo came from the observation that a mouse erythroleukemic cell line deficient in A1 possesses shortened telomeres (LaBranche et al., 1998). Reexpression of A1, or UP1, a proteolytic fragment of A1, in the deficient cell line reestablishes the normal telomere length (LaBranche et al., 1998). While both A1 and UP1 bind to vertebrate telomeric repeats directly and with specificity, only UP1 can recover telomerase activity from a cell lysate, suggesting that UP1 recruits telomerase to the ends of chromosomes (LaBranche et al., 1998). Hence, UP1 may bind telomeric repeats and serve as a bridge between telomeres and telomerase, to permit the enzyme's functions more efficiently. Also, other proteins could have the same role in different organisms (for example, Cdc13p in *S. cerevisiae*, Nugent et al., 1996).

In addition to single-strand, terminus-specific binding proteins, specific proteins bind telomeric DNA along the length of the double-stranded telomeric region. The best-characterized double-stranded telomere binding protein is the Repressor Activator Protein (Rap1p) from *S. cerevisiae*. RAP1 is a sequence-specific DNA-binding protein that binds to many promoters, to two elements that silence mating-type genes, and to (C1-3A) n tracts at telomeres (for reviews see: Shore, 1994; Fang and Cech, 1995; Brun et al., 1997). There is a Rap1p binding site approximately every 18bp in yeast telomeric repeat sequences (Gilson et al., 1993). Rap1p appears to be involved in a protein-counting mechanism that regulates telomere length (Marcand et al., 1997). Immuno-localization experiments show that Rap1p antibodies localize to the ends of meiotic chromosomes (Klein et al., 1992). In mitotic cells, Rap1p is concentrated in a discrete number of foci near the nuclear periphery (Palladino et al., 1993), which correspond to clusters of telomeres.

In human cells, Telomeric Repeat binding Factor1 (TRF1) binds duplex telomeric DNA and contains a particular Myb motif called the telobox, which is required for telomeric repeat recognition (Zhong et al., 1992; Chong et al., 1995; Bilaud et al., 1996). TRF1

binds DNA as a dimer using a large conserved domain near the N-terminus of the protein for TRF1-TRF1 interactions (Bianchi et al., 1997). TRF1 was recently shown to be involved in the regulation of telomere length in human cells (van Steensel and de Lange, 1997). Another telomeric protein, TRF2, containing a single Myb sequence has been identified recently. TRF2 is bound specifically to duplex TTAGGG repeats in vitro and is localized to all human telomeres in metaphase chromosomes (Bilaud et al., 1996, 1997). Like TRF1, TRF2 carries a C-terminal Myb motif and a large TRF1-related dimerization domain near its N-terminus (Broccoli et al., 1997). However, the dimerization domains of TRF1 and TRF2 do not interact. TRF2 differs from TRF1 in that its N-terminus is basic rather than acidic. Because TRF1 and TRF2 show differences, these factors probably have distinct functions at telomeres. In cells containing a dominant negative allele of the gene encoding TRF2 there is a loss of G-rich overhangs and there are end-to-end chromosome fusions detectable in metaphase and anaphase cells (van Steensel et al., 1998). These results suggest that maintaining a terminal G-rich overhang is absolutely essential for the protection of chromosomes from fusion events.

Telomere chromatin

In all organisms tested so far, the bulk chromatin and subtelomeric repeats are constituted of a nucleosomal array. In lower eukaryotes, it has been shown that the double strand telomeric DNA exhibits, after nuclease digestion, a non-nucleosomal organization called telosome (Gottschling and Cech, 1984; Gottschling and Zakian, 1986; Wright et al., 1992). It has been suggested that the telosome is part of a heterochromatin domain, located at the ends of the chromosomes. Consistent with this idea, in yeast, genes placed near telomeres are subject to transcriptional repression, known as Telomeric Position Effect (TPE) (Gottschling et al., 1990). As a corollary to TPE, these domains are replicated late in S phase (Ferguson and Fangman, 1992). In yeast, the end-binding factor Ku, together with Rap1p, Sir2p, Sir3p Sir4p and Rlf2p as well as histone H3 and histone H4, are required for TPE. This phenomenon, like Position Effect Variegation (PEV) in *Drosophila* (Henikoff, 1990), extends from the transcriptionally inactive, repetitive elements at the chromosomal ends, to adjacent euchromatin (Renauld et al., 1993). In contrast to the telomeres of lower eukaryotes, most of the much longer mammalian telomeres are organized into nucleosomal arrays (Makarov et al., 1993; Tommerup et al., 1994) and the telomeres feature nucleosomal components and an organization similar, but not identical, to those of the bulk chromatin (Tommerup et al., 1994; Bedoyan et al., 1996). However, the formerly mentioned double-strand-specific telomere DNA binding proteins, TRF1 and TRF2 have been proposed to be part of a special telomeric chromatin structure (Chong et al., 1995; van Steensel et al., 1998).

Telomere cytology: telomere-nuclear envelope associations

In 1885, Rabl described his cytological observations of mitotic anaphase chromosomes in living and fixed cells from salamander and newt larvae (Rabl, 1885). He observed that centromeres and telomeres where positioned at opposite poles in the cell nucleus, forming a V-shaped chromosome. Since this particular arrangement reappeared at the prophase, he concluded that chromosomes maintained the polarized arrangement induced by anaphase throughout interphase. A Rabl conformation has since been observed in a variety of organisms (Dernburg et al., 1995).

An indication that telomeres might contribute to maintain the polarization seen by Rabl came from the observation that, in most organisms, telomeres are found at or near the nuclear periphery (Dernburg et al., 1995, Fig. 2). This association has led to speculation about its purpose. Since telomeres are heterochromatic and regions of *Drosophila* polytene chromosome that

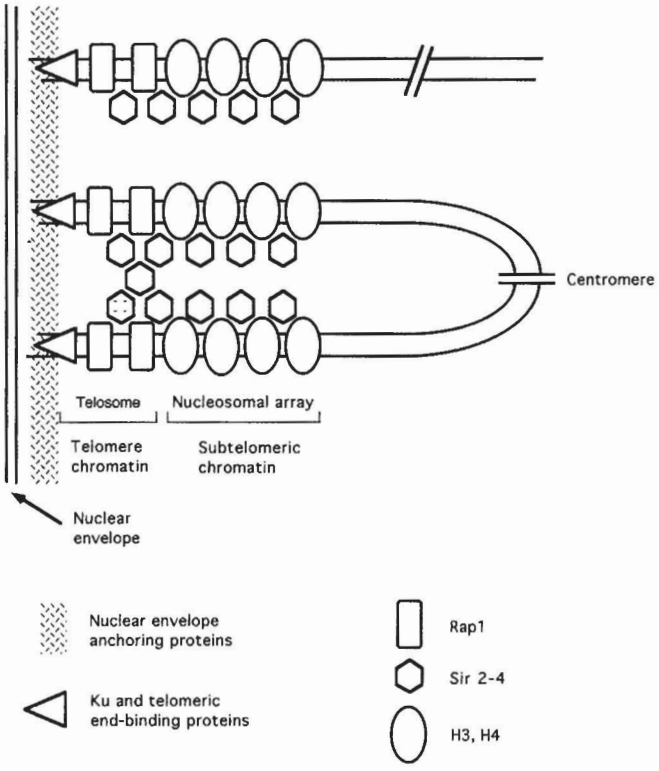

Fig. 2. A schematic model of telomere interactions in the nucleus. Telomeric repeat DNA is associated with a complex of specific proteins forming the telosome. This schematic model illustrates how protein interactions may lead to telomere-telomere and telomere-nuclear envelope interactions. Telomeres are known to cluster near the nuclear envelope. This peripheral location appears to be achieved via association of telomere end binding proteins, like Ku, with some nuclear structures (matrix) and the resulting anchoring may be crucial for the formation or maintenance of a heterochromatic structure. Note that in humans, telomeres may only be anchored to the matrix, but not the nuclear lamina (Luderus et al., 1996; and see text).

interact with the nuclear envelope map to heterochromatin (Mathog et al., 1984), the peripheral location of telomeres may be crucial for the formation or maintenance of a heterochromatic structure. Additionally, it is possible that by associating with the nuclear envelope, telomeres may serve to anchor the chromosomes, thereby providing a resistance to large scale reorganization during interphase and preventing the chromosome arms from becoming entangled. Finally, linking telomeres to the nuclear envelope might simplify homology searches between chromosomes, important for the successful onset of meiosis. However, in humans, biochemical fractionation of TRF and telomeric DNA did not reveal an interaction with the nuclear lamina in interphase (Luderus et al., 1996). Furthermore, ultrastructure analysis indicated that the mammalian telomeric complex is distributed throughout the nuclear volume arguing against a role for the nuclear envelope in telomere function during interphase.

Telomere-telomere associations

Many cytological studies have provided evidence for telomeric clustering in interphase nuclei (Dernburg et al., 1995). In *S. cerevisiae*, immunofluorescence microscopy with antibody against Rap1p, a telomeric DNA binding protein, was used to detect telomeres. This resulted in the detection of fewer signals than the number of telomeres (8-16 spots for 64 telomeres) and suggested that yeast telomeres are aggregated or juxtaposed near the nuclear envelope (Klein et al., 1992). However, this aspect of telomere biology is unlikely to be a conserved feature since no such clustering is observed in somatic mammalian cells (Luderus et al., 1996). Why do telomeres associate with each other? An answer to this question has recently been proposed (Kirk et al., 1997). In *Tetrahymena*, a mutant telomeric DNA sequence generated by the expression of an altered telomerase RNA causes a severe delay or block in anaphase. In these cells, the sister chromatids begin to separate but are unable to segregate to the daughter poles, suggesting that telomere-telomere associations are required for sister chromatid cohesion until anaphase and that resolution of this association in anaphase is abrogated by the mutant telomeric DNA sequence. The mechanisms mediating these associations are unknown but it is possible that some of the alternatively folded structures of telomeric DNA observed in vitro may play roles in interactions between chromosomes ends (reviewed in Wellinger and Sen, 1997). Alternatively, the involvement of telomere binding proteins was suggested. For instance, in *Drosophila melanogaster*, mutations in the gene UbcD1 result in frequent telomere-telomere associations during both mitosis and male meiosis that are not seen in wild type cells (Cenci et al., 1997). UbcD1 gene encodes a class I ubiquitin-conjugating enzyme that is involved in selective protein degradation mediated by the ubiquitin-proteasome pathway. The telomeric associations observed in UbcD1 mutants suggest that ubiquitin-mediated proteolysis of a telomere-associated protein is normally needed to ensure proper telomere behavior during *Drosophila* cell division. The results from *Tetrahymena* and *Drosophila* studies demonstrate that telomeres play an important role in sister chromatid cohesion and these associations have to be resolved to ensure the completion of anaphase.

Telomeres and meiotic chromosomes

During prophase of the first meiotic division, telomeres of most chromosomes are attached to a small region of the nuclear envelope. This particular arrangement, called the "bouquet", is thought to promote proper chromosome pairing by reducing the freedom of movement of sequences near telomeres and by concentrating the chromosome attachment sites in a limited region of the nucleus (Dernburg et al., 1995). Recently, a direct role for telomere in meiotic chromosome synapsis and segregation has been revealed in *S. cerevisiae* by the study of NDJ1 (nondisjunction1), a gene encoding a protein that accumulates at telomeres during meiotic prophase (Conrad et al., 1997; Chua and Roeder, 1997). In ndj1Δ cells, initiation of synaptonemal complex formation, a structure that connects homologous chromosomes along their length, is delayed and elevated nondisjunction of homologues ensues. However, there is no effect caused by the absence of Ndj1p on the segregation of a telomere-less ring chromosome, indicating that Ndj1p is not required for meiotic chromosome separation per se but is essential to separate chromosomes that have telomeres.

A novel role for telomeres in premeiotic nuclei has recently been uncovered in fission yeast. *S. pombe* nuclei assume a characteristic elongated nuclear morphology called "horsetail" during a certain period preceding meiotic division. During this period, the telomeres are always observed as a single cluster, a structure reminiscent of the "bouquet" stage. A combination of molecular localization with imaging techniques gave detailed insights into the nuclear movements associated with meiotic prophase (Funabiki et al., 1993; Chikashige et al., 1994). At this stage, the nucleus moves back and forth in the cell, apparently mediated by a bundle of cytoplasmic microtubules. Unexpectedly these microtubules are associated with the clustered telomeres and this complex appears to lead the movement of chromosomes, the centromeres trailing at the opposite end of the moving nucleus. Chikashige et al. (1994) suggested that the movement of the nucleus may aid pairing, recombination and segregation of homologous chromosomes by shuffling them with their ends tied together. Similar roles for the clustered telomeres in human and mouse cells in meiotic prophase have been proposed recently (Sherthan et al., 1996; Bass et al., 1997).

Future prospects

Although the telomere field progressed enormously over the last few years, many questions remain

unanswered. For example, how do telomere proteins and telomerase coordinate their activities through the cell cycle? What is the relevance of telomere localization inside the nucleus? How do telomere factors interact with other cellular proteins? As mentioned previously, human telomeres undergo progressive shortening in somatic cells and telomerase activity is not detectable in these cells. In contrast, in most human tumors, a mechanism that restores and maintains telomeres, such as activation of telomerase, occurs. This dependence of immortalized human cells to maintain a functional telomere suggests that approaches designed to interfere with telomere function may lead to novel strategies for cancer treatment. Such procedures may target telomere replication (anti-telomerase) or other proteins implicated in telomere maintenance.

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