Advanced Information

# Maintenance of chromosomes
# by telomeres and the enzyme telomerase

*The 2009 Nobel Prize in Physiology or Medicine is awarded to Drs. Elizabeth H Blackburn, Jack W Szostak and Carol W Greider for their discovery of how chromosomes are protected by telomeres and the enzyme telomerase. They solved a long-standing fundamental problem in biology; how can the ends of chromosomes be maintained and spared from erosion or rearrangement during repeated cellular divisions? By ingenious genetic experiments they demonstrated that chromosomal termini have an evolutionarily conserved structure and function. Subsequently, meticulous biochemical studies revealed the existence of a previously predicted enzyme, named telomerase, responsible for synthesis of chromosomal DNA ends, with the novel feature of dependence on an intrinsic RNA template. Deficiency of telomerase results in a gradual shortening of telomere repeats upon successive cell divisions, limiting viability and ending with cell death in a process called replicative senescence. In humans, mutations in genes encoding components of the telomerase complex cause hereditary disease characterized by cancer predisposition and defects in stem cell renewal and tissue maintenance. Being able to proliferate indefinitely most cancer cells maintain telomeres via increased telomerase activity. The discovery of telomerase has deeply influenced biomedical research and led to the development of cancer therapies presently under evaluation.*

## Introduction

In the beginning of the previous century it was recognized that chromosomes, present in the cell nucleus, carry the genetic information (Thomas H Morgan; Nobel Prize in Physiology or Medicine 1933). Hermann Muller (Nobel Prize in Physiology or Medicine 1946) and Barbara McClintock (Nobel Prize in Physiology or Medicine 1983) both found that broken chromosomes were unstable and prone to rearrangements and fusion whereas the chromosome ends were protected from such events[1,2]. The special nature of chromosomal ends was noted and they were named telomeres (from the Greek words telos (end) and meros (part)) by Muller. However, the molecular nature of the telomeres was unknown.

Another interesting problem presented itself after the structure of DNA was unravelled[3] (Nobel Prize in Physiology or Medicine to Francis Crick, James Watson and Maurice Wilkins 1962) and DNA polymerases were discovered[4] (Nobel Prize in Physiology or Medicine to Arthur Kornberg 1959). Since DNA polymerases are dependent on a preformed primer to initiate replication it appeared paradoxical that complete synthesis of the ends of linear DNA present in eukaryotic chromosomes can be achieved. James Watson and Alexsei Olovnikov independently recognized this problem and pointed out that the newly formed DNA molecule will be left with a single-stranded tail[5,6]. Moreover, it was postulated that such incomplete replication would lead to a shortening of chromosomes with time, resulting in reduced viability. Olovnikov also pointed out

the possible link between chromosome shortening and the limited replicative lifespan of human somatic cells observed in culture[6,7]. The "end-replication problem" (Fig. 1) must of course have a satisfactory solution in order to allow faithful replication of chromosomes in every cell division. This is a particular requirement for eukaryotic life as we know it. In most prokaryotes and viruses the DNA making up the genome is circular thereby circumventing this difficulty.
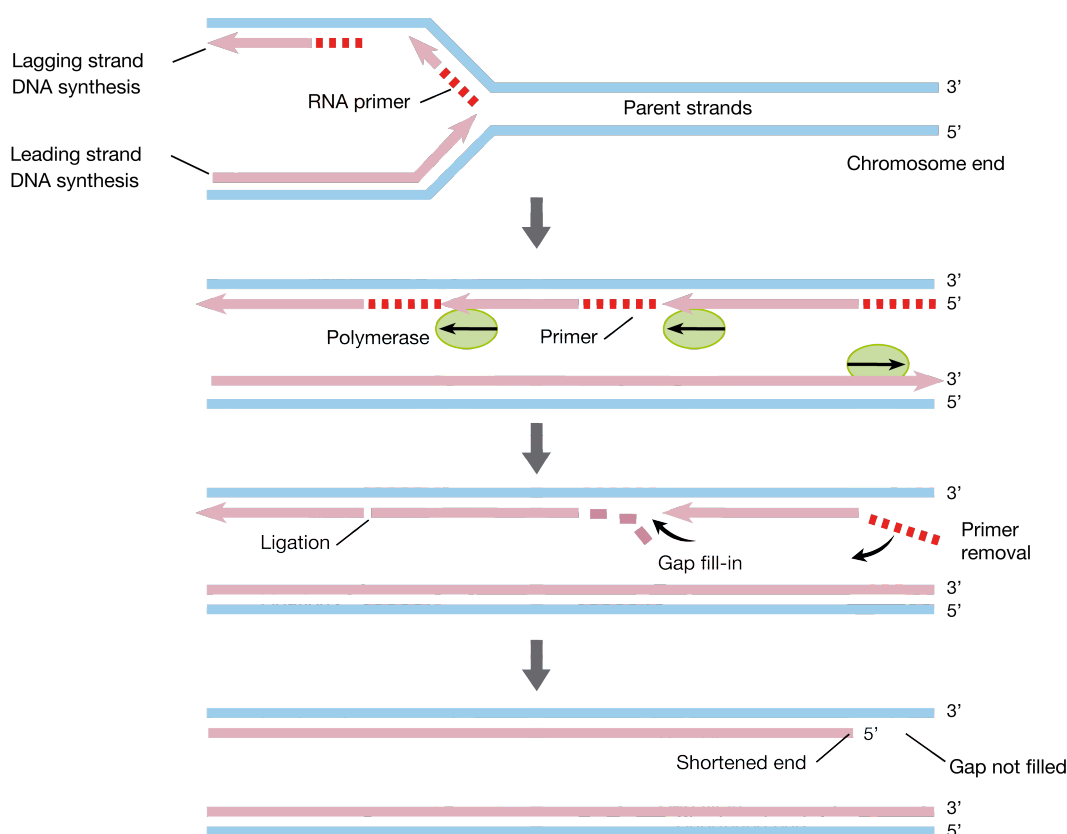


Figure 1. The end-replication problem. Copying of the lagging strand of a linear DNA molecule by DNA polymerase occurs in a stepwise fashion dependent on an RNA primer that is subsequently degraded. The gap is filled-in and ligated to produce an intact DNA molecule. At the very end, DNA polymerases are not able to fill the gap with the result that the end will become shorter upon each round of replication.

## Setting the stage for the discoveries

The presence of DNA repeats near or at the ends of linear eukaryotic chromosomes had been recognized by the early 1970s[8,9] but detailed end sequence information did not become available until 1978. In 1975 Elizabeth Blackburn arrived in the laboratory of Joseph Gall at Yale after completing her Ph.D. studies in the laboratory of Frederick Sanger in Cambridge (Nobel Prize in Chemistry 1958 and 1980), where she learned to master an early version of DNA sequencing. Joseph Gall had an interest in genes encoding ribosomal RNA (rDNA) and had shown that in certain organisms extrachromosomal rDNA genes are heavily amplified. Specifically, using the unicellular ciliate *Tetrahymena thermophila* as a model organism he found that the amplified rDNA was present as linear DNA[10]. Elizabeth

Blackburn set out to determine the end sequence of the rDNA molecules in order to provide information relevant to the amplification and replication mechanisms. The results showed that the rDNA molecules contained tandem repeats of the hexanucleotide CCCCAA at each end[11]. In addition it was noted that the number of repeats varied between 20 and 70.

By the late 1970s Jack Szostak had established his own laboratory at Harvard Medical School and was working on the mechanisms underlying homologous recombination in yeast. He had found that the ends of linear plasmid DNA molecules introduced into yeast cells were highly recombinogenic, promoting rearrangement or interaction with homologous chromosomal DNA and integration, and long-term maintenance was not possible[12]. These findings were in line with the early observations of Muller and McClintock mentioned above that broken chromosomes are highly unstable. This related to another interest of his laboratory, concerning the possibility of constructing artificial yeast chromosomes, for which stability and faithful replication is required.

## The Discoveries

### *Stabilization of chromosomes by telomere DNA is evolutionarily conserved*

A crucial step was initiated when Elizabeth Blackburn and Jack Szostak attended the same Gordon Conference in 1980. They decided to collaborate to investigate if adding *Tetrahymena* chromosome end repeat DNA sequences to linear yeast plasmids could stabilize the ends to allow replication. A 1.5 kb fragment from *Tetrahymena* rDNA was ligated to both ends of a linearized plasmid containing a yeast derived origin of replication and the ligated molecule was used to transform yeast cells in an experiment transcending eukaryotic kingdom boundaries (Fig. 2).

The approach worked beautifully, with a majority of transformants maintaining the hybrid plasmid in a linear form. By replacing one of the ends derived from *Tetrahymena* and adding fragments of yeast DNA, followed by selection, they could also clone yeast sequences
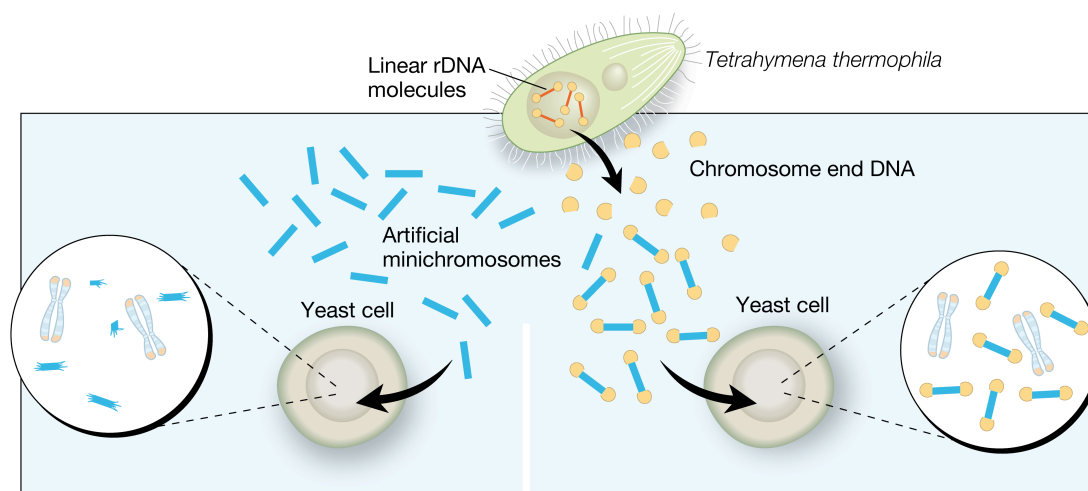


Figure 2. The DNA end-sequence of linear DNA molecules from *Tetrahymena* added to artificial minichromosomes allows their long-term stable maintenance in yeast. Conservation of the structure and function of chromosomal termini between very distant species is striking.

functionally equivalent to the *Tetrahymena* rDNA end fragment. The yeast end fragments showed similarities to the *Tetrahymena* counterparts and were present in the yeast genome in a copy number consistent with a contribution to every chromosome. Szostak and Blackburn published the results of the study in *Cell* in 1982[13].

**A key conclusion emerging from these experiments was that the striking and evolutionarily conserved functional properties of the *Tetrahymena* rDNA end sequences provided chromosomal stability. This, combined with the presence of functionally similar sequences in yeast, allowed the deduction that such end sequences corresponded to functional telomeres.**

Meanwhile it was shown that CCCCAA hexanucleotide arrays found on rDNA molecules were present in many locations within the *Tetrahymena thermophila* genome, consistent with a general function. Moreover, similar repeats were also present in three other *Tetrahymena* species[14] as well as at the ends of rDNA molecules in the molds *Physarium* and *Dicytostelium* [15,16]. Studies in the now independent laboratory of Elizabeth Blackburn at the University of California, Berkeley and from other research groups showed that the ends of linear rDNA and other DNA molecules varied in length and could be extended[17,18].

In a follow-up study the laboratories of Blackburn and Szostak identified the yeast telomere tandem repeat sequence as $C_{1-3}A$ and showed that telomere sequences from both *Tetrahymena* and yeast could be extended upon maintenance in yeast[19]. Remarkably, yeast telomeric repeats, which displayed an identifiable difference in sequence to the *Tetrahymena* counterpart, were added also to the *Tetrahymena* derived plasmid end.

Several different models were advocated to explain the complete replication of telomeres. These included the involvement of recombination or transposition events, the presence of palindromes or hairpin structures or perhaps a specific enzymatic activity synthesizing telomere DNA de novo. Such an enzyme had to be different from any known enzyme.

### Telomerase synthesizes chromosome ends de novo

At this point Carol Greider had started as a graduate student in Blackburn's laboratory at Berkeley and together they embarked on an exploration of how telomere ends were synthesized, employing a biochemical approach. She developed an assay using *Tetrahymena* cell extracts taking advantage of the fact that on *Tetrahymena* rDNA molecules the required free 3'OH group is present on the G-rich DNA strand complementary to the strand containing the CCCCAA repeats. A synthetic DNA oligonucleotide, $(TTGGGG)_4$ , was thus used as a primer to initiate synthesis. Using this assay she could show addition of TTGGGG repeats in vitro, thus indicating the presence of enzymatic activity in her cell extracts, Fig 3. The terminal transferase-like enzymatic activity that added single nucleotides in a stepwise fashion was proteinaceous and unique in that it added only the specific telomere sequence in an apparently untemplated manner. Functional conservation of the enzymatic process was demonstrated in a critical experiment where *Tetrahymena* type TTGGGG hexanucleotide repeats were added to a primer corresponding to the distinct yeast telomeric DNA ending with GTGGG. It was also observed that an extra G was first added to the yeast primer containing three G residues at the end in order to allow the subsequent correct addition of *Tetrahymena* TTGGGG repeats with four G residues. Greider and Blackburn published the results in *Cell* in 1985[20]

Figure 3. Primer specificity of the elongation reaction.
Different synthetic single-stranded oligomers were added
to reactions containing a *Tetrahymena* cell extract,
radioactively labelled dGTP and unlabeled dTTP.
Primers were (TTGGGG)$_4$ (lane 5), the yeast telomeric
oligomer (lane 6), (CCCCAA)$_4$ (lane 7), a pBR322 sequence
oligomer (lane 8) and no oligomer (lane 9). The sizes of
input synthetic oligomers are shown in lanes 1-4.
The ladder of radioactively labelled bands corresponds to
DNA molecules containing increasing numbers of TTGGGG
repeats.
Figure taken from reference 20.
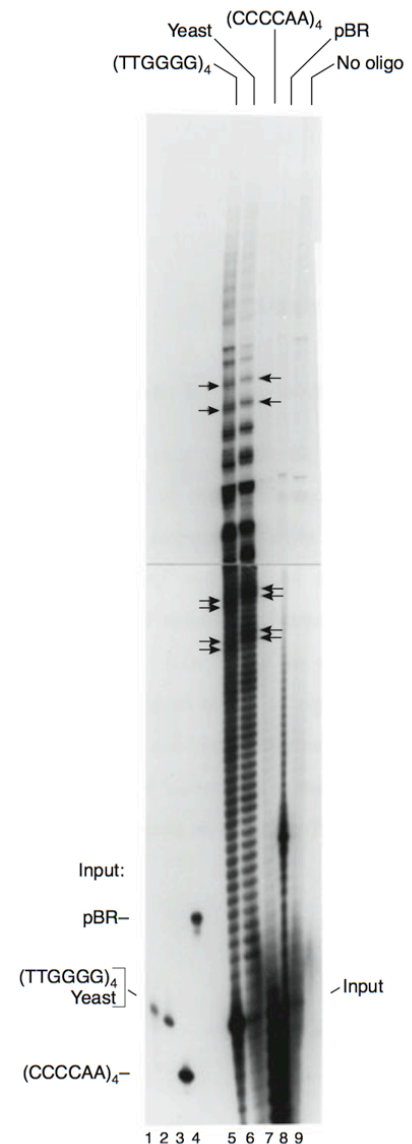
In a following study Greider and Blackburn showed the enzyme to be a ribonucleoprotein
complex critically dependent on both the protein and RNA component[21]. In this second
paper the name telomerase was adopted to denote the enzyme. With respect to a potential
role of the RNA component Greider and Blackburn wrote; " *It is tempting to speculate that
the RNA component might be involved in determining the sequence of the telomeric repeats
that are synthesized and/or the specific primer recognition. If the RNA of telomerase contains
the sequence CCCCAA, this sequence could act as an internal guide sequence*".

As an independent scientist at Cold Spring Harbor Laboratory Carol Greider continued to
work on telomerase in collaboration with Blackburn in an effort to clone and characterize
the small RNA that co-purified with the telomerase. As reported by Greider and Blackburn in
an article in *Nature* in 1989 the RNA component was indeed found to contain the sequence
CAACCCCAA, suggested to be the template for the synthesis of TTGGGG repeats[22]. Blocking
availability of this sequence motif compromised telomerase enzymatic activity. Final
proof of the role of the CAACCCCAA RNA sequence as a template for telomere synthesis was

provided when Blackburn's group showed that mutations in the RNA sequence resulted in corresponding alterations in the DNA sequence being synthesized[23].

**At this point the salient key features of the telomerase enzyme as a unique type of reverse transcriptase with a catalytic protein component and an intrinsic RNA template had been elucidated. The basic mechanism of telomere synthesis and maintenance was thus established. (Fig 4.)**
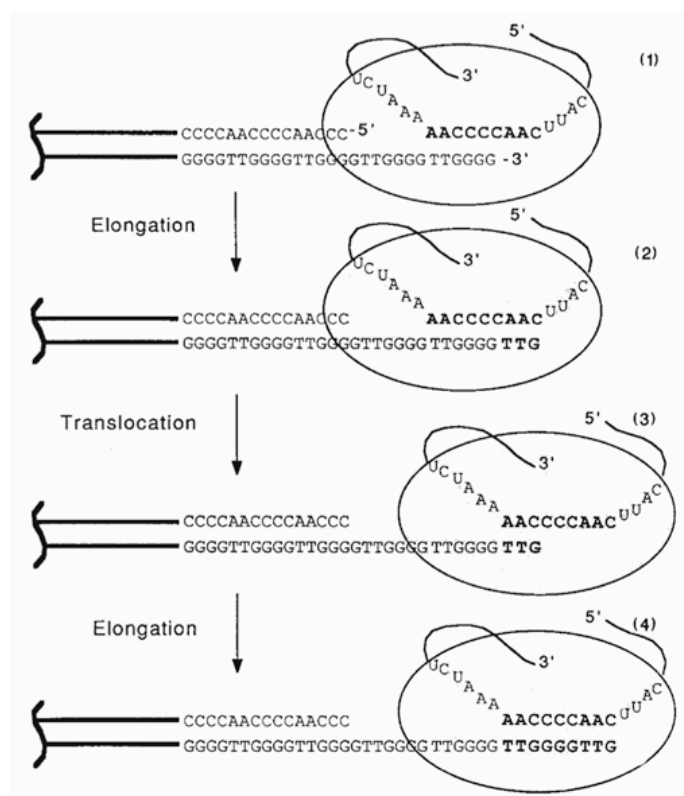


Figure 4. Model for elongation of telomeres by telomerase. The *Tetrahymena* telomere is shown containing a 13-base overhanging TTGGGG strand. (1) After recognition of the TTGGGG strand by telomerase, the 3' most nucleotides are hybridized to the CAACCCCAA sequence in the RNA. (2) The sequence TTG is then added one nucleotide at a time. (3) Translocation then repositions the 3' end of the TTGGGG strand such that the 3' most TTG nucleotides are hybridized to the RNA component of telomerase. (4) Elongation occurs again, copying the template sequence to complete the TTGGGGTTGG sequence. This mechanism explains how oligonucleotides with 3' ends terminating at any nucleotide with the sequence TTGGGG are correctly elongated to yield perfect tandem repeats of (TTGGGG)$_n$.
Figure taken from reference 22.

It is worth noting that the discoveries of general principles of chromosomal maintenance in eukaryotes, including mammals and primates, were all made by studying relatively simple model organisms.

## Further developments

Szostak's laboratory, in a parallel effort to identify the telomerase, had embarked on a genetic approach in yeast and isolated a mutant named *EST1* (ever shorter telomeres). This mutant displays a progressive loss of telomere sequences accompanied by an increased frequency of chromosome loss and a delayed senescence phenotype[24]. This finding suggested an important role of telomere maintenance for genome stability and cell viability. With respect to cell division it was stated; "*a loss of telomeric DNA during each somatic cell division may play a role in determining the number of cell divisions possible in a given cell lineage*". In the study of mutated telomerase RNA in *Tetrahymena* Blackburn's laboratory found one mutant with a corresponding phenotype also involving senescence and nuclear defects, further supporting the notion that telomere maintenance is important for genome stability and cell viability[23].

We now know much more about the structure and function of telomeres, the telomerase multiprotein complex and the regulatory role of transcribed telomeric RNA[25,26] as well as about epigenetic modulation of telomeres[27]. It is noteworthy that the gene corresponding to the *EST1* yeast mutant has been shown to encode a protein that is part of the telomerase complex and serves a regulatory function[28]. Several non-canonical functions of telomerase components have emerged, a recent example being the interaction between the Telomerase Reverse Transcriptase (TERT) catalytic subunit and the RNA component of a proposed mitochondrial RNA processing endoribonuclease RMRP. This ribonucleoprotein complex contains an unexpected RNA-dependent RNA-polymerase activity involved in the production of dsRNA molecules[29]. A continued flow of new data is expected.

## Significance of the discovery of telomere function and telomerase

These discoveries have significant medical implications for many fields, including cancer, ageing, stem cell maintenance and hereditary disease syndromes.

### *Cancer*

It was soon discovered that telomeres in many cancer cell lines are abnormal[30,31] and that telomerase activity is increased in 80-90% of cancers[32,33]. Tumours without telomerase activity often exhibit activation of an Alternative Lengthening of Telomeres (ALT) mechanism[34], consistent with the infinite replicative capacity of cancer cells. These findings are also consistent with observations that too short or unprotected telomeres induce senescence and ultimately cell death, which is now believed to be an important protective mechanism preventing unlimited cell growth and cancer development, Fig. 5.

The above observations have led to development of numerous therapeutic strategies targeting telomerase, either by inhibition of enzyme activity or by employing a cancer vaccine approach to kill telomerase overexpressing tumour cells. At present a number of clinical trials in a wide variety of cancer forms are ongoing, mostly involving telomerase based cancer vaccines (clinicaltrials.gov). However, no conclusive results are as yet available.

There are also a number of potential hurdles, one being that senescence and apoptosis induced by impaired telomere function appears to be p53 dependent[35] and most tumours present with inactivating p53 mutations. An additional complicating factor is the observation in mouse models that tumours can develop in the absence of telomerase activity and that short telomeres may in some situations enhance genomic instability and accelerate tumour development. This is consistent with the cancer predisposition observed in familial disease syndromes characterized by defects in telomerase function[36]. Greider's group recently reported that in mice the presence of short telomeres initiates telomere recombination and other telomere maintenance mechanisms, both in non-transformed primary cells and in tumour cells[37], suggesting that drug resistance may show up as a problem. Yet another factor of potential concern is that normal adult stem cells have been reported to have unusually long telomeres and possess telomerase activity[38]. This again implies a risk of unwanted side effects of anti-telomerase therapies.

Genome-wide association studies have very recently implicated TERT as a susceptibility gene for the development of many cancer types including lung cancer and glioma[39-41] and genetic TERT variants that reduce telomerase activity are reported as risk factors for acute myeloid leukemia[42].

Taken together this means that there is still much to learn about the basic biology behind anti-telomerase therapy and whether or not such therapy will be effective against human cancer.
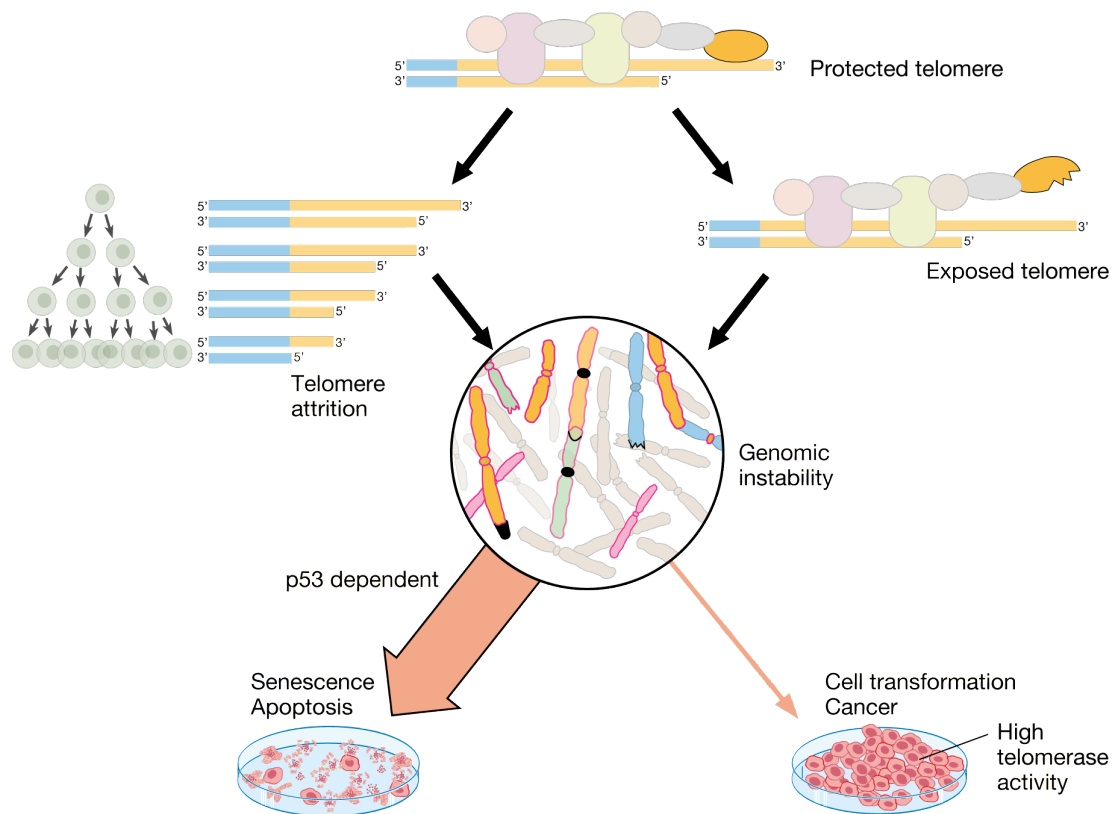
Figure 5. Schematic model of how short or unprotected telomere ends cause genomic instability followed by cell proliferation arrest and cell death. Sometimes the genetic changes allows escape from this response and instead lead to cell transformation and cancer.

Regardless of the complexities described above the studies of the role of telomere maintenance in tumours have proved to be very valuable for our understanding of basic cancer biology.

### *Ageing*

Early experiments showed a striking association between telomere shortening and a reduction of the replicative life span of cultured human cells[43], consistent with the early genetic evidence obtained in model systems that short telomeres induce senescence[23,24]. Conversely, introduction of telomerase into normal human cells in culture extends life span in the apparent absence of other cellular alterations[44]. However, other studies indicate that in epithelial cells secondary events such as lowering p16/INK4a expression by mutational inactivation or promoter methylation play an important role for the acquisition of apparent immortality[45,46]. The interpretation of the results from studies overexpressing the protein component of the protein of human telomerase,TERT, is complicated by the fact that we now know that TERT has several other activities unrelated to telomere maintenance, potentially contributing to increased cell proliferation[33].

A link between organismal ageing and both telomerase activity and telomere length is supported by studies in telomerase deficient mice[47] and in mice overexpressing the TERT telomerase component[48]. However, the association between telomere length and cell maintainance is not absolute and compromised telomere function can cause tissue degeneration in the absence of telomere shortening[49]. Quite surprisingly, recent studies

show that TERT may also stimulate tissue progenitor cell proliferation independent of its reverse transcriptase activity. Instead, TERT mediated activation of a Myc- and Wnt-related transcriptional program takes place and illustrates a non-canonical mechanism of action[50,51]. Moreover, it has now been found that induced pluripotent stem cells (iPS) show telomere characteristics similar to embryonic stem cells with respect to epigenetic marks and that the decreasing reprogramming efficiency of cells from telomerase deficient mice can be rescued by telomerase reintroduction[52]. Undoubtedly, maintenance of telomere length and protection are important factors in the control of cellular life span but organismal ageing is a highly complex process influenced by many different factors. Research activity in this area is intense.

### *Hereditary disease syndromes*

Germ line mutations in the genes encoding the TERT or the Telomerase RNA Component (TERC) subunits of human telomerase, or in genes encoding some telomere-binding proteins, have been genetically linked to acquired and congenital aplastic anemias[53]. The syndrome X-linked Dyskeratosis Congenita (DKC) presents with mutations in the gene DKC1, encoding a protein that associates with ribosomal RNA and TERC[54,55]. Other forms of autosomal dominant dyskeratosis congenita are due to mutations in TERC , TERT or other genes encoding proteins involved in telomere maintenance, providing convincing evidence that telomerase deficiency is an underlying cause[56]. The key role of telomerase dysfunction in DKC provides a link between short telomeres and a human disease characterized by signs of premature aging such as hair loss/greying, dental loss and osteoporosis, in addition to the defining features of nail dystrophy, oral leukoplakia and abnormal skin pigmentation. Additional related syndromes have been associated with TERC mutations[55]. Heterozygous germ line mutations in TERT and TERC as well as short telomeres have been found in some patients with familial idiopathic pulmonary fibrosis[57,58] .

As expected for a broadly conserved cellular function of profound importance, genetic defects compromising telomere and telomerase integrity are disease causing. Knowledge about the underlying molecular mechanisms provides diagnostic possibilities and raises the hope of developing future therapies.

## Conclusion

Elizabeth Blackburn, Carol Greider and Jack Szostak have discovered how the conserved function of chromosomal telomere repeat sequences protects against degradation and recombination events and have identified a new enzyme complex, telomerase, that is responsible for the synthesis of telomere DNA. Studies of telomerase and telomere maintenance have provided very important insights into areas of high medical relevance such as cancer, ageing and hereditary disease syndromes although the connections are more complex than initially anticipated. The discoveries have also led to the development of new therapeutic strategies for cancer treatment based on the targeting of telomerase activity or expression that are now undergoing clinical testing.

The discoveries concerning telomere function and maintenance that are recognized by this year's Nobel Prize in Physiology or Medicine have solved one of the fundamental problems in biology and opened a whole new research field.

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