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Telomeres: Cancer to Human Aging

Sheila A. Stewart[1] and Robert A. Weinberg[2,*]

[1]Departments of Cell Biology and Physiology and of Medicine, Washington University School of Medicine, St. Louis, Missouri 63110;
email: sheila.stewart@cellbiology.wustl.edu

[2]Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142;
email: weinberg@wi.mit.edu

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*Corresponding author.

Key Words

senescence, telomerase, crisis, tumorigenesis

Abstract

The cell phenotypes of senescence and crisis operate to circumscribe the proliferative potential of mammalian cells, suggesting that both are capable of operating in vivo to suppress the formation of tumors. The key regulators of these phenotypes are the telomeres, which are located at the ends of chromosomes and operate to protect the chromosomes from end-to-end fusions. Telomere erosion below a certain length can trigger crisis. The relationship between senescence and telomere function is more complex, however: Cell-physiological stresses as well as dysfunction of the complex molecular structures at the ends of telomeric DNA can trigger senescence. Cells can escape senescence by inactivating the Rb and p53 tumor suppressor proteins and can surmount crisis by activating a telomere maintenance mechanism. The resulting cell immortalization is an essential component of the tumorigenic phenotype of human cancer cells. Here we discuss how telomeres are monitored and maintained and how loss of a functional telomere influences biological functions as diverse as aging and carcinogenesis.

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Contents

Telomere: noncoding DNA and associated proteins located at the termini of linear chromosomes

INTRODUCTION

Since Barbara McClintock's original description in the 1940s, telomeres have been recognized as important capping structures that play a central role in distinguishing the true ends of a linear chromosome from a bona fide double-stranded DNA (dsDNA) break (McClintock 1941). In recent years, we have learned a great deal about the complex nucleoprotein structures located at the ends of chromosomes. Emerging from these studies is an understanding that telomere function is inextricably linked to cell cycle control, cellular immortalization, and tumorigenesis. We are only now beginning to understand the molecular details of how a telomere is monitored, regulated, and modified and how these functions permit continued cell cycle progression. In this review, we attempt to cover a broad range of topics concerning telomere biology, including information on the structure of a telomere, its relation to cellular lifespan, and its role in tumorigenesis. Each of these subjects warrants its own separate review, and so we would like to apologize from the outset to our many colleagues whose work could not be included in the present one.

TELOMERES AND CELLULAR LIFESPAN

Replicative Senescence

Early researchers in the area of replicative senescence assumed that individual cells from multicellular organisms possess an immortalized growth phenotype, i.e., that they are able to proliferate indefinitely. In the 1960s, however, Leonard Hayflick's pioneering work changed this paradigm and thereby initiated a new field of study. Hayflick found that fibroblasts isolated from an individual possess only a limited proliferative potential in vitro: A lineage of these cells could pass through only a predetermined number of growth-and-division cycles (Hayflick 1965). Cells that had reached the allowed limit of replication and thus halted further proliferation were termed senescent; such cells were characterized by a flat, extended shape and remained active metabolically but no longer divided. Indeed, we now know that such senescent cells, if properly treated, can remain viable for

years (S. Stewart & R. Weinberg, unpublished data). Importantly, Hayflick's work demonstrated that cells isolated from the same individual on multiple occasions recapitulated this finite growth phenotype.

These seminal observations led to the hypothesis that cells possess an internal clocking mechanism that is capable of (*a*) tracking the number of cellular divisions through which their lineage has passed and (*b*) halting any further division after a predetermined number of cell divisions (also known as the Hayflick limit). This work led to the further hypothesis that a limited proliferative capacity plays an important role in both aging and tumor suppression (Hayflick 1976). Since its original description, the role of cell senescence in tumor suppression has received much support, whereas the role of cell senescence in organismic aging remains unclear.

A link between cell senescence and the telomere was suggested in the 1990s, when two researchers described what would eventually become known as the telomere hypothesis. In the 1970s both Watson and Olovnikov had described the end replication problem, in which they suggested that linear chromosomes would be unable to replicate the extreme 3′ ends faithfully (Olovnikov 1973, Watson 1972). Thus, even if the initial RNA primer were synthesized at the extreme 3′ end of the template strand, a small portion of the chromosomal DNA would be lost following completion of replication. Such underreplication would create a problem if important genetic information were located at the end of the chromosome. This potentially catastrophic problem was solved by the evolutionary development of the telomere, which was eventually shown to be a repetitive sequence of noncoding DNA (reviewed in Blackburn 1991). In mammals, the telomere consists of thousands of tandem repeats of the hexanucleotide sequence TTAGGG. Fluorescent in situ hybridization showing human telomeres is shown in **Figure 1**.

To determine the fate of the chromosome ends, Harley, Greider, and colleagues began to examine the telomeric DNA of human chromosomes during successive rounds of DNA replication and cellular division (Allsopp et al. 1992). To do so, they utilized four-base-pair restriction enzymes to digest nontelomeric chromosomal DNA into small pieces (with an average DNA size of 126 base pairs); the telomeric DNA remained intact because its repeating hexanucleotide sequences were not recognized by these enzymes. The researchers then analyzed the size of this surviving telomeric DNA by Southern blot analysis, a procedure now termed the TRF (telomere restriction fragment) Southern blot.

Harley, Greider, and colleagues (Allsopp et al. 1992) initially observed a smear representing the heterogeneous lengths of the telomeres within their cell populations. Extending these analyses, they then examined a population of cells throughout its replicative lifespan and demonstrated that mean telomere lengths were reduced progressively with

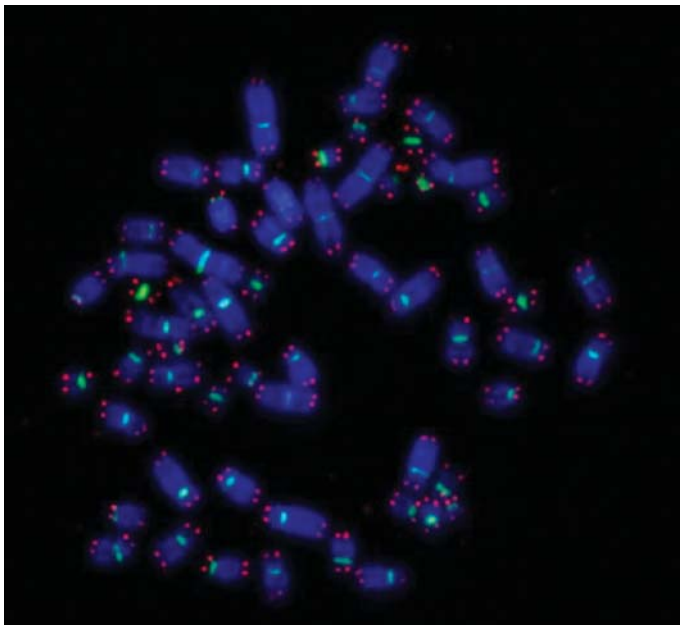

Figure 1

Telomere and centromere localization of metaphase chromosomes by fluorescence in situ hybridization (FISH). Use of FISH, together with telomere- and centromere-specific probes, reveals the telomeres at the ends of each HeLa cell metaphase chromosome (*red*) and a centromere located in its middle (*green*).

Tumorigenesis: process by which normal human cells are transformed into tumor cells

Senescence: p53- and Rb-dependent permanent growth arrest

TRF: telomere restriction fragment

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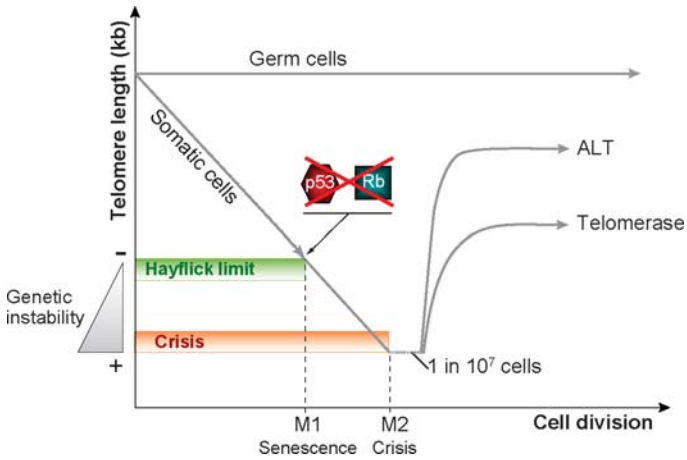

Figure 2

The telomere hypothesis. Telomere length (*ordinate*) is progressively lost during successive rounds of cellular division (*abscissa*), eventually leading to p53- and Rb-dependent permanent growth arrest, referred to as senescence. Inactivation of p53 and Rb function allows continued cellular division and further telomere shortening. Telomeres eventually erode to a length at which they are unable to protect chromosome ends, resulting in crisis, i.e., end-to-end chromosome fusions and apoptotic cell death. Rare clones (~1 in 10^7) may emerge from a population of cells in crisis. These clones maintain stable telomere lengths through the activation of a telomere maintenance mechanism, i.e., human telomerase catalytic subunit (hTERT) expression or the alternative lengthening of telomeres (ALT) mechanism.

each subsequent division (**Figure 2**), exactly as Watson's (1972) and Olovnikov's (1973) models had predicted. Importantly, when cells isolated from the same individual were followed in several independent cultures, these cells entered into senescence with roughly the same average telomere lengths. This observation suggested that telomere shortening serves as the genetic clocking mechanism originally described by Hayflick (1965) and that the telomeres tracked the number of cellular divisions through which an individual cell lineage had passed. In addition, it was hypothesized that once the telomeres shortened to a certain predetermined length, these DNA sequences were responsible for triggering entrance into senescence (Greider & Blackburn 1996 and references therein).

LT: SV40 large T antigen

Since these path-finding discoveries, we have learned that telomeres can indeed control replicative lifespan. However, they do so through mechanisms that are more complex than simple maintenance of telomere length above a critical threshold level. Instead, it is the maintenance of a properly functional, or capped, telomere structure (which can be influenced by telomere length) that is critical to continued cellular division (reviewed in Blackburn 2000). Indeed, as described below, the induction of replicative senescence can often be separated from the erosion of telomere length below a certain threshold level.

Cell Senescence: Man Versus Mouse

The permanent growth arrest that characterizes replicative senescence is dependent on the downstream effectors p53 and Rb, which impose this state once telomeric DNA has undergone certain changes (Shay et al. 1991). The key role of these two tumor suppressor pathways in senescence was demonstrated by expressing in presenescent cells the SV40 viral Large T antigen (LT) or the human papillomavirus (HPV) proteins E6 plus E7. LT functionally eliminates p53 and Rb, as do HPV E6 plus E7. Analyses of human cells that were destined to undergo senescence demonstrated that abrogation of both the p53 and Rb pathways was necessary for cells to bypass senescence (Shay et al. 1991), whereas inactivation of either p53 or Rb did not suffice to bypass senescence. This was not found, however, to be the case for murine cells, which need lose only the function of either the p53 or Rb pathways to bypass senescence (Sherr & DePinho 2000 and references therein). It is still unclear why, to escape senescence, both pathways must be inactivated in human cells, whereas murine cells need lose function of only one pathway. Nonetheless, these observations indicate that these regulatory pathways are organized differently in cells of the two species.

The importance of the p53 and Rb tumor suppressor pathways is not the only characteristic that distinguishes human and murine senescence. Unlike in human cells, progressive telomere shortening is not observed in

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cultured mouse embryonic fibroblasts (MEFs) (Blasco et al. 1997, Zijlmans et al. 1997). In addition, human embryonic fibroblasts typically divide 50–100 times before entering senescence. In contrast, MEFs divide only approximately ten times before entering senescence. Because murine telomeres are on average 50 kb, whereas their human counterparts are approximately 15–20 kb (Blasco et al. 1997, Zijlmans et al. 1997), the above observations raised questions about the importance of telomere erosion to replicative senescence in the mouse.

To explain these apparent discrepancies, some suggested that murine cells were more sensitive to in vitro growth conditions that resulted in nontelomeric damage and a distinct type of senescence often termed stress-induced senescence (Itahana et al. 2004). Indeed, this was found to be the case: Murine cells are exquisitely sensitive to high oxygen tensions, notably those experienced under standard conditions of cell culture (~20%). Accordingly, when MEFs are propagated at physiological (rather than ambient) oxygen tensions (~3%), they do not undergo early senescence in vitro (Parrinello et al. 2003). These observations indicated that cell-physiological mechanisms unrelated to telomere length are able to induce senescence in culture.

Telomere Dysfunction and Senescence

Telomere shortening is thought to lead to loss of structural integrity of the telomere nucleoprotein, resulting in activation of the p53 and Rb tumor suppressor pathways and cellular senescence (Shay & Wright 2005). The telomere is a complex nucleoprotein that contains both single- and double-stranded DNA and associated proteins that interact to maintain a stable structure. Electron microscopy has revealed that the telomeric DNA forms a higher-order structure (Griffith et al. 1999), in which the overhanging, single-stranded tail of the G-rich strand inserts itself back into the double-stranded telomeric repeats, thereby yielding a displacement loop that is known as a T-loop (owing to the presence of the telomeric DNA repeat sequence) (**Figure 3**).

TRF2 is a telomere-associated protein that facilitates T-loop formation (Stansel et al. 2001), and overexpression of a dominant-negative form of TRF2 (TRF2ΔBΔM) is thought to result in T-loop loss in vivo. Moreover, introduction of TRF2ΔBΔM results in

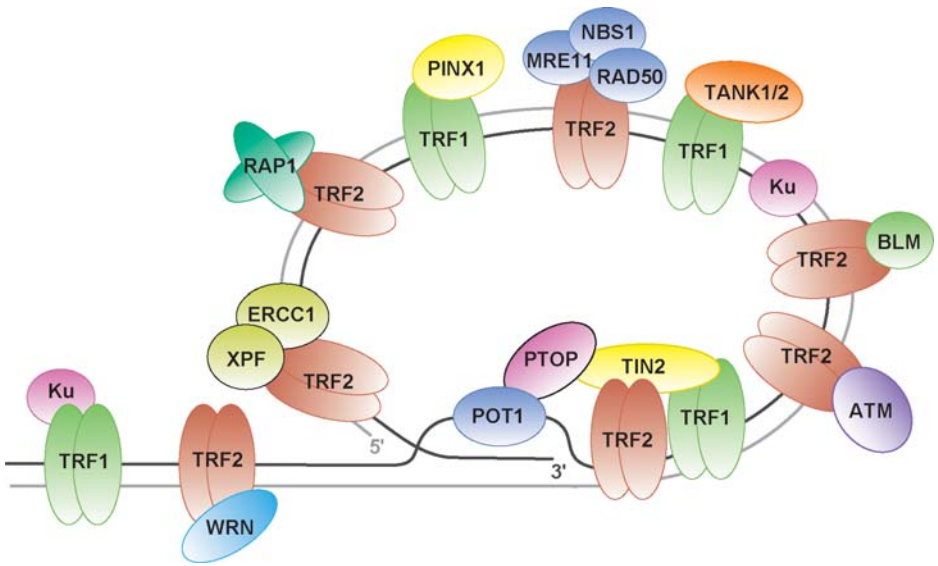

Figure 3

The T-loop schematic. Representation of the T-loop, in which the single-stranded, G-rich overhang inserts into the double-stranded telomeric DNA, creating a displacement loop. The DNA and associated telomeric proteins create a capped, or functional, telomere.

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RNAi: RNA interference

siRNA: small interfering RNA

the rapid induction of senescence in wild-type human fibroblasts (Karlseder et al. 2002). As in replicative senescence, TRF2ΔBΔM-induced senescence activates both the p53 and Rb pathways. Importantly, cells undergoing TRF2ΔBΔM-induced senescence display reduction in the G-rich single-stranded overhang without an associated loss of overall double-stranded telomeric DNA repeats. Because TRF2 is critical to T-loop formation, maintenance of a specific telomere-associated molecular structure—the T-loop—may be critical for continued cellular proliferation and the avoidance of senescence. Moreover, such observations dissociate the signal that triggers entrance into senescence from the overall length of telomeric DNA.

The unique structure of the telomere, described in part above, is thought to distinguish it from the bona fide dsDNA breaks that occasionally affect chromosomal DNA. If so, loss of telomere integrity should erase these differences, causing the degraded telomere to appear much like a dsDNA break and leading to the same DNA damage response that usually follows the formation of dsDNA breaks, including activation of the p53 damage response pathway. Indeed, researchers have reported such a finding, supporting the importance of telomere integrity in the suppression of the DNA damage response.

Overexpression of TRF2ΔBΔM in human cells results in activation of the ATM and ATR kinases (which signals the presence of dsDNA breaks in a cell) as well as the downstream effectors, p53 and p16 (Karlseder et al. 1999, 2002). These observations suggest that uncapped or dysfunctional telomeres can elicit a classic DNA damage response, raising the possibility that a similar mechanism operates during replicative senescence, when loss of telomere integrity results in an inability to protect the chromosomal ends. In agreement with this notion, some have demonstrated that human fibroblasts undergoing replicative senescence exhibit DNA damage foci at their telomeres (d'Adda di Fagagna et al. 2003). These foci, referred to as TIFs (telomere dysfunction–induced foci), contain many of the classic DNA damage response proteins, including γH2A.X, 53BP1, MDC1, NBS1, and phosphorylated SMC1.

TIFs have been found in the majority of cells undergoing replicative or TRF2ΔBΔM-induced senescence (d'Adda di Fagagna et al. 2003, Takai et al. 2003), suggesting that a continuing DNA damage response is responsible for maintaining the senescent phenotype. Indeed, RNA interference (RNAi) and microinjection studies support this contention. For example, delivery of small interfering RNA (siRNA) constructs targeting Chk1/2 or microinjection of nonfunctional, dominant-interfering ATM or ATR kinase proteins resulted in S phase entry (indicating emergence from senescence), in spite of the presence of defective telomeres in a cell (d'Adda di Fagagna et al. 2003, Takai et al. 2003). This demonstrates that the classic DNA damage response mechanism mediates the G1 arrest observed in senescent cells.

Although the data above clearly support a role for the DNA damage response in telomere dysfunction and replicative senescence, many senescing cells within a population do not display TIFs. In these populations, those cells that did not display TIFs instead expressed high levels of the cell cycle inhibitor $p16^{INK4A}$ (Herbig et al. 2004). This observation suggested that senescing populations of cells are biologically heterogeneous: Some cells experience telomere dysfunction, whereas others are affected by a telomere-independent mechanism that induces an arrest phenotype indistinguishable from senescence—the stress-induced senescent state, which is discussed in more detail below.

Stress-Induced Senescence

Recently reported data have highlighted a number of conditions that can lead to stress-induced senescence. These include low serum or growth factor concentrations, exposure to high levels of DNA damage, inappropriate

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conditions of growth (including those that induce the expression of the $p16^{INK4A}$ and $p21^{Waf1}$ cell cycle inhibitors), and high oxidative stress levels (Ramirez et al. 2001, Sherr & DePinho 2000, Wei et al. 2001, Wright & Shay 2002, and references in all). Exposure to these various agents or signals results in a phenotype that is indistinguishable from that shown by cells that have reached the Hayflick limit and enter replicative senescence.

Ectopic expression of the human telomerase catalytic subunit (hTERT)—the cellular enzyme that functions to restore and extend telomeric DNA—bypasses replicative senescence (Bodnar et al. 1998, Vaziri & Benchimol 1998). In contrast, overexpression of hTERT has no detectable effect on stress-induced senescence, causing some to argue that replicative senescence and stress-induced senescence are distinct biological processes. This would imply that stress-induced senescence represents a response to stimuli or conditions that originate outside of the cell (i.e., extrinsic), whereas replicative senescence is exclusively a cell-autonomous mechanism (i.e., intrinsic). More work in this area will be needed to answer this possibility definitively.

Senescence: A Tumor Suppressor Mechanism?

Senescence limits the proliferative potential of a cell and consequently has been argued to function as a potent tumor-suppressing mechanism (reviewed in Campisi et al. 2001). More specifically, senescence may prevent the outgrowth of cell populations at risk of evolving into neoplasias. Several lines of evidence support this notion. For example, as detailed above, damaged telomeres are a potent inducer of senescence; these may arise in cell lineages that have passed through an excessive number of growth-and-division cycles, which may occur during the long, multistep formation of neoplastic cell populations. In addition, as already mentioned above, certain types of DNA damage; inappropriate growth stimuli, including oncogene activation; and perturbations in chromatin structure can all lead to stress-induced senescence (Sherr & DePinho 2000, Wei et al. 2001, Wright & Shay 2002, and references in all).

hTERT: human telomerase catalytic subunit

Molecular proof demonstrating that senescence blocks the growth of neoplastic cells in vivo recently was supplied through study of both murine cancer models and human cancer. Thus, some researchers have utilized the EμN-Ras mouse model to demonstrate that N-Ras overexpression leads to a senescent growth arrest that delays the appearance of tumors (Braig et al. 2005). Loss of wild-type p53 in this model results in the bypass of senescence and rapidly developed invasive T cell lymphomas. This study demonstrated that senescence delayed the appearance of malignant cells (Braig et al. 2005). In another study, loss of the tumor suppressor PTEN in the prostate resulted in a p53-mediated senescent growth arrest that, once again, blocked further tumor progression (Chen et al. 2005).

Further support of the role of senescence in tumor prevention comes from the findings of other investigators who have demonstrated that human nevi display a senescent phenotype (Michaloglou et al. 2005). Nevi are benign growths of melanocyte origin and often contain mutations in the BRAF oncogene, which is a protein kinase that functions downstream of Ras. Interestingly, overexpression of oncogenic BRAF V600E in melanocytes leads to senescence, suggesting that the expression of this oncogene results in senescence in vivo and inhibits transformation. Yet other researchers have utilized the conditional K-Ras V12 transgenic mouse to demonstrate that nontumorigenic lung adenomas are senescent, displaying the classic markers of this state, including senescence-associated β-galactosidase (SA-βgal) and high levels of $p16^{INK4A}$ expression (Collado et al. 2005). In this model, progression to adenocarcinoma resulted in loss of all the senescence markers, suggesting that progression required premalignant cells to bypass senescence. Taken together, these studies strongly

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support a role for senescence in blocking the formation of tumors.

Crisis: cellular death characterized by end-to-end chromosomal fusions

ALT: alternative lengthening of telomeres

Telomerase: cellular, RNA-dependent DNA polymerase that adds telomeric repeats to the ends of chromosomes

Crisis

Cell populations that succeed in bypassing senescence through the inactivation of the Rb and p53 signaling pathways continue to divide until their telomeres become critically short (**Figure 2**) and no longer protect the chromosome ends from the cell machinery charged with the detection and repair of ds-DNA breaks. When this occurs, the population of cells enters a second proliferative block referred to as crisis, which is characterized by short telomeres, end-to-end chromosomal fusions, anaphase bridges, and cell death by apoptosis (Shay & Wright 2005, Wright & Shay 1992). This second arrest state is distinct from senescence in two fundamental ways: (*a*) Rampant genomic instability is observed, which is associated with (*b*) widespread cell death.

On occasion, however, a rare clone of cells (1 in 10^7 human cells) can emerge from a population of cells in crisis; such cell clones are invariably immortal. Analyses of telomeric DNA in such variant clones indicate that telomere lengths are maintained in the face of ongoing rounds of DNA replication and cellular division (Wright & Shay 1992). Moreover, unlike populations of precrisis cells, the great majority of these immortalized cell populations demonstrate expression of the telomerase reverse transcriptase (hTERT) enzyme, which was mentioned in passing earlier. Those cell clones that do not activate hTERT expression resort instead to activating a poorly understood telomere maintenance mechanism termed alternative lengthening of telomeres (ALT), which, at the molecular level, may in fact represent several distinct mechanisms (discussed below) (**Figure 2**). Taken together, these observations provide strong support for the hypotheses that escape from crisis and entrance into an immortal growth state depends on acquisition of telomere maintenance functions and that this can be achieved by activation of either telomerase function or an ALT mechanism.

The causal role of telomerase in cellular immortalization was demonstrated formally following the cloning of the gene encoding hTERT (Meyerson et al. 1997, Nakamura et al. 1997). Telomerase is a cellular reverse transcriptase that utilizes an associated RNA molecule as a template to add telomeric repeats to the ends of telomeres, thereby extending them. By studying structure-function relationships in the distantly related HIV-1 reverse transcriptase, two research groups were able to create a dominant-negative allele of hTERT (DN-hTERT) and demonstrate its essential role in both telomere maintenance and cellular immortality (Hahn et al. 1999b, Zhang et al. 1999). Thus, inhibition of hTERT activity following DN-hTERT expression in already immortalized cells resulted in the progressive loss of telomeric repeats during successive rounds of DNA replication and cellular division. Eventually telomeres became critically shortened, and as observed in crisis, end-to-end chromosomal fusions became evident and were followed by widespread cell death. Strikingly, the preexisting length of telomeres at the beginning of each of these experiments primarily dictated the time required for cell populations to enter crisis. For example, when the DN-hTERT allele was introduced into cells that initially possessed telomeres in the range of 2–3 kb, these cells entered crisis following fewer cell divisions than did cells possessing initial telomere lengths of 6–8 kb (**Figure 4**).

The critical role of telomere maintenance in cellular immortality was further supported by the introduction of wild-type hTERT into precrisis fibroblasts, endothelial cells, and retinal pigment epithelial cells (Bodnar et al. 1998, Rufer et al. 1998, Vaziri & Benchimol 1998, Yang et al. 1999). Introduction of hTERT resulted in telomere stabilization and cellular immortality. Indeed, resulting telomerase-expressing fibroblasts have been

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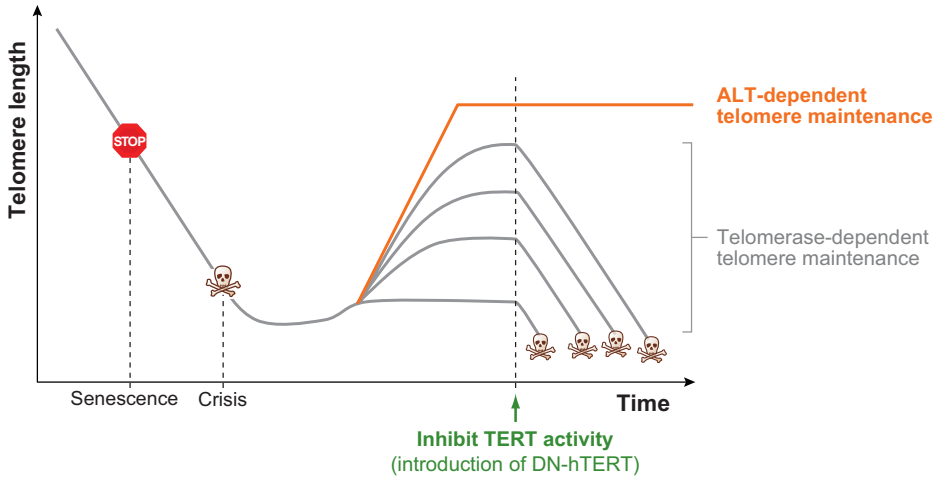

Figure 4

Telomere length dictates entrance into crisis. Immortal cells that have bypassed senescence and crisis maintain stable telomeres. Cell clones can possess different telomere lengths, as the graph shows. Introduction of a dominant-negative hTERT allele (DN-hTERT) into immortal telomerase-positive cells (see text for details) inhibits telomerase activity, resulting in a growth arrest reminiscent of crisis, which is characterized by telomere loss, end-to-end chromosomal fusions, and apoptotic cell death. Initial telomere lengths in an individual cell clone dictate the timing of the entrance into crisis, following inactivation of telomerase activity. In other words, cells that maintain longer telomeres require more rounds of DNA replication and cellular division before telomeres reach a critically short length that results in crisis. Cells that maintain their telomeres through the ALT mechanism are unaffected by introduction of the DN-hTERT allele.

maintained in culture for years, further supporting the idea of telomere maintenance as a prerequisite for cellular immortality. Importantly, cells immortalized through hTERT expression appear normal and are responsive to growth stimuli and growth inhibitors, indicating that whereas hTERT can immortalize cells, it does not, on its own, cause cell transformation (Hahn et al. 1999a, Jiang et al. 1999, Morales et al. 1999).

Fibroblasts immortalized by ectopic expression of hTERT display gene expression profiles that are very similar to that of their normal early-passage counterparts. This observation soon led to the notion that hTERT immortalization may allow experimenters to immortalize human cells while maintaining their normal karyotype and gene expression profiles. Such cell lines should, in principle, allow one to study normal cellular processes in the absence of the genomic instability inherent in the previous models of cell immortalization that utilized viral oncogenes such as SV40 LT to effect immortalization. However, this initial excitement regarding the possibilities of hTERT-mediated cell immortalization faded after it was shown that many epithelial cell types did not become immortalized in response to hTERT (Rheinwald et al. 2002) and that, after extended in vitro culture, hTERT-immortalized cells do indeed display changes in their gene expression profiles (Choi et al. 2001; S. Stewart, unpublished data). In fact, it is difficult to know whether these changes are due to telomerase expression or to the long-term growth of cells in tissue culture dishes in the presence of bovine serum. Despite these potential pitfalls, many groups have utilized hTERT-mediated immortalization to study the normal biological functions of cells showing only minimal genomic instability in vitro.

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CELL SENESCENCE AND HUMAN AGING

Two major hypotheses have attempted to explain the molecular basis of cellular and thus organismic aging (reviewed in Aviv 2004, Ben-Porath & Weinberg 2004, and Vijg & Suh 2005). The first suggests that aging is the result of the slow accumulation of damage that leads to cellular and eventually tissue deterioration. The second suggests that aging is the result of a cellular program that is governed by a biological clock, such as telomere length. Examination of human cells propagated in culture has indeed shed light on the cellular bases of aging.

As described above, when grown in culture, normal human cells will undergo a limited number of divisions before entering a state of replicative senescence in which they remain viable but are unable to divide further (reviewed in Campisi 1996). Some have proposed that this limited replicative capacity contributes to the phenotypes associated with aging, such as reduced wound healing and weakened immune systems (Effros 2000, Paradis et al. 2001, Rubin 2002). Similarly, diseases characterized by high cellular turnover, such as acquired immunodeficiency disease (AIDS) and cirrhosis, are thought to be at least partially the result of cellular mortality (Effros 2000, Paradis et al. 2001, Rubin 2002), i.e., the limited ability of tissues to regenerate themselves through cell proliferation.

The role of telomere homeostasis and cell senescence in human aging has been supported by studies demonstrating a relationship between donor age and telomere lengths, correlations between in vitro growth capacity and donor age, and reduced in vitro growth capacity of cells isolated from patients suffering from various types of progeria (conditions characterized by premature aging) when compared with normal, age-matched control cells (Dimri et al. 1995, Faragher et al. 1993, Martin et al. 1970). Although these observations are intriguing, their correlative nature makes it difficult to conclude that telomere loss is the central driving force behind human cell and thus tissue aging.

Arguably, some of the most persuasive data on this point come from patients suffering from dyskeratosis congenita (Dokal 2001, Mason et al. 2005). There are two forms of this disease (Dokal 2001; Vulliamy et al. 2001a,b). The first form is autosomal recessive and results from mutations in the human *TERC* gene (which encodes the RNA subunit of the telomerase holoenzyme). The second is an X-linked autosomal dominant form of the disease and is the result of a mutation in the dyskerin gene, which compromises ribosome biosynthesis and seems also to affect assembly of the telomerase holoenzyme, resulting in loss of enzyme function.

Analyses of cells from dyskeratosis congenita patients reveal telomere shortening and dysfunction when compared with telomeres in the cells of age-matched controls. Patients suffering from this disease manifest several distinct abnormalities, including abnormal skin pigmentation, nail dystrophy, mucosal leukoplakia, bone marrow failure, and cancer predisposition (Dokal 2001). Interestingly, this disease also appears to demonstrate anticipation (symptoms increasing in severity with each succeeding organismic generation), although more patient families must be analyzed to confirm this (Vulliamy et al. 2004). Many of these symptoms are reminiscent of aged humans and, as discussed below, are also observed in mice lacking telomerase function. Nevertheless, the fact that aspects of human aging can be phenocopied by specific defects in the telomere-maintenance machinery does not prove that telomere erosion is normally a primary causal force that drives human aging.

TELOMERASE AND CANCER

The development of neoplasia is a multistep process that involves accumulation of a number of genetic and epigenetic alterations that collaborate to produce transformed cells.

In addition, studies of human tumor cell genomes reveal an ever-increasing number of genetic abnormalities that can contribute to tumorigenesis, underscoring the complexity of this process. Despite this complexity, early studies in murine and avian models suggested that cancer may be the result of only a few mutations, raising the possibility that many of the observed mutations in human cancer cells were not responsible for the transformed phenotype (Hanahan & Weinberg 2000, Land et al. 1983). For example, in the 1980s, researchers demonstrated that the introduction of two cooperating oncogenes was sufficient to transform murine cells (Land et al. 1983, Ruley & Fried 1983). Subsequent studies in transgenic mice corroborated these original observations. Despite such successes in transforming rodent cells, similar experiments consistently failed to transform human cells to a tumorigenic state, calling into question the relevance of animal models to human carcinogenesis.

In fact, epidemiologic studies in human systems have suggested that four to six rate-limiting events are required for cancer formation (Armitage & Doll 1954, 2004; Ruley & Fried 1983). These original estimates were eventually echoed by experiments that followed the cloning of the *hTERT* gene, which encodes the catalytic subunit of the telomerase holoenzyme. Because hTERT is expressed at significant levels in 90% of human tumors and because normal cells express extremely low levels of this enzyme, it seemed reasonable that telomerase activity would be required to transform a human cell. Indeed, Hahn et al. (1999a, 2002) demonstrated that a normal human cell can be converted to a fully tumorigenic cell with a set of introduced, defined genetic elements, including a clone of the *hTERT* gene. In particular, inactivation of the p53 and Rb tumor suppressor pathways through expression of the SV40 LT, abrogation of a PP2A (protein phosphatase 2A) function through expression of SV40 small t antigen (ST), mitogenic stimulation through oncogenic H-Ras expression, and cellular immortalization achieved through hTERT expression together produced a transformed human cell capable of colony formation in soft agar and tumor formation in immunocompromised host mice (Hahn et al. 1999a, 2002) (**Figure 5**).

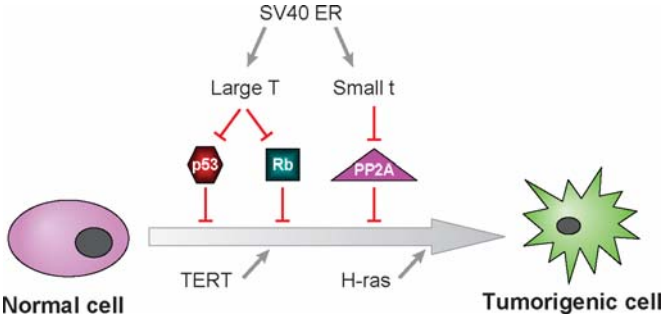

Figure 5

In vitro transformation of human cells. Normal human cells can be transformed with a set of introduced, defined genetic elements. Specifically, introduction of clones specifying the SV40 Large T and small t antigens (which functionally inactivate p53, Rb, and PP2A), oncogenic H-Ras, and the hTERT catalytic component of the telomerase holoenzyme results in anchorage-independent growth and tumor formation in immunocompromised mice.

The study by Hahn et al. (1999a) underscored the requirement for cellular immortalization in the transformation process. In addition, a subsequent study demonstrated that aneuploidy was not a prerequisite for the tumorigenic cell phenotype (Zimonjic et al. 2001). Since this original report by Hahn et al. (1999a), numerous groups have applied this same transformation strategy and found that, indeed, these five changes suffice to transform a variety of human cell types. Current efforts are now focused on "humanizing" the mutations, i.e., utilizing mutant alleles that are commonly found in human tumors. For example, in humans, SV40 LT is not associated with tumorigenesis, whereas p53 mutations and loss of $p16^{INK4A}$ are commonly observed. Moreover, by introducing genetic mutations that are documented to be present in human tumors, researchers are now building cellular models that may be useful in drug discovery and development.

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Telomeres and Cancer: Lessons from the Mouse

As mentioned above, normal human somatic cells express low to undetectable levels of telomerase activity that are insufficient to maintain telomere lengths, whereas more than 90% of human tumor cell populations that have been assayed express high levels of telomerase activity and maintain telomere lengths (Hahn 2005, Shay & Bacchetti 1997). Because tumor cells typically display telomere lengths that are shorter than neighboring, normal cells, telomerase activation is thought to be a relatively late event in the transformation process that, once achieved, may stabilize existing telomere lengths but fails to restore telomeres to the lengths seen in the nearby normal cells (reviewed in Maser & DePinho 2002).

Telomere loss and the ensuing genomic instability are believed to be driving forces in the transformation process. This notion has received substantial support from the studies, noted above, that utilize telomerase knockout mice, which display progressive telomere shortening, similar to the behavior of telomeres in normal human cells (Blasco et al. 1997). As mentioned above, these animals do not exhibit any apparent pathological phenotypes in the early generations, presumably because their cells still possess large telomere reserves. However, late-generation mice do display pathologies associated with aging and demonstrate an enhanced transformation phenotype. Significantly, when mated to cancer-prone mice, the telomerase-negative animals recapitulate some of the phenotypes observed in human neoplasias (Maser & DePinho 2002).

Late-generation *mTERC*$^{-/-}$ mice develop lymphomas and teratocarcinomas with high frequency, and these are associated with extensive anaphase bridge formation and genetic instability (reviewed in Maser & DePinho 2002). Such anaphase bridges are expected to form following loss of telomeres and resulting end-to-end fusions of chromatids. When the *mTERC*$^{-/-}$ mice are mated to *Ink4a*$^{-/-}$ mice, the spectrum of tumor types is altered slightly, and mice develop lymphomas and fibrosarcomas (Greenberg et al. 1999). Mice deficient in both mTERC and p53 develop an even more varied tumor spectrum, displaying lymphomas, sarcomas, and adenocarcinomas (Artandi et al. 2000). Importantly, tumor formation in late-generation *mTERC*$^{-/-}$*Ink4a*$^{-/-}$ and *mTERC*$^{-/-}$*p53*$^{-/-}$ mice is suppressed, suggesting that telomere reserves decline to a level that is incompatible with further cellular proliferation.

Of greater interest is the tumor spectrum observed in *mTERC*$^{-/-}$*p53*$^{+/-}$ mice. These mice still develop lymphomas and sarcomas, but they also develop epithelial carcinomas that are similar to the majority of human tumors (Artandi et al. 2000). Again, tumor development is reduced in late-generation mice, but the altered tumor spectrum suggests that tumors in these animals more closely resemble those found in humans. The breakage-fusion-bridge cycles occurring in these mice following telomere collapse presumably contribute to generalized instability in their cells, which in turn favors the inception of the observed tumors.

EXTRATELOMERIC FUNCTIONS OF TELOMERASE

The ability of the telomerase enzyme to reverse telomere attrition, impart cellular immortality, and contribute to tumorigenesis indicates that this enzyme's effects derive from its ability to add telomeric repeats to the ends of the chromosomal DNA and thereby stabilize telomere lengths and the structural integrity of entire chromosomes. Although this model has been borne out by numerous studies, it has also become clear that the role of telomerase in cellular biology is more complex then simply one of telomere length maintenance.

Accumulating evidence suggests that telomerase also influences normal cellular

physiology, even in cells that have long telomeres. For example, studies have demonstrated that mice lacking functional telomerase are unable to maintain proper tissue homeostasis, particularly in tissues of high cell turnover, such as the bone marrow, skin, liver, and gastrointestinal tract (Herrera et al. 1999, Lee et al. 1998, Rudolph et al. 1999). These phenotypes may be exacerbated in aged mice or following an infectious challenge, even in animals that do not demonstrate critically short telomeres (Rudolph et al. 2000).

Other studies have demonstrated that ectopic expression of telomerase in cells that are already telomerase positive results in increased resistance to apoptosis (Holt et al. 1999). Another study has demonstrated that ectopic expression of wild-type hTERT allows nontumorigenic ALT cells (cells that possess long telomeres but do not express endogenous hTERT) to form tumors (Stewart et al. 2002). Furthermore, use of an hTERT mutant that is unable to extend telomere lengths recapitulated this phenotype, leading to the argument that the tumorigenic phenotype in this model was not the result of telomere elongation (Stewart et al. 2002). In agreement with this study, mice with relatively long telomeres displayed an enhanced tumorigenesis rate once an mTERT transgene was expressed in certain tissues (Artandi et al. 2002). Once again, it is difficult to understand how increased telomerase expression could enhance tumorigenesis simply by increasing the lengths of already long telomeres.

More recently, telomerase expression has been found to influence hair growth in a transgenic mouse model (Sarin et al. 2005). Ectopic expression of mTERT in the skin epithelium caused a rapid transition from the telogen phase (resting phase of the hair follicle cycle) to the anagen phase (active growth phase). This transition resulted in robust hair growth. Examination of the stem cells localized to the bulge region of the hair follicle revealed that this transition was due to increased proliferation of the normally quiescent, multipotent stem cells. Importantly, stem cell proliferation did not depend on the concomitant presence of *mTERC* (the RNA component of the telomerase enzyme), indicating that this proliferation did not depend on telomere elongation by the ectopically expressed telomerase enzyme.

Recently reported data also suggest that telomerase may play an important role in maintaining chromosomal structure in domains other than telomeres (Masutomi et al. 2003). Thus, normal human fibroblasts transiently express low but detectable levels of telomerase during each S phase. In spite of this expression, telomeres shorten during each round of DNA replication and cell division, indicating that this transient telomerase expression does not suffice to prevent telomere erosion. This raised the question of the precise function of telomerase expression in these cells.

RNAi-directed knockdown of hTERT in normal, presenescent fibroblasts led to loss of the single-stranded overhang and to an accelerated entrance into senescence, suggesting that telomerase expression is required to rebuild the overhang and allow proper telomere assembly and function, even when telomeres are quite long. Further work is needed to demonstrate conclusively that this is indeed the role of telomerase expression in these cells, and it is not clear whether this proposed function would explain the apparent telomere-independent roles of telomerase in normal cells. In any event, it is already apparent that telomerase plays a key role in normal cell physiology that is independent of its effects on overall telomere length.

A recent study has added further complexity by suggesting that hTERT plays a key role in DNA repair (Masutomi et al. 2005). RNAi-directed knockdown of hTERT in normal human cells led to abrogation of the usual DNA damage response, following radiation, in the absence of apparent effects on the telomeres. Analysis of cells following radiation treatment revealed that they did not form the DNA damage foci that are usually seen following irradiation. Accordingly,

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APB: ALT-associated PML body

PML: promyelocytic leukemia

normal cells devoid of hTERT were radiosensitive and demonstrated chromatin fragmentation in response to irradiation. The precise role that hTERT plays in the repair of radiation-induced dsDNA breaks remains to be clarified. Nonetheless, it is clear once again that this enzyme influences cellular processes that are unrelated to maintenance of telomere length.

TELOMERE MAINTENANCE IN THE ABSENCE OF TELOMERASE

As discussed above, more than 90% of all human tumors utilize telomerase for telomere maintenance, whereas the telomerase-independent telomere maintenance (ALT) mechanism mentioned above is observed in only 7–10% of tumors (Bryan et al. 1997, Shay & Bacchetti 1997). When tumors are analyzed by tissue type, the ALT mechanism appears to be more prevalent in tumors arising from mesenchymal tissues and the central nervous system, raising the possibility that there are intrinsic differences in cells isolated from various tissue lineages that dictate whether telomerase or ALT is activated as a means of escaping crisis during multistep tumor progression. Uncovering these putative differences will be an important advance in our understanding of the cellular immortalization process.

Several markers have been associated with the ALT phenotype. In particular, individual telomeres in ALT cells are highly heterogeneous in length and are subject to rapid decreases and increases in this length. Indeed, some chromosomes that completely lack telomeric repeats can be detected in a population of ALT cells (Londono-Vallejo et al. 2004). Some have proposed that the rapid changes in telomere lengths are due to the ALT mechanism itself.

In yeast, a mechanism similar to the ALT mechanism in mammalian cells becomes activated in response to the absence of telomerase, and in these cells, telomerase-independent telomere maintenance depends on homologous recombination (Lundblad & Blackburn 1993). In fact, recently reported data do support homologous recombination as a key mechanism in mammalian ALT cells (see below) (Londono-Vallejo et al. 2004, Varley et al. 2002). In addition to highly variable telomere lengths, mammalian ALT cells also contain promyelocytic leukemia bodies (APBs), which are not observed in telomerase-positive cells (Yeager et al. 1999). APBs contain the promyelocytic leukemia (PML) protein; telomeric DNA; telomere-binding proteins, including TRF1 and -2; and numerous proteins involved in genetic recombination (Yeager et al. 1999).

One study that has suggested that ALT cells may utilize a recombination-based mechanism demonstrated that a unique DNA sequence inserted into the subtelomeric region of one chromosome in an ALT cell line was rapidly transmitted to other telomeres (Dunham et al. 2000). This suggested that ALT occurs either through interchromosomal homologous recombination or through a copy-choice mechanism of template switching during DNA replication. From this observation, one may speculate that ALT cells exhibit a higher incidence of generalized homologous recombination than telomerase-positive cells. One study addressed this possibility directly and found no evidence that supported such a contention (Bechter et al. 2003). This may indicate that the elevated recombination rates in ALT cells are limited to the telomeres and do not represent a global change in homologous recombination. Although it is not clear how changes in telomere-specific recombination may occur, it is attractive to speculate that a telomere-binding protein, such as TRF2, may be altered in ALT cells, resulting in telomere-specific recombination events.

Three additional studies have supported the hypothesis that ALT is recombination based. The first demonstrated that Sp100 can influence the presence of APBs and telomere maintenance (Jiang et al. 2005). Sp100 is a component of PML nuclear bodies.

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Overexpression of this protein sequesters MRE11, Rad50, and NBS1 away from APBs, resulting in progressive telomere shortening. Importantly, ectopic expression of Sp100 also suppressed the rapid telomere-length fluctuations that are characteristic of the ALT phenotype, suggesting that the ALT mechanism was suppressed in these cells. The second study demonstrated that expression of a TRF2 mutant (TRF2$^{\Delta B}$) protein resulted in massive telomeric deletions that were dependent on the activities of XRCC3, a protein that is involved in Holliday junction resolution (Y. Liu et al. 2004). Interestingly, the telomeric deletions corresponded to the appearance of T-loop-sized telomeric circles, suggesting that homologous recombination contributed to the telomeric loss associated with TRF2$^{\Delta B}$ expression. Electron microscopic analysis of ALT cells revealed the presence of telomeric circles (Cesare & Griffith 2004, Tokutake et al. 1998) like those observed following TRF2$^{\Delta B}$ expression, suggesting that a similar mechanism was active and responsible for telomere maintenance.

The third and most recent study regarding the recombination-based mechanisms of ALT has suggested that chromatin modulation may be a key determinant of telomerase activation or lack thereof. The chromatin environments surrounding the *hTERT* and *hTERC* promoters were compared among telomerase-positive human tumor cell lines, normal human cells, and human cells utilizing ALT (Atkinson et al. 2005). In ALT cells, histone H3 and H4 were hypoacetylated, and H3, lysine 9 and H4, lysine 20 were methylated. In contrast, hTERT expression was associated with hyperacetylation of H3 and H4 and methylation of H3, lysine 4. Treatment of normal cells and ALT cells with 5-azadeoxycytidine and trichostatin A resulted in chromatin remodeling and activation of hTERC and hTERT expression, supporting a role for the chromatin environment in governing telomerase expression. This study suggests that normal cells suppress telomerase activity through chromatin modification, which needs to be reversed for cells to activate telomerase during tumorigenesis. This raises the question of why ALT cells do not simply remodel the chromatin surrounding these promoters and thereby activate telomerase function. Further studies regarding control of chromatin modulation may help explain whether certain cell types are more adept at remodeling their chromatin and may thereby reveal the controls governing activation of ALT versus telomerase.

hTERC: human telomerase RNA subunit

TELOMERE-BINDING PROTEINS

Over the past ten years, a substantial list of telomere-specific proteins has been assembled, and it is now appreciated that these proteins, together with the telomeric DNA, form a functional, or capped, telomere. In fact, as Blackburn (2000) points out, the telomeric proteins and DNA operate in a mutually reinforcing fashion to maintain a properly folded and functional structure. This section details the known functions of the core telomeric proteins, collectively referred to as either the Shelterin complex (de Lange 2005) or the telosome (D. Liu et al. 2004a), as well as other proteins that are important to telomere function but are known to play other roles in DNA maintenance distinct from repairing or maintaining telomeric DNA.

Telomeric Core Proteins

The first mammalian telomere-binding protein, TRF1 (telomere repeat–binding factor), was initially described in 1992 (Zhong et al. 1992). This protein was identified on the basis of its binding specificity for the vertebrate telomeric hexanucleotide repeats, TTAGGG. A second protein, TRF2, was later identified as a TRF1 paralog (Bilaud et al. 1997, Broccoli et al. 1997). Like TRF1, TRF2 displays high sequence specificity for telomeric repeats and contains a Myb DNA-binding domain. Later, yeast two-hybrid experiments demonstrated that the Tin2 and Rap1

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MRN: complex consisting of MRE11, Rad50, and NBS1

proteins also interact with TRF2 (Kim et al. 1999, Li et al. 2000). More recently, several groups have used a variety of approaches to identify TPP1 (also referred to as TINT1, PTOP, and PIP1) as a Tin2- or Pot1-interacting protein. (Houghtaling et al. 2004, D. Liu et al. 2004b, Ye et al. 2004). Finally, Pot1, the telomeric single-strand-binding protein, was identified by sequence homology to telomere-binding proteins in unicellular organisms (Baumann & Cech 2001) and appears to be evolutionarily the most well-conserved protein of the group. Below, we highlight only some of the important functions of these proteins. The literature on telomere-binding proteins has grown rapidly in recent years, and a single review could easily be dedicated to them.

Telomeres and DNA Repair/Replication Proteins: Antagonistic or Synergistic Relationships?

In addition to the known core telomere-binding proteins, DNA repair proteins are also found at the telomeres. A simplistic view might suggest that the presence of DNA repair enzymes would compromise the telomere's task of concealing the chromosome ends from the DNA repair machinery. The available evidence, on the contrary, suggests that the presence of these repair proteins is vital to telomere maintenance. These proteins include the Ku complex, the MRN complex (including MRE11, Rad50 and NBS1), XPF/ERCC1, ATM, BLM/WRN, Rad51D, and Rad54 (reviewed in de Lange 2005). The precise function of each of these proteins at the telomere is only now being uncovered, and far more work is required. We briefly discuss some of the known functions below.

The Ku complex is central to the nonhomologous end-joining machinery and binds to the telomere through an interaction with TRF2 (Song et al. 2000). Loss of Ku activity in humans leads to immunodeficiency and cancer predisposition (Doherty & Jackson 2001). Deletion of the Ku homologs, Ku70 or Ku86, in the mouse results in premature aging, aberrant telomere lengths, and loss of telomere capping (Espejel & Blasco 2002, Espejel et al. 2002, Goytisolo et al. 2001, Jaco et al. 2004, Samper et al. 2000). Rad54 is involved in homologous recombination, and its absence in murine cells results in short telomeres and loss of capping function (Jaco et al. 2003). Rad51D loss yields a similar outcome (Tarsounas et al. 2004).

Loss of XPF/ERCC1 results in the cancer predisposition syndrome termed xeroderma pigmentosum (Boulikas 1996, Lehmann 2003, Wood 1999). A biochemical screen has demonstrated that this nucleotide excision repair complex associates with the telomere through an interaction with the telomere-binding protein TRF2 (Zhu et al. 2003). Importantly, it was suggested that XPF/ERCC1 is able to modulate the 3′ single-stranded telomeric overhang during telomere uncapping. As described above, introduction of the dominant-negative allele of TRF2 (TRF2DN) results in rapid telomere uncapping and, in the absence of functional p53 and Rb, leads to end-to-end chromosomal fusions (Karlseder et al. 1999). These fusions, as mentioned above, are the result of nonhomologous end joining; however, prior to end-to-end chromosomal fusions, the telomere single-stranded overhang must be processed, a task carried out by XPF/ERCC1. When the TRF2DN allele was introduced into cells from mice deficient in ERCC1, the cells displayed fewer chromosomal fusions, supporting XPF/ERRC1's role in telomere processing (Zhu et al. 2003). Still, this leaves unanswered the question of XPF/ERCC1's normal function at the telomere. One possibility is that XPF/ERCC1 is required to process the telomere overhang to aid in disassembling the T-loop, thereby providing access to the replication machinery during the S phase of the cell cycle.

WRN and BLM are ReqQ helicases (Nakura et al. 2000). As noted in the supplemental material (Supplemental Text and

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Supplemental Figure 1; follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org**), mutations of their encoding genes result in progeria and cancer predisposition (Dyer & Sinclair 1998). Interestingly, loss of these genes in the mouse results in premature aging phenotypes only when mice lacking these genes are bred with *TERC* knockout animals (Chang et al. 2004, Lombard et al. 2000). In other words, the phenotype is only seen when telomere lengths are limiting, a situation that is thought to recapitulate the human condition of progeria. BLM and WRN are structure-specific helicases that resolve complex structures, such as Holliday junctions, which are reminiscent of the T-loops located at the ends of the telomere. Both proteins are recruited to the telomere through an interaction with TRF2 (Opresko et al. 2002, Stavropoulos et al. 2002).

BLM's role at the telomere remains obscure. More is known about WRN: A recent report indicates that it is indispensable for telomere replication, playing a central role in lagging-strand synthesis at the telomere (Crabbe et al. 2004). When WRN activity was compromised, cells displayed large sister telomere losses and subsequent chromosomal fusions. Furthermore, cell cycle analysis demonstrated that WRN specifically localized to the telomere only during S phase of the cell cycle, supporting its role in telomere replication. This observation raises the possibility that WRN activity is required to resolve complex structures, such as the G-quadruplexes hypothesized by some investigators to form at telomeric sequences. If this were indeed WRN's role, then loss of WRN activity, if not properly resolved, might result in stalled replication forks that would lead to telomere loss and fusions.

Cells from WRN patients do display sister chromatid telomere losses that are rescued by telomerase expression (Crabbe et al. 2004). How might telomerase rescue such a defect? Teixeira et al. (2004) reported that telomerase shows a preference for shorter telomeres; these authors suggest that in WRN cells telomerase may protect chromosomes undergoing sister telomere loss by adding telomeric repeats back to them before they can be processed by the DNA repair machinery. Confirmation of this model requires additional work.

ATM is a PI3 kinase homolog that plays a central role in DNA damage sensing and repair (Khanna & Jackson 2001). Indeed, ATM activity is required to initiate many DNA repair processes and to activate p53 function in response to DNA damage—a role mentioned earlier in this review. Loss of ATM in the human results in ataxia telangiectasia, whereas loss in the mouse results in radiosensitivity (Shiloh & Kastan 2001). When bred to *mTERC* knockout mice, mice showing ATM loss have premature aging phenotypes, again highlighting a requirement for adequate telomere reserves to forestall these processes (Wong et al. 2003). ATM interacts with the telomere through the TRF2 protein, and early work suggested that TRF2 suppressed ATM function at the telomere (Karlseder et al. 2004). However, ATM may have an alternative role at the telomere: In one study, ATM activity was required during the G2 phase of the cell cycle (Verdun et al. 2005). During this time, ATM activated a DNA damage response mechanism at the telomere, leading to recruitment of MRE11 and NBS1. Degradation of MRE11 or NBS1 or loss of ATM function resulted in telomere dysfunction, indicating that this recruitment was critical to normal telomere maintenance. This led, furthermore, to the proposal that a localized DNA damage response is required to process the telomere end and allow it to fold properly, resulting in a capped, functional configuration. This observation appears to contradict the widely held notion that the telomere operates to disguise or protect the telomere from the DNA damage response and thereby highlights the complex nature of telomere maintenance that we are only now beginning to understand. The coming years are likely to supply us with a more detailed understanding of how the DNA repair enzymes work to maintain telomere

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homeostasis and how these functions become compromised in tumorigenesis and aging.

EPIGENETIC MODULATION OF TELOMERE LENGTH

Telomere homeostasis is the result of a delicate balance of sufficient double-stranded and single-stranded telomeric DNA and the correct complement of telomere-binding proteins. More recent work has also suggested that modulation of the histones associated with telomeric DNA can govern telomere maintenance as well. Like many regions of the bulk genomic DNA, telomeric DNA is associated with arrays of nucleosomes (Tommerup et al. 1994) that are characteristic of heterochromatin. In many respects, the telomeric heterochromatin is similar to heterochromatin found around pericentric regions and, as such, demonstrates an enriched binding of the heterochromatin protein 1 (HP1) isoforms HP1α, HP1β, and HP1γ (Garcia-Cao et al. 2004, Peters et al. 2001, Schotta et al. 2004). In addition, telomeric DNA possesses high levels of histone 3, lysine 9 and histone 4, lysine 20 trimethylation. These chromatin modifications appear to be directed by members of the Rb family of proteins, which are able to bind and direct the actions of certain chromatin-modifying enzymes: suppressor of variegation 3–9 homolog (SUV39H) and suppressor of variegation 4–20 homolog (SUV4–20H). Both proteins are histone methyltransferases. Modulation of telomeric chromatin leads to alteration in telomere length regulation (Garcia-Cao et al. 2004).

TELOMERE-BINDING PROTEINS: BEYOND THE TELOMERE

As illustrated by the above examples, the early view that the telomere disguised or protected the ends of the chromosomes from DNA repair enzymes addresses only part of its function. Thus, the DNA repair proteins and pathways are integral to normal telomere homeostasis. It therefore should not be surprising that proteins previously thought to function exclusively at the telomeres exert functions that are unrelated to the maintenance of normal telomere function.

For example, as mentioned in the supplemental text, tankyrase inhibits TRF1 binding to telomeric DNA and promotes TRF1 degradation, resulting in telomere elongation (Chang et al. 2003, Smith & de Lange 2000, Smith et al. 1998). A recent report showed that tankyrase also plays a key role in sister chromosome resolution (Dynek & Smith 2004). Thus, RNAi-directed loss of tankyrase expression resulted in cell cycle arrest and loss of sister chromosome resolution. And analysis of the arrested cells demonstrated that the sister chromosomes lined up on the metaphase plate but were unable to segregate because they remained attached via their telomeres.

TRF2 has been found to be localized to dsDNA breaks immediately following their formation. However, unlike proteins found in classic DNA damage foci, such as γH2A.X, localization of TRF2 was lost in a matter of minutes (Bradshaw et al. 2005). A subsequent study demonstrated that the transient association of TRF2 with sites of DNA damage requires ATM activity (Tanaka et al. 2005). ATM phosphorylated TRF2 at an ATM consensus site in response to DNA damage, and inhibition of ATM activity resulted in loss of TRF2 phosphorylation and DNA damage localization following irradiation. Interestingly, the TRF2 found at sites of DNA damage did not bind DNA, suggesting that TRF2 was tethered indirectly to the DNA, ostensibly through a protein-protein interaction. The precise role of TRF2 in DNA damage response/repair remains to be determined. Nevertheless, this observation highlights yet another link between telomere-binding proteins, telomere homeostasis, and DNA damage response and repair. These observations suggest that we need to reevaluate the relationship between DNA repair/replication proteins and those responsible for telomere maintenance and that a number of these

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proteins likely play important roles in both normal cellular processes.

In addition to its apparent role in the DNA damage response, TRF2 may be involved in tumorigenesis. Thus, in a transgenic murine model, overexpression of TRF2 in the skin resulted in a severe phenotype in response to light (Munoz et al. 2005). These animals displayed hyperpigmentation, premature skin deterioration, and a condition that resembled xeroderma pigmentosum. The telomeres had marked telomere shortening and displayed single-stranded telomeric DNA overhang loss, which may reflect the ability of TRF2 to bind the XPF protein. This observation is in contrast to what was observed when TRF2 was overexpressed in human cells: increased telomere shortening with concomitant protection of the single-stranded overhang (Karlseder et al. 2002). It is not clear why the human and mouse differ in regard to the effects of TRF2 on the single-stranded overhang of the telomere. In addition, the transgenic mice displayed increased telomere shortening when compared with age-matched controls and increased genomic instability. Moreover, analyses of human basal and squamous cell carcinomas have revealed an increase in TRF2 expression levels in approximately 10% of cases (Munoz et al. 2005), consistent with a role for TRF2 in human carcinogenesis.

Yet another study that examined human mammary epithelial cell lines and cell lines derived from breast carcinomas also suggested that TRF2 plays a role in tumorigenesis. Analysis of TRF2 expression revealed upregulation of the protein at the posttranscriptional level (Nijjar et al. 2005). In addition, upregulation of TRF2 protein was associated with altered TRF2 localization. TRF2 was found throughout the nucleus as well as at telomeres. Whether this relocalization represents the movement of TRF2 to sites of DNA damage has not been determined.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Is aging the price we pay for remaining cancer free during our early life? And are telomeres and replicative senescence at the center of this ongoing battle? Recent advances support the role of senescence in tumor suppression and the loss of telomere integrity as a driving force in the transformation process (Braig et al. 2005, Chen et al. 2005, Collado et al. 2005; reviewed in Maser & DePinho 2002, Michaloglou et al. 2005). If we have learned anything, it is that the telomere and its associated proteins play a critical role in genomic integrity. Importantly, understanding how the telomere interacts with the DNA repair machinery in normal as well as pathological conditions will further our general understanding of telomere biology. In addition, it is now clear that cell senescence is critical for tumor suppression, and accumulating data suggest that the former may also influence many of the pathologies associated with aging. The coming years are sure to shed much light on telomere homeostasis and, in turn, on the roles of the telomere in human aging.

SUMMARY POINTS

1. Normal human somatic cells possess a limited replicative potential that is dictated by the proper maintenance of the telomere and by their responses to certain cell-physiological stresses.
2. Senescence is often triggered by cell-physiological stresses that cells suffer in vitro and possibly in vivo.
3. Senescence functions as an important tumor suppressor mechanism.

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4. In the event that cell lineages circumvent the proliferative block imposed by senescence, the telomeres of these cells will continue to shorten, leading to crisis, in which chromosomal fusions, genetic instability, and widespread cell death occur.
5. Telomere homeostasis, which is essential for the normal function of telomeres to protect the ends of chromosomes, is dictated by both the configuration of telomeric DNA and the functions of telomere-binding proteins.
6. DNA repair enzymes associate with telomeres and play critical roles in telomere maintenance in still-unclear ways.
7. The two canonical tumor suppressor pathways—involving the Rb and p53 tumor suppressor proteins—impose senescence in the event that telomeric DNA is disrupted in certain ways.
8. Cells can acquire replicative immortality either through the expression of telomerase or through the activation of the alternative lengthening of telomeres (ALT) pathways.

FUTURE ISSUES

1. How is senescence activated at the molecular level, and what pathways govern activation of the p53 and Rb tumor suppressor pathways, which impose a senescent growth state?
2. What are the molecular mechanisms that enable the ALT pathway to maintain telomeres, and will this pathway constitute an important escape pathway for cells subject to antitelomerase therapies?
3. Do telomere shortening and cell senescence contribute to human aging?
4. What is the precise relationship between DNA repair and telomere homeostasis?

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These observations, which demonstrate that telomere length is an important parameter of cellular immortality, eventually led to the formulation of the telomere hypothesis and its role in limiting cellular proliferation.

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