

New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin

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Telomeres are stabilized, and telomeric DNA is replenished, by the action of the ribonucleoprotein reverse transcriptase telomerase. Telomere capping functions include the ability of telomeres to protect chromosome ends from cellular DNA-damage responses such as cell cycle arrest or apoptosis. This property of telomeres is especially important for cancer cells, which continue proliferating despite chromosome aberrations. Telomere capping is influenced by multiple, mutually reinforcing factors including telomere length, although telomere length is only one of several determinants of telomere functionality. For example, many cancer cells express high levels of telomerase yet maintain relatively short telomeres. We consider three aspects of telomere capping that have emerged relatively recently: (1) a new role for telomerase in telomere capping independent of its function in telomere elongation. Support for this novel function comes from experiments showing an increase in replicative potential with the reactivation of telomerase, without net telomere lengthening; (2) the role at telomeres of DNA damage proteins. We propose a model in which two factors specifically target telomeres for the action of telomerase, as opposed to recombination or non-homologous end-joining: binding by telomeric proteins that limits DNA damage responses at telomeres, and the affinity of the telomerase RNP for telomeric proteins and DNA; and (3) we discuss a potential protective role of amplified subtelomeric DNAs, which may aid capping of telomeres maintained by non-telomerase based mechanisms through the formation of heterochromatin.

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Introduction

Telomeres in human cancer cells, as in most eukaryotic organisms, consist of a tandem array of telomeric DNA repeats bound to specialized telomeric proteins. In human cancer cells, telomeric DNA is usually maintained by the action of the ribonucleoprotein enzyme telomerase. After

the nature of the cellular DNA polymerases became understood, it was predicted in the early 1970s that linear DNA molecules, including those of eukaryotic chromosomes, would require mechanisms in addition to conventional DNA polymerases to complete the replication of their very termini (Watson, 1972). Accordingly, it was predicted that without such a mechanism, terminal attrition of DNA would eventually lead to loss of genetic information and prevent cells from continuing to multiply, resulting in cellular ‘senescence’ (Olovnikov, 1971, 1973).

The enzymatic activity of telomerase, documented at first *in vitro* and then *in vivo*, was shown to lengthen terminal regions of eukaryotic telomeric DNA by RNA-templated addition of repeated DNA sequences (Greider and Blackburn, 1985, 1987; Yu *et al.*, 1990), thus solving this problem. Essential conserved core molecular components of telomerase include the reverse transcriptase protein family member TERT, and the telomerase RNA TER. Other proteins are associated with telomerase and at least some of them are required for its action *in vivo* (see Evans and Lundblad, 2000 for references). Cancer cells, with their unchecked proliferation, typically contain active telomerase, which is frequently upregulated to high levels (Kim *et al.*, 1994). Understanding the basic biology of telomeres will help clarify the role that upregulation of telomerase plays during the genesis of cancer cells. In particular, telomere capping—the ability of telomeres to protect chromosome ends from DNA damage responses—is important because altered chromosome dynamics in cancer cells can lead to aneuploidy and other potentially transforming abnormalities.

When telomere capping fails, the cellular responses that occur are symptomatic of DNA damage, such as cell cycle arrest or, in human cells, for example, cell death (Karseder *et al.*, 1999; Smith and Blackburn, 1999; Yu *et al.*, 1990). Telomeric DNA is then also subjected to molecular processes normally applied to double-stranded breaks within chromosomes: end-to end fusions, degradation, and recombinational events that in some cases fuse telomeric ends. Certain molecular changes at telomeres that compromise their capping can also unleash unregulated telomerase action at the uncapped telomere, in contrast to the normally tight regulation of its action on capped telomeres (McEachern and Blackburn, 1995).

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The most obvious potential genomic consequences of loss of telomere capping are the formation of dicentric chromosomes, subsequent breakage of the dicentric chromosomes in anaphase and consequent aneuploidy. There is also the potential for loss of genetic information through degradation of the terminal region of a chromosome whose telomere becomes uncapped. The two essential functions of telomeres—protecting chromosome ends and facilitating their complete replication through elongation by telomerase—are linked by two observations: First, a certain minimal length of telomeric DNA is required to prevent recognition of telomeres as DNA damage. As discussed below, what this minimal length is depends on multiple factors, including the species and cell type. Second, the initially paradoxical finding that telomeres require several DNA damage response proteins for end-protection and for elongation by telomerase again shows that these processes are interconnected (Gasser, 2000).

This review will focus on the interplay between telomerase and some of the molecular properties of telomeres that play roles in telomeric capping. This leads us to some speculations on possible roles of telomerase in cancer. We next discuss the functional tie-in of DNA damage response pathways to telomere maintenance by telomerase. Finally, we discuss some provocative observations on cells lacking telomerase that may provide insights into a hitherto relatively neglected question in connection with telomere capping: the roles of subtelomeric regions.

Telomeric length dynamics and capping

In cells with active telomerase, such as cancer cells, the telomere-telomerase complex comprises a dynamic system in which telomeric DNA length is continually being built up and shortened in a regulated way that maintains telomere length homeostasis and retains telomere functionality. The action of telomerase is regulated by sequence-specific DNA binding proteins that bind the telomeric DNA repeats. Through separate domains, these proteins bind additional proteins to assemble a higher-order complex on the telomeric DNA. Repeated DNAs in subtelomeric regions of the chromosome often have heterochromatic properties that may modulate telomerase activity *in vivo*. In proliferating cells that either naturally or through various experimental manipulations lacked telomerase, the predicted telomere shortening was observed. Such cells, as diverse as ciliate, yeast, and human primary cells in culture, eventually exhibited senescence (Harley *et al.*, 1990; Lundblad and Szostak, 1989; Yu *et al.*, 1990). The model that telomere shortening caused cellular senescence became widely accepted. While in some situations this is experimentally borne out, it is far from the whole story. Rather, multiple lines of evidence from a variety of eukaryotes and cell types suggest a useful formulation of telomere functionality as being determined by the capped status,

or otherwise, of a telomere. Telomere uncapping causes a set of molecular and cellular consequences. So, what exactly are the factors that contribute to a capped telomere?

While uncapping of a telomere can be affected by telomere length, it is also affected by other factors. Multiple, mutually reinforcing mechanisms determine the probability of whether a telomere switches into the uncapped (nonfunctional) or capped (functional) state, thereby uncoupling telomere length from uncapping in some situations. As reviewed recently elsewhere, at least four distinct structural components contribute to telomere capping: (1) the higher order telomeric DNA-protein complex, whose overall length (i.e. the number of telomeric repeats) dictates whether telomerase or nucleases can access telomeric DNA; (2) the protein complex found on the terminal few repeats; (3) the DNA-protein complex on the single stranded G-rich DNA extension of the telomere, which is most likely important for preventing DNA damage response and regulating the cell cycle dependent structure of the telomere; and (4) telomerase itself (Blackburn *et al.*, 2000; Blackburn, 2001). A key feature of capping is that often, compromising or abrogating one component of the capping system can be harmless; loss or compromise of more than one component can cause failure of one or more capping functions. Only if all these factors are kept constant does the probability of switching change directly with telomere length – there is a higher probability of switching to a functionally uncapped state with shorter telomeres. Thus the idea of a critically short telomere becomes only one special case of a more generally applicable concept: the probability that a telomere will switch between a functionally capped and a functionally uncapped state.

The role of telomerase in telomere capping

Telomeres are typically short in cancer cells

A central assumption of many studies on human cells has been that the average loss of telomeric DNA that occurs each time a cell divides is fixed. Telomere length then became used as a criterion to deduce cellular proliferation history and future potential. Progressive telomere loss has been experimentally demonstrated using non-immortalized cells in culture that lack detectable telomerase (Harley *et al.*, 1990; Vaziri *et al.*, 1993). However, in such cells the rate of loss varies with the cell type and, for fibroblasts, is increased when proteins such as ATM and WRN are disabled (Huffman *et al.*, 2000; Schulz *et al.*, 1996; Vaziri *et al.*, 1997). An even more confounding problem with extrapolating a rate of telomere loss to division potential in the human body occurs when telomerase is activated; the degree of shortening of telomeric DNA can now no longer be taken as a measure of the number of cell divisions, since telomerase action can elongate telomeres. In some studies, average telomere length in T cells was used as an indicator of the

number of times these cells had divided. But normal human T and B cells (and a variety of other normal human somatic cell types) may contain active telomerase depending on their developmental status and whether they have been stimulated to divide. Indeed, telomeres of human B cells lengthen as the cells proliferate during at least one stage in their development, when telomerase is normally activated (Weng *et al.*, 1997).

In human cancer cells, telomerase is typically highly upregulated (Kim *et al.*, 1994). However, telomeres in proliferating cancer cells are often quite short (de Lange *et al.*, 1990; Hastie *et al.*, 1990). The shortness of these telomeres can be reconciled with their apparent functionality by taking into account the complexity of telomere length regulation. Over 25 different genes can affect telomere length regulation in *Saccharomyces cerevisiae*. For example, yeast telomere length can be directly altered by changing the level of functional telomerase RNA (Roy *et al.*, 1998). Telomerase level is also limiting for telomere length maintenance in the mouse, since cells lacking one copy of the TERT gene have shortened telomeres relative to cells from mice with the normal gene dosage of two TERT gene copies (Liu *et al.*, 2000). Manipulating levels of telomere binding proteins such as the negative length regulator Rap1p in yeast, or human proteins that also act as negative regulators of telomere length – TRF1, TRF2 and the TRF1-binding protein TIN2 – can alter telomere length (Conrad *et al.*, 1990; Kim *et al.*, 1999; Krauskopf and Blackburn, 1996, 1998; Marcand *et al.*, 1997; Smogorzewska *et al.*, 2000). The relative levels and activity of these various proteins are predicted to be among the determinants of telomere length *in vivo*; it remains to be determined which are the most crucial in determining telomere length in human cancer cells.

A new role for active telomerase in protecting telomeres and allowing cell proliferation

Active telomerase itself helps functionally cap chromosome ends and prevent telomere fusions. This was shown in experiments in which the presence of functional telomerase allowed cells to remain competent for proliferation, even with short telomeres (net elongation of short telomeres was not seen). Yeast cells or primary mammalian cells in culture that lack telomerase activity (including cells that express telomerase point mutants lacking catalytic activity) are initially capable of normal healthy growth, typically for many tens of population doublings. Therefore, active telomerase is not needed for cell proliferation while the telomeres are sufficiently long. During the initial robust growth phase of mammalian primary cells or yeast in culture, a high fraction of the cell population is cycling. However, as the cell population propagates, an increasing fraction of the cells, and eventually essentially all of the population, exit the cell cycle in a process termed replicative senescence (Karatzas and Shall, 1984; Karatzas *et al.*, 1984; Lundblad and

Szostak, 1989; McEachern and Blackburn, 1996). Interestingly, those mammalian cells remaining in the cycling pool of cells divide at a similar rate in both early and later in the passaging; it is mainly the increasing fraction of cells exiting the cell cycle that slows the overall population doubling rate, rather than an increase in the time it takes the individual cells to complete each cell cycle (Karatzas and Shall, 1984).

Ectopic expression of the limiting component of telomerase (hTERT) in cultured human primary cells, which normally completely lack telomerase activity, conferred an apparently indefinite extension of proliferative lifespan. Telomeres were greatly lengthened in these initial experiments (Bodnar *et al.*, 1998). Similar results were seen in spontaneously immortalized, SV40 T-antigen transformed cells made to ectopically express hTERT (Counter *et al.*, 1998). Expression of a different, hypomorphic hTERT construct produced normal telomerase activity (as assayable *in vitro*) and the same lifespan extension of the SV-40 T-antigen transformed human fibroblast cell type. However, with this hTERT construct, telomere length continued to shorten for several tens of population doublings, eventually stabilizing at a relatively short length. The notable point was that these telomeres were shorter than those in the control cells at the crisis point, which had received either no hTERT gene, or a point mutant hTERT gene encoding enzymatically inactive telomerase (Figure 1; Zhu *et al.*, 1999). Similar results have been found for other human cell types transformed with an apparently hypomorphic hTERT construct: primary endothelial cells (Yang *et al.*, 1999) and primary fibroblasts (M Kim and EH Blackburn, unpublished work). Human fibroblast cells transformed with SV-40 T-antigen lack certain checkpoint functions, and have a high frequency of telomere fusions as they approach crisis (Chang *et al.*, 1997; Ducray *et al.*, 1999). The ectopic expression of telomerase in these cells also caused a decrease in the frequency of telomere fusions. This rescue of telomere function occurred not only in the experiments in which telomeres elongated (Counter *et al.*, 1998), but also in those where the hypomorphic hTERT was expressed, in which telomeres continued shortening as cells proliferated well past the normal crisis point (Zhu *et al.*, 1999).

These results suggested that active telomerase allows telomeres to remain functional even at lengths that, in the absence of telomerase, would have caused cells to stop dividing or led to telomere-telomere fusions (Figure 1). An alternative hypothesis that was advanced to account for these and similar human cell results was that the hypomorphic hTERT, while it did not lengthen telomeres overall, selectively lengthened the shortest telomeres in the cell, which otherwise would have become uncapped and signaled cells to cease proliferating or caused telomeric fusions to occur (Ford *et al.*, 2001; Zhu *et al.*, 1999). However, while a single short telomere may be hard to detect in human cells, which have on the order of 100 telomeres per cell, the budding yeasts *S. cerevisiae* and *Kluyveromyces*

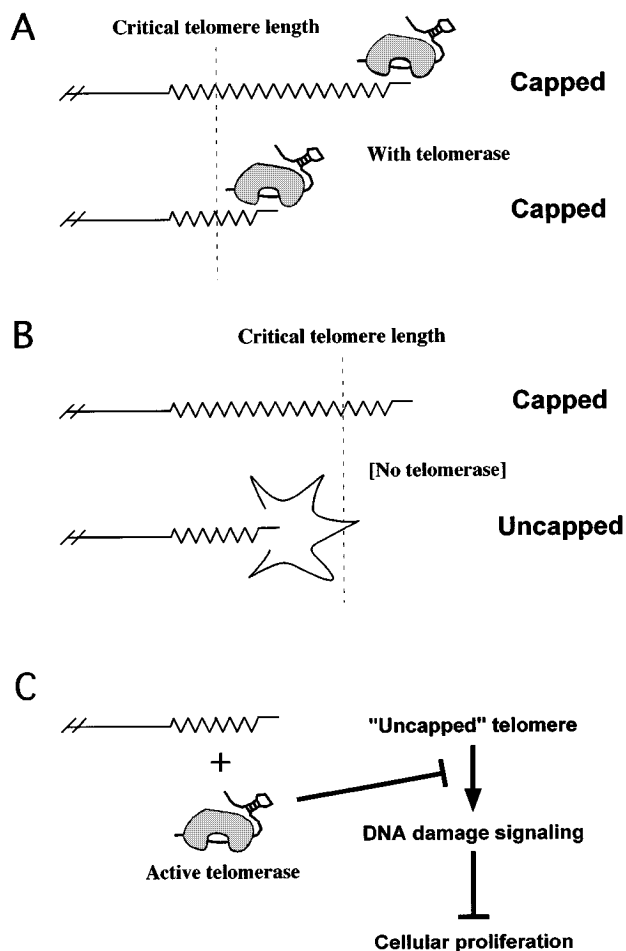


Figure 1 Telomerase exerts a capping function independent of its role in telomere elongation. (a) and (b) critical telomere length is influenced by many factors, including the presence of active telomerase. (c) A model for the role of active telomerase in telomere capping. Uncapped telomeres elicit a DNA damage response which halts cellular proliferation. Active telomerase forms a structure at the telomere that prevents chromosome ends from being sensed as DNA damage

lactis have respectively only 32 and 12 telomeres per cell and a single short telomere can be readily detected in a relatively low fraction of the cell population. Results comparable to those described above for human cells have also been obtained in these yeast species, supporting the interpretation that telomerase can protect cells from the effects of even very shortened telomeres. Various telomerases carrying mutations in the RNA component of telomerase were tested in *S. cerevisiae* and *K. lactis*. These telomerases had normal *in vitro* enzymatic activity, but were hypomorphic *in vivo*, by the criterion that telomeres became shortened, although they were stably maintained at the short length. Cell growth remained normal and robust, with no decline over time. Strikingly, telomeres in these healthily growing cells were significantly shorter than the shortest detectable telomeres in isogenic control cells lacking active telomerase (produced by specific

telomerase RNA mutations) (Prescott and Blackburn, 1997b, 2000; J Roy, Y Tzfati and EH Blackburn, unpublished work). At the point of comparison, over 99.9% of the latter cells had exited the cell cycle following the prior period of progressive telomere shortening. In these yeast experiments, regardless of whether the telomerase was enzymatically active or not, telomerase RNA levels were stable, and the telomerase RNP was indistinguishable from wild-type in terms of chromatographic, gel filtration and gel electrophoretic properties.

Telomerase thus appears to permit cell proliferation by stabilizing short telomeres that would be unstable in the absence of functional telomerase. Notably, these results revealed that it is possible to experimentally uncouple rescue by telomerase of telomeric function (as judged by the ability to allow continued cell proliferation and avoid telomeric fusions) from net lengthening of telomeres. Hence these results define another aspect of telomerase RNP function in addition to telomere elongation. The finding that the same results were found with very different types of specific telomerase mutations, and in different cell types (two different yeast species and two human cell types), strongly suggests that the common feature that determined the ability of cells to grow was the presence of enzymatically competent telomerase.

How does active telomerase achieve a telomere-protective effect?

The mechanism of this capping function of telomerase is not known, but two general models can be considered. These models are not mutually exclusive. First, telomerase may physically interact with the telomere at a critical time in the cell cycle, beyond the time required just for polymerization, and protect the exposed single-stranded DNA terminus (Figure 1a). Such a model is suggested by the finding that *in vitro*, telomerase from two budding yeasts remains stably associated with its telomeric DNA extension product following a round of polymerization (Fulton and Blackburn, 1998; Prescott and Blackburn, 1997a). Yeast telomerase acts as a dimer, and is a discrete complex containing at least two functionally interacting RNAs (Prescott and Blackburn, 1997a). Recent results suggest that, similarly, human telomerase contains two functionally coupled active sites (Beattie *et al.*, 2001; Wenz *et al.*, 2001). These findings have significant implications for understanding telomerase action. For example, it was proposed that two telomeric DNA termini, such as the two newly replicated sister chromatid ends, could be elongated on a dimeric telomerase (Prescott and Blackburn, 1997a).

Alternatively, we propose that telomerase itself has a proliferation-promoting effect on cells. This may take the form of signaling to the cell-cycle machinery, and can be envisaged as potentially 'reassuring' cells, even those with short telomeres, that telomerase is present

so that telomere integrity will be intact (Figure 1c). Since the specific stabilization of very short telomeres was not seen in cells expressing a stable but enzymatically inactive telomerase RNP, it may be that telomerase has to assume an enzymatically competent conformation (perhaps while bound to its telomeric DNA substrate), in order to signal to the cell to keep dividing. A model in which the telomerase RNP plays a signaling role is attractive because cancer cells, which have been selected for their proliferative capability (among other properties), typically have significantly elevated levels of telomerase. If active telomerase feeds into the signaling pathways that determine cell proliferative or death responses, the high level of active telomerase might override anti-proliferative or cell death signals. Thus cancer cells with high levels of telomerase might gain a selective growth advantage.

Circumstantial support for this view comes from other observations. First, Stampfer and colleagues showed that cultured human breast epithelial cells that were telomerase-positive resisted the anti-proliferative/growth-inhibiting effects of TGF- β (Stampfer *et al.*, 2001). By contrast, the control telomerase-negative cells were sensitive to TGF- β . Experimentally, ectopic expression of enzymatically competent hTERT in such telomerase-negative, TGF- β responsive cells was sufficient to convert them into a TGF- β -resistant state. Second, de Lange, Varmus and colleagues showed that telomerase activity was upregulated early in the course of tumor development in a mouse mammary tumor model, even though the loss of telomeric DNA that had occurred at that point was not expected to disrupt telomere functionality (Broccoli *et al.*, 1996). Third, results obtained in pre- and post-mitotic rodent hippocampal neurons also hint at a cell-protective role for telomerase. Blocking endogenous hTERT expression in these cells increased apoptosis. Conversely, experimentally activating ectopic hTERT expression in cells of neuronal origin protected against apoptosis (Fu *et al.*, 2000; Zhu *et al.*, 2000). While telomerase activity

in various neuronal cell types was repressed as development progressed, hTERT mRNA and protein levels persisted for much longer in the postmitotic cells in the brain and spinal cord, often at significant levels, implying a potential for another role of the hTERT protein in the absence of detectable enzymatic telomerase activity (Klapper *et al.*, 2001). Lastly, Blasco and colleagues have shown that *in vivo* over-expression of the mouse telomerase reverse transcriptase mTERT in basal keratinocytes promotes proliferation of these cells despite the fact that the keratinocyte telomeres, already very long, were not appreciably changed in length upon expression of mTERT (Gonzalez-Suarez *et al.*, 2001).

The ability of telomerase to allow continued proliferation of cells with very short telomeres highlights a central question that is still unanswered: when all other variables are fixed, what critical structural difference(s) between long and short telomeres determine whether the telomeres is capped or not? This question is fascinating because telomeric DNA, and by implication telomere-binding proteins, are still present when telomeres become short enough to lose capping function. These results suggest that to achieve capping, a higher-order DNA-protein complex of unknown structure must form at the telomere – the ability of this structure to form is telomere length-dependent. In human or mouse cells, critically short telomeres can still contain thousands of base pairs of telomeric TTAGGG repeats. Yet, by still unknown mechanisms, such telomeres are insufficient to prevent cellular checkpoint responses and their downstream consequences. Such responses to uncapped telomeres are discussed next.

DNA damage response proteins and telomere maintenance

The discovery that DNA damage response proteins were important for normal telomere maintenance has

Table 1 Differences between cellular responses to a double-stranded break and a short telomere

	Double-stranded break	Short telomere
Molecular outcome	DNA repair via homologous recombination or non-homologous end-joining	Elongation by telomerase
Cellular outcome	Cell cycle arrest	No cell cycle arrest (until very short and uncapped)
DNA sequence	For random DSBs, no specific sequence	Telomeric repeats or degenerate telomeric sequence
Double-stranded binding proteins	DSBs are random, so no sequence specific proteins	Rap1p complex ¹ or TRF complex ²
Single-stranded binding proteins	RPA (replicative ssb) ³ , Rad51/52 for homologous recombination ⁴	Cdc13p complex ⁵ or Pot1 ⁶ or T-loop junction ⁷
DNA damage proteins that sense, signal and respond	ATM, MRX, Ku, BRCA1, histone H2A-X, Mrt-2/Rad17 ⁸	ATM, MRX, Ku, Mrt-2/Rad17 ⁹
Terminal DNA structure	Processed into very long 3'-overhang ¹⁰	Processed into short, regulated 3'-overhang ¹¹ , T-loop ⁷
Affinity for telomerase	Low ¹²	High ¹²

¹(Conrad *et al.*, 1990). ²(Broccoli *et al.*, 1997; Li *et al.*, 2000). ³(Lee *et al.*, 1998). ⁴(Shinohara *et al.*, 1992). ⁵(Nugent *et al.*, 1996). ⁶(Baumann and Cech, 2001). ⁷(Griffith *et al.*, 1999). ⁸Reviewed in (Zhou and Elledge, 2000). ⁹Reviewed in (Gasser, 2000). ¹⁰(Sugawara and Haber, 1992). ¹¹(Wellinger *et al.*, 1996). ¹²(Greider and Blackburn, 1985; Hammond *et al.*, 1997; Harrington and Greider, 1991; Lee and Blackburn, 1993)

given important insight as to which molecules sense short telomeres and signal to facilitate telomerase action (reviewed in Gasser, 2000). However, a crucial paradox is also raised by these findings – how does signaling through the same proteins lead to very different responses at a double-stranded break (cell cycle arrest, up-regulation of damage-related transcription, and homologous recombination or non-homologous end-joining (NHEJ) to repair the break) and at a short telomere (cell cycle progression, telomerase action at the telomere)? Here we present a general model for how these two responses might be directed, then discuss in more detail the roles in telomere maintenance of a particularly interesting set of DNA damage proteins: the ATM family kinases and their downstream targets.

Specific similarities and differences between double-stranded breaks and telomeres that need to be considered are listed in Table 1. In our model, DNA repair (by homologous recombination or NHEJ) and elongation by telomerase are competing responses at telomeres and at non-telomeric DNA ends. Initially, at both sites, DNA damage proteins sense double-stranded breaks and short telomeres in a similar way and fulfill the same molecular roles. Two factors favor the telomerase response at telomeres: (1) Limitation of the action of DNA damage response proteins by sequence-specific telomere-binding proteins; and (2) Attraction of telomerase by its affinity with telomere-specific proteins and/or the telomeric DNA sequence.

Experimental evidence exists that favors the hypothesis that some of the known DNA damage proteins act on the same downstream target molecules in both contexts. For example, there is genetic evidence in *S. cerevisiae* that Tel1p (a homologue of human ATM) acts at telomeres through the Mre11/Rad50/Xrs2 (MRX) complex – a multifunctional entity with roles in many different responses to double-stranded breaks in both meiosis and in mitosis (Haber, 1998; Ritchie and Petes, 2000). Experiments with human cells imply that activation of the MRX complex by ATM is a conserved mechanism: ATM phosphorylates the Xrs2p homologue NBS1 in DNA damage responses (Gatei *et al.*, 2000; Lim *et al.*, 2000; Zhao *et al.*, 2000). Hence it is likely that Tel1p/ATM phosphorylates the Xrs2p (= hNBS1) component of the MRX complex at double stranded breaks and at telomeres. Furthermore, a mutant of the Mre11p protein that is defective for nuclease activity *in vitro* is defective in meiotic recombination but competent for both NHEJ and for telomere maintenance, suggesting that the last two processes share mechanistic similarities (Moreau *et al.*, 1999).

A double-stranded break created in the middle of a chromosome is rapidly processed to create very long 3' single-stranded overhangs (Sugawara and Haber, 1992). Direct evidence that telomeric DNA protects free DNA ends from such degradation came from experiments placing a short stretch of telomeric DNA next to the break site, which limited this rapid degradation, even in the absence of telomerase (Diede and Gottschling, 1999). *De novo* telomere formation on

linear plasmids transformed into *S. cerevisiae* is enhanced by even short terminal sequences of telomeric DNA (Lustig *et al.*, 1990). This provides further molecular evidence supporting the notion that the action of DNA damage proteins is limited by telomeric sequences. At least some of these protective effects can be attributed to the major double-stranded telomere-binding protein Rap1p, because artificially tethering Rap1p C-termini near a DNA end can also enhance *de novo* telomere formation.

Telomerase is attracted to short, single-stranded 3' telomeric overhangs at least partly by the interaction between Est1p and Cdc13p, a process that would occur much less efficiently at overhangs of random sequence (Evans and Lundblad, 1999). The sequence preference of the telomerase reverse RNP is an additional requirement that favors the telomerase response when the single-stranded overhang is composed of telomeric sequence. Single-stranded DNA is most likely to be elongated by telomerase when it contains telomere-like DNA sequences, which enhance binding both to the templating domain of the telomerase RNA by base-pairing, and to the 'anchor site' distal to the template via poorly understood molecular interactions between the telomeric DNA and the telomerase RNP (Greider and Blackburn, 1985; Hammond *et al.*, 1997; Harrington and Greider, 1991; Lee and Blackburn, 1993).

An analogy between our model and programmed genome rearrangement in ciliated protozoan *Euplotes crassus* is instructive. During development of the somatic nucleus in *Euplotes*, the genome undergoes stereotyped site-specific fragmentation followed either by rejoining of broken DNA ends (with concomitant elimination of the DNA sequences originally located between the breakage sites) or by *de novo* telomere addition, to form small linear minichromosomes (Blackburn, 1995). Intriguingly, cleavage sites within the genome that are processed by such DNA rejoining/sequence elimination, or *de novo* telomere formation onto the cut DNA end, share a common sequence element (Baird and Klobutcher, 1989; Jaraczewski and Jahn, 1993). In both cases, the element is located exactly 17 bp from the cleavage site. This sequence conservation suggests that a common factor directs the placement of a double-stranded break at both types of sites. Then, flanking sequence elements that differ between the two types of sites probably serve to direct the chromosome break into the appropriate developmental path – i.e. end-joining or telomeric DNA addition by telomerase (Yu and Blackburn, 1991).

Mutations in the ATM gene cause a cancer-prone phenotype in humans and mice. In yeasts, two of the most important DNA damage proteins required for telomere maintenance are the ATM family kinases Tel1p and Mec1p (homologous to human ATM and ATR respectively). In both *S. cerevisiae* and the fission yeast *Schizosaccharomyces pombe* the combined deletion of both kinases causes telomere shortening and eventual replicative senescence that is phenotypically quite similar to a telomerase null mutation (Naito *et al.*, 1998; Ritchie *et al.*, 1999). In budding yeast, Tel1p

plays the major role in telomere maintenance—*tell* deletion mutants have very short telomeres while *mec1* deletion mutants have telomeres of wild-type length (when viability is rescued by the *sml1* mutation). Genetically, Tel1p acts through the telomerase pathway (Ritchie and Petes, 2000). In the absence of Tel1p and Mec1p, the *in vitro* catalytic activity of the telomerase ribonucleoprotein is identical to wild-type. However, altering various aspects of telomere structure allows telomerase to maintain telomeres in *tell mec1* cells, and replicative senescence of the cells does not occur (Chan *et al.*, 2001). These experiments suggested that ATM family kinases are required to modulate the structure of the telomere, either to facilitate telomerase access and/or to create a suitable substrate for the enzyme. The finding that ATM kinases were not required for telomerase catalytic activity corroborated genetic experiments showing that Tel1p acts via the MRX complex.

The function of the MRX complex at telomeres or double-stranded breaks is not well understood. In human cells, telomere association by components of the MRX complex has been shown by immunolocalization and TRF2 immunoprecipitation experiments. MRX components also accumulate at sites of DNA damage (Maser *et al.*, 1997; Mirzoeva and Petrini, 2001). Processing of double-stranded breaks in a 5′–3′ direction to yield single-stranded overhangs is reduced in *rad50* mutant cells (Sugawara and Haber, 1992), suggesting that the role of the complex is to create an appropriate substrate for telomerase or the DNA recombination machinery (DuBois *et al.*, 2000; Willinger *et al.*, 1993). This hypothesis is supported by the fact that a telomerase RNA template mutation (*tlc1-476A*) that increases single-stranded telomeric DNA is sufficient to bypass the requirement for Tel1p and Mec1p *in vivo* (Chan *et al.*, 2001). Mre11p has nuclease and DNA hairpin cleavage activity *in vitro*, but a point mutation abolishing its single-stranded endonuclease activity in the presence of Mn²⁺ (Moreau *et al.*, 1999) still has normal telomere maintenance, suggesting that the hypothetical DNA-processing role does not depend on this activity. Rad50p has structural features in common with the SMC family of ATPases (Hopfner *et al.*, 2000), proteins that are required for chromosome condensation and sister chromatid cohesion – the role of the MRX complex at telomeres may require Rad50p to bridge two telomeres, perhaps for efficient action by a dimeric telomerase RNP.

As alluded to above, a major unanswered question about the function of DNA damage-response proteins, including MRX, in telomere maintenance is how their action is differently regulated at double-stranded breaks and at telomeres. In human cells, ATM kinase and the MRX complex seem to have an additional role in telomere end protection; cells lacking ATM or the Xrs2p homologue NBS1 show rapid telomere shortening, even in the presence of endogenous levels of telomerase (Metcalf *et al.*, 1996; Vaziri *et al.*, 1997). Interestingly, overexpression of hTERT can counteract the loss of ATM or NBS1

in human fibroblasts (Ranganathan *et al.*, 2001; Wood *et al.*, 2001).

The role of Mec1p/ATR at telomeres is even less well understood than those of ATM and the MRX complex. In *S. pombe* cells, deletion of Rad3p (the Mec1p homologue) causes noticeable telomere shortening, whilst *S. pombe tell* cells have wild-type telomere length (Naito *et al.*, 1998). This situation is the reverse of that seen in *S. cerevisiae*. Thus, the Mec1/ATR dependent factors that facilitate telomerase action at telomeres may prove just as critical as the MRX complex in human cells – although the fact that ATR is an essential gene in mammalian cells has complicated a genetic test of this question. Mec1p has many potential substrates, and yeast genetics should be helpful in unraveling which of its downstream targets are important for telomere maintenance. One potentially important substrate was uncovered by recent work showing that Mec1p was shown to phosphorylate histone H2A in response to DNA double strand-breakage (Downs *et al.*, 2000). Mutating the Mec1p phosphorylation site on H2A to mimic a constitutive phosphorylation state of H2A caused a bulk decondensation of chromatin. In the absence of the yeast ATM homologue Tel1p, the kinase Mec1p still allows telomerase to maintain telomeres, but at short lengths (Ritchie *et al.*, 1999). A testable hypothesis is that the short telomeres in a *tell* mutant elicit local phosphorylation of H2A by Mec1p, decondensing the subtelomeric chromatin to promote access by telomerase. This hypothesis is attractive because, as discussed next, a growing amount of evidence indicates that heterochromatin formed by repeated subtelomeric DNA may contribute to telomere functions.

Why are many telomeres heterochromatic?

Heterochromatic properties of telomeric regions

The subtelomeric regions in many species contain tandem arrays of a variety of complex sequences. Cytologically, telomeres in a variety of plants and animals are heterochromatic, implying a high degree of DNA folding (Dernburg *et al.*, 1995; Suja and Rufas, 1994). Experimental evidence suggests that folding of the terminal region of the telomere into a more internal subtelomeric region can contribute to telomere capping. Chromatin immunoprecipitation experiments in *S. cerevisiae* showed that telomerically bound proteins such as Rap1p were cross-linked not only to telomeric repeat tract DNA, but also to subtelomeric DNA sequences several kb towards the centromere (de Bruin *et al.*, 2000; Grunstein, 1998; Lieb *et al.*, 2001). In addition, a yeast transcriptional activation element, UAS_G, placed downstream of (or even somewhat distant from) a reporter gene, can activate transcription specifically in a subtelomeric location (de Bruin *et al.*, 2001). This result suggested that folding back within the subtelomeric chromatin can in effect position the element ‘upstream’ of its associated gene, consistent with the chromatin immunoprecipitation findings.

Experimentally placing a gene in a telomeric heterochromatic region in *Drosophila melanogaster*, or up to

several kb away from the telomeric repeat tract in *S. cerevisiae*, imposes position effect variegation on that gene (Aparicio *et al.*, 1991; Gottschling *et al.*, 1990; Pardue, 1995; Renaud *et al.*, 1993). Position effect variegation is characterized by epigenetic heterochromatin states that are semi-stable for several cell divisions, with a gene under its influence switching between silenced or non-silenced states. Telomeric silencing in yeast shares several molecular requirements with silencing of the chromosome-internal mating type loci (Aparicio *et al.*, 1991), including the recruitment of Sir3p and Sir4p proteins by the C-terminal domain of Rap1p (Kyriou *et al.*, 1992). This suggested a molecular mechanistic link between the telomeric structures underlying telomere capping/uncapping status and the two functional states – repressed or active – that characterize heterochromatin. The Ku heterodimer is another important link between silencing and telomere function. In yeast, Ku associates with the telomere and has a role both in promoting telomerase activity (potentially by binding to telomerase RNA) and in telomeric silencing (Boulton and Jackson, 1998; Gravel *et al.*, 1998; Mishra and Shore, 1999; Peterson *et al.*, 2001).

A proposed role for heterochromatin in telomere capping

As mentioned above, a defining feature of telomere behavior *in vivo* is mutual reinforcement between the different molecular components that determine telomere capping. One such component that appears to become crucial for telomere capping when telomerase function is lost or compromised is heterochromatinization of the subtelomeric region. In *Drosophila*, the simple tandem repetition of a sequence is sufficient for it to gain heterochromatic properties (Dorer and Henikoff, 1997). Hence the large, complex-sequence repeats at telomeric regions in *D. melanogaster*, *Drosophila yakuba*, certain mosquitoes, the midge *C. tentans* and plants from the *Allium* family (onions and garlics) could form a nucleosomally based local heterochromatin structure that stabilizes telomeres (Pardue and DeBaryshe, 1999). *Drosophila* telomeres appear to have no absolute requirement for specific sequences for capping, as strains have been isolated containing a chromosome end that has lost all the normal telomeric elements from that end. Telomere fusions were not seen at these novel telomeres, although eventually Het-A retrotransposon sequences (which are found at all normal *Drosophila* telomeres) did sporadically reappear at the broken end (reviewed in McEachern *et al.*, 2000). *Drosophila* is capable of an uncapped DNA end response, since a different, newly generated chromosome break can cause cell cycle arrest and apoptosis (reviewed in McEachern *et al.*, 2000). It has therefore been suggested that protein assemblages at *Drosophila* telomeres or ends are formed *de novo* relatively inefficiently (to provide the opportunity for cells to repair broken DNA ends), but once formed are highly heritable (reviewed in McEachern *et al.*, 2000).

The sub-telomeric Y' tandem repeats in *S. cerevisiae* are 5–6 kb elements flanked by TG₁₋₃ repeat tracts.

Normally, only four or fewer copies of the subtelomeric Y' elements are present at a telomere, and some sub-telomeres lack Y' elements and instead contain a subtelomeric X element family sequence (Figure 2). Y' elements may contribute to capping, but this potential role is only unmasked when some aspect(s) of telomere maintenance is compromised. First, subtelomeric sequences were found to influence telomere regulation in *S. cerevisiae* mutants in which telomere length was affected: such mutations had different effects on Y'-telomeres versus telomeres containing the X subtelomeric element (Craven and Petes, 1999). Second, in telomerase-negative yeast cells, RAD52-dependent Y' repeat amplification into large tandem arrays on all telomeres permitted cells to survive loss of telomerase function and to grow quite well (Lundblad and Blackburn, 1993). In one model, the presence of the terminal TG₁₋₃ tract in these 'survivor' cells is the determinant that confers telomere capping function. If this is true, Y' amplification simply serves as a source of terminal telomeric DNA; Y' DNA will periodically become terminal because of incomplete replication, and this DNA will be degraded until a stretch of TG₁₋₃ is reached. However, in such survivors the terminal TG₁₋₃/AC₁₋₃ repeat tract was still no longer than it was at the point of the initial senescence, when over 99% of the cells ceased to divide (Lundblad and Blackburn, 1993). Therefore, an alternative model is that the Y' tandem arrays form nucleosome-based heterochromatin. This can then substitute for or augment the telomere capping that is usually conferred primarily by the telomeric repeat tracts.

We can consider two models by which Y' element amplification allows formation of a heterochromatic structure that is important for telomere function in cells lacking telomerase. In one, the TG₁₋₃ repeats separated by Y' elements are the critical sequence element. These stretches of telomere-like DNA come together in a cluster mediated by Sir-Rap1p interactions (Figure 2). The prediction is that survivors should fail to arise through Y' amplification in rap1 mutants lacking interactions with Sir3p and Sir4p, because telomeric and subtelomeric TG₁₋₃ repeat tracts would not be able to recruit the Sir complex. In the second model, it is the 5 or 6 kb Y' tandem repeats themselves, regardless of their flanking TG₁₋₃ repeat tracts (which are typically only one to a few hundred base pairs in length), that form subtelomeric heterochromatin regions on all telomeres upon becoming amplified into tandem arrays, as described for any tandem repeat array in *D. melanogaster* (Figure 2). In this model, survivor formation via the Y' amplification mechanism might show more dependence on histone deacetylation, which would affect formation of heterochromatin in general, than on the Rap1p C-terminal domain, which would be specific for telomeric repeat arrays in this context.

A similar potential role for subtelomeric repeat arrays is suggested by recent work with immortalized SV40-transformed human cells that lack telomerase. As in yeast survivors of telomerase deletion that form Y' amplifications, the terminal TTAGGG repeat tracts in

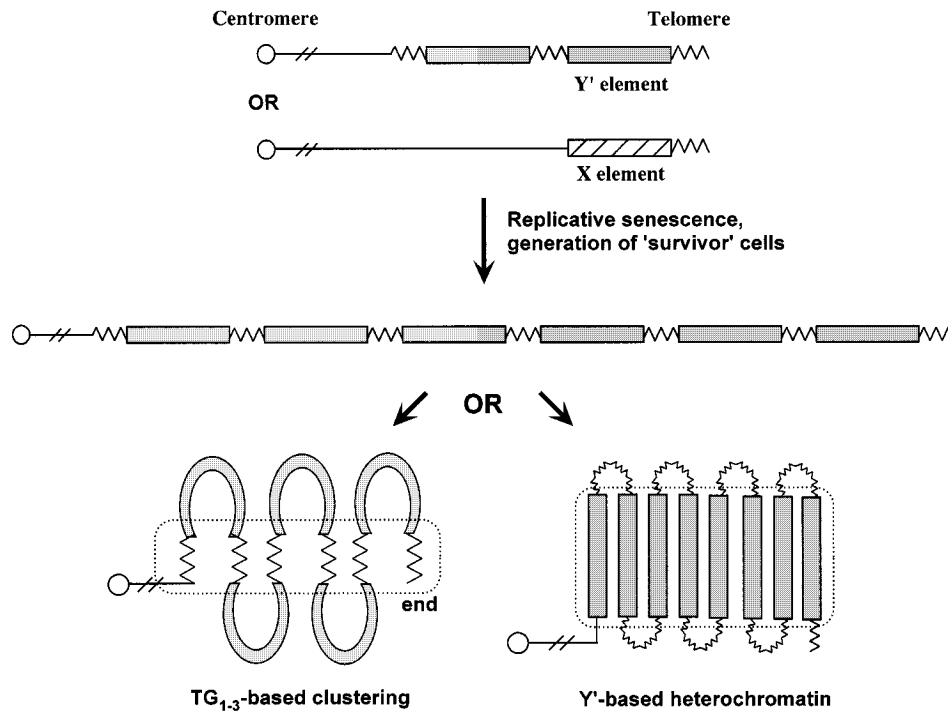


Figure 2 Two models for how amplified Y' elements might aid telomere capping in *S. cerevisiae* through the formation of heterochromatin. See text for details

these human cells were short, but the subtelomeric regions contained tandem arrays of repeat units, each consisting of a segment of SV40 DNA plus a TTAGGG repeat tract (R Marciniak, personal communication). The striking similarity between this situation and the one in Y'-amplifying yeast survivors is another line of evidence suggesting that subtelomeric heterochromatinization may be another mechanism that reinforces the capping of telomeres.

Outlook and future questions

The factors contributing to telomere stability, some of which we have discussed in this review, show that telomere capping involves multiple, intersecting molecular pathways. The mutual reinforcement and redundancies among the different mechanisms that play

into telomere capping may reflect the importance of having functional safety nets for protecting a cellular entity as crucial as the genome. Telomerase, DNA damage response pathways and the different components of telomeres are the critical players in this cellular drama – how do they act together to ensure telomeric and hence genomic stability? Answering this question will involve defining the precise molecular nature of the capped and uncapped telomeric states.

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