

## How to Protect the Chromosomal Ends?

Telomerase, Chromosome Stability and Aging

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The 2009 Nobel Prize in Physiology or Medicine was jointly awarded to three scientists – Elizabeth H Blackburn, Carol W Greider and Jack W Szostak – for their work during early 1980s and 1990s which helped unravel the molecular mechanisms of protection of chromosomal ends by telomeres and the enzyme telomerase. Their discovery has had major implications in the fields of ageing and cancer research. Blackburn and Greider are the ninth and tenth women scientist, to get this award in physiology or medicine and this is the first time that two women scientists have shared the prize.

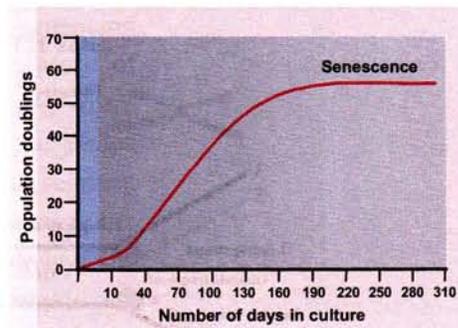
### The History

During the 19th century, when scientists had just begun to address the cellular basis of life, August Weismann proposed that “death takes place because a worn-out tissue cannot for ever renew itself and because a capacity for increase by means of cell-division is not everlasting, but finite”. He suggested that in future it would be shown that somatic cells would have *finite* capacity to divide, whereas the germ-line cells would have *indefinite* capacity. Perhaps this was the first mention of the concept of finite lifespan and immortalization. Alexis Carrel (Nobel Prize 1912) opposed this idea and believed that vertebrate cells are immortal in culture and can divide indefinitely. In fact, he performed a well-known experiment at the Rockefeller Institute for Medical Research, in which he could grow chick heart fibroblasts for over 34 years, which is much longer than the lifespan of the chicken itself! However, his experiment could never be completely replicated, casting doubts on his observation. In 1961, in contradiction to Alexis Carrel’s idea, Leonard Hayflick and Paul Moorhead demonstrated that cultured normal diploid human fibroblasts grew for only 60–80 population doublings after which



they stopped dividing and underwent a growth arrest, termed as ‘senescence’ (Figure 1) [1]. This limitation to cellular proliferation is termed as the ‘Hayflick limit’ and represents the first proposal for a cellular counting mechanism.

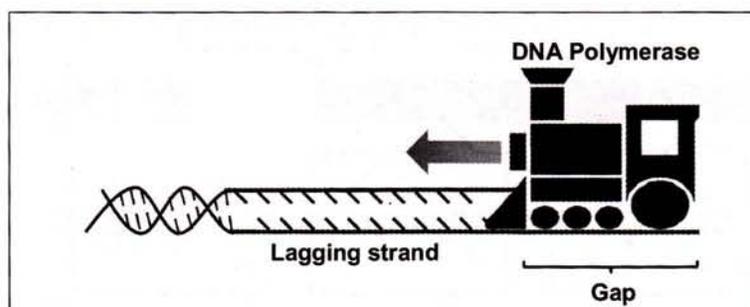
In the 1930s, independent of studies on cell immortality, Hermann Müller (Nobel Prize in 1946) and Barbara McClintock (Nobel Prize in 1983) observed that chromosome ends had special properties that protected them from end to end fusion. Müller called these structures as “telomeres” (from Greek, *telos* for end and *meros* for part). Even though these structures were recognized to be critical for the integrity of DNA at chromosomal ends, their detailed structure remained unknown. In the 1970s, as the mechanism of DNA replication became clearer, James Watson (Nobel Prize in 1962) noted that DNA polymerase, the enzyme responsible for DNA replication, could not fully synthesize the 3’ end of linear DNA (Box 1). In 1973, while walking into the Moscow railway station after hearing some of Hayflick’s work being discussed, a Russian theoretical biologist, Alexey Olovnikov, saw an analogy between the train representing the ‘DNA polymerase’ and the track representing the DNA (Figure 2). He mused that if the ‘train’ replicated the DNA track underneath the car, the first segment of DNA would not be replicated because it was underneath the engine at the start. Together, Watson and Olovnikov termed this as the ‘end-replication problem’ (Box 1). Olovnikov immediately recognized that the end-replication problem would result in telomere shortening with each round of



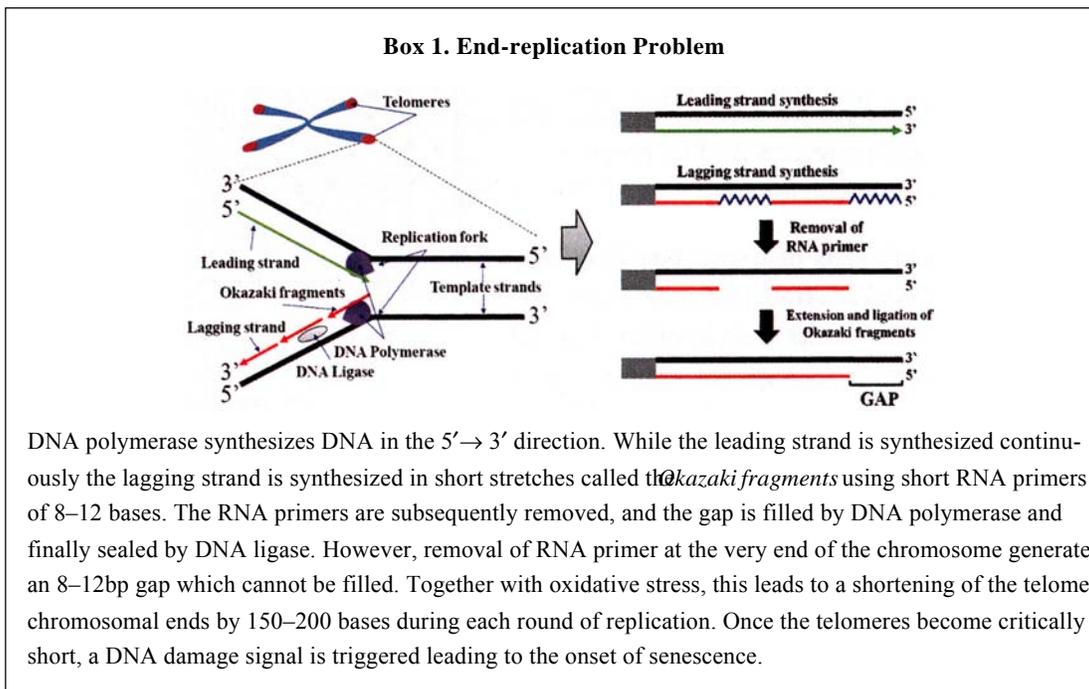
**Figure 1. Fate of human somatic cells in culture.**

#### Keywords

Chromosomes, telomeres, telomerase, immortalization, cancer. Nobel Prize in Physiology or Medicine.



**Figure 2. Alexey Olovnikov's hypothetical model of end-replication problem.**

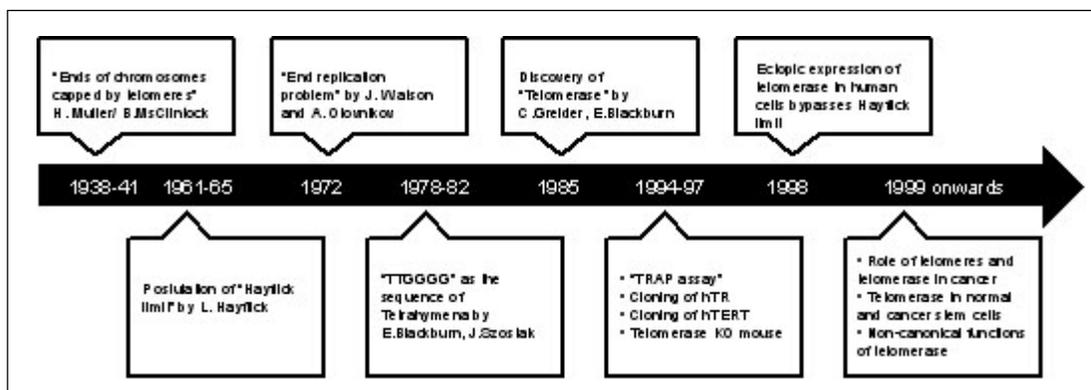


replication and further proposed ‘telomere erosion’ as a cause for the finite replicative potential observed by Hayflick in his experiments.

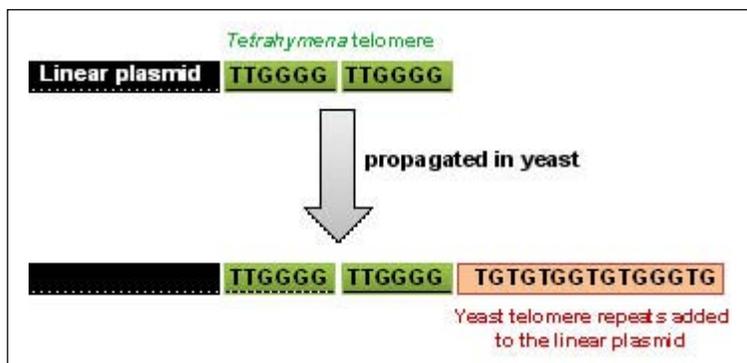
### The Discovery

With these findings and speculations in the background (Figure 3), during the mid 1970s, Elizabeth Blackburn, who had finished her PhD under the guidance of Fred Sanger at the University of Cambridge, wanted to apply new sequencing

**Figure 3. Timeline of telomere and telomerase research.**



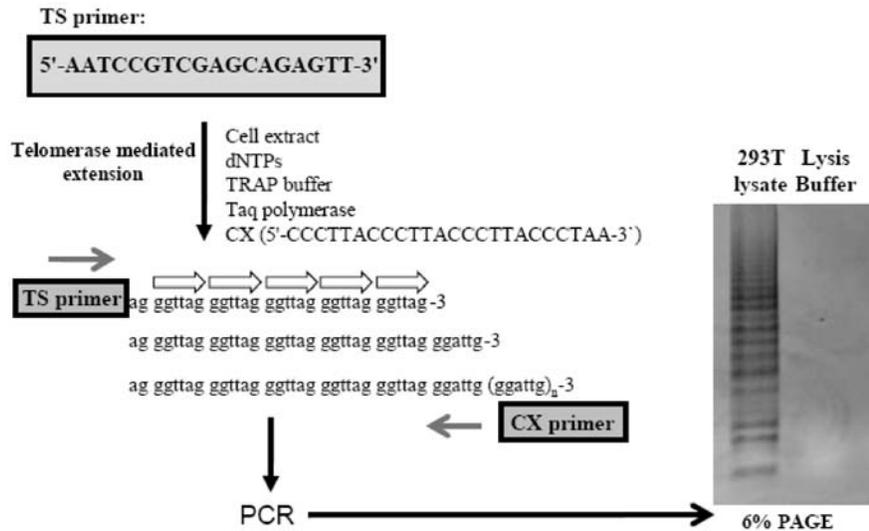
approaches to study telomeres. She joined the laboratory of Joseph Gall at Yale University as a postdoctoral fellow, where she sequenced the telomeres of a single-celled protozoan called *Tetrahymena* and found that they contained a six-base segment TTGGGG that repeated 20–70 times. Later, while starting her own laboratory at the University of California, Berkeley, she teamed up with Jack W Szostak who was at that time working on yeast cells at Harvard Medical School and constructed artificial yeast linear plasmids. In 1982 they found that when *Tetrahymena* telomeric DNA sequence was added to these engineered chromosomes and introduced into yeast cells, the sequence was able to protect the linear chromosomes even in distantly related yeasts [2]. They showed subsequently that yeast could extend the ends of inserted *Tetrahymena* telomeres by adding new DNA bases that resembled the yeast telomere sequence rather than that of *Tetrahymena* (Figure 4) [3]. Based on these observations Blackburn and Szostak speculated that an activity intrinsic to yeasts was responsible for adding nucleotides and extending the ends of *Tetrahymena* telomeres. They termed this function as the *telomere terminal transferase* or *telomerase* activity. The challenging task of identifying and characterizing the molecular entity that extends the ends of chromosomes became the PhD thesis topic for Carol W Greider, PhD student in Blackburn's laboratory. In 1985, Greider and Blackburn demonstrated the addition of repeats of six-base segments of DNA to synthetic GT-rich primers that represented either the *Tetrahymena* or yeast telomeric ends in cell-free extracts (Box 2) [4]. Their subsequent



**Figure 4. The cross-kingdom experiment.**



## Box 2. How to Detect Telomerase Activity



In 1985, Greider and Blackburn identified a telomere terminal transferase activity in *Tetrahymena* cell-free extracts that could add 6 base repeats onto a synthetic telomere primer under *in vitro* conditions [1]. They developed an assay in which they added a synthetic telomere primer (TTGGGG)<sub>4</sub> to *Tetrahymena* cell-free extract along with  $\alpha$ -<sup>32</sup>P-dNTPs and unlabelled dNTPs. After incubation for 90 minutes at 30°C, the reaction mixtures were phenol extracted, ethanol precipitated, run on a 6% polyacrylamide, 7M urea sequencing gel and analyzed by autoradiography. They observed a 6 base pair repeating pattern which confirmed the addition of 6 base pair repeats by the then so-called 'telomere terminal transferase' activity, or *telomerase* activity. This represented the first of the many such 6 base pair ladder pictures developed since then demonstrating telomerase activity. Assaying for telomerase enzymatic activity was later improvised by various groups, most notable being TRAP (Telomeric Repeat Amplification Protocol) assay [5] as shown in the figure. In this modified assay, the TS primer acts as a substrate for telomerase-mediated addition of TTAGGG repeats. Together, the TS and CX primers amplify the extended products in a PCR reaction, and the products are then fractionated on a polyacrylamide gel.

studies showed that the *telomerase* is a ribonucleoprotein and utilizes a telomeric sequence present within its RNA component as the template for adding the necessary bases to the ends of the telomeres [5, 6].

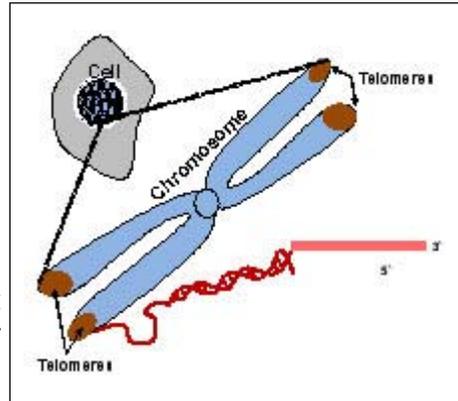
### Telomeres and Telomerase

Telomeres are nucleoprotein structures found at the ends of linear chromosomes (*Figure 5*) which play an important role in chromo-



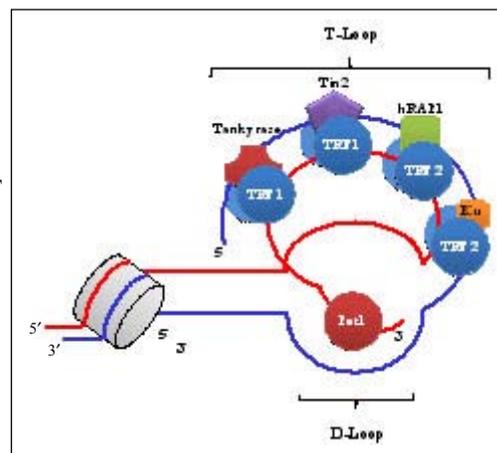
some structure and function. In most of the eukaryotic cells, telomeric DNA consists of G-rich, tandem repeating sequences running from 5' to 3' direction. For e.g., the *Tetrahymena* telomeric DNA contains repeats of TTGGGG, the yeast contains  $TG_{(1-3)}$  and mouse and human telomeres contain repeats of TTAGGG. The length of telomeres is highly variable across species with some ciliates having 50 bases long telomeres while mice have unusually long hundreds of kilobases (kb) of telomeres. The length of telomeres can vary from tissue to tissue, and even within a single cell each chromosome might have telomeres of different sizes. In humans, the telomeres are comprised of several TTAGGG repeats covering 5–20 kb in size. Generally the vertebrate telomeres end with a 3' single-stranded stretch, few hundred nucleotides long. This single-stranded 3' overhang folds back onto the double-stranded DNA to form a larger T-loop with lariat structure, thus protecting the chromosomal ends. This 3' overhang anneals within the double-stranded DNA by displacing one strand and forms a smaller D-loop (Figure 6). Various telomere-associated proteins interact with both the single and double stranded regions of the telomeres and their interplay has an important role in maintaining the telomere structure and function (Table 1).

Telomerase is an RNA-dependent DNA polymerase that adds telomeric repeat units to the ends of linear chromosomes to elongate it. It is made up of essentially two components: one is the RNA component termed hTR (human Telomerase RNA), which serves as a template for telomeric DNA synthesis, and the other is the catalytic subunit termed hTERT (human Telomerase Reverse Transcriptase) which has a reverse transcriptase activity (synthesizing RNA from DNA is called transcription, whereas synthesizing DNA using RNA as template is called *reverse* transcription). While hTR is found ubiquitously in all tissues, the expression of hTERT



**Figure 5. Telomeres: the protective ends of chromosomes.**

**Figure 6. Structure of telomeric end along with associated telomere-binding proteins.**



Protein name	Function
<b>TRF1</b> Telomere repeat binding factors	Negative regulator of telomeric length, telomerase repressor
<b>TRF2</b> Telomere repeat binding factors	Negative regulator of telomeric length, telomerase repressor
<b>Pot1</b> Protection of telomeres 1	Transfer TL information ss- to ds-DNA
<b>Tankyrase1</b> TRF1-interacting ankyrin-related ADP-ribose polymerases 1	TRF1 down-regulation
<b>Tin2</b> TRF1 interacting nuclear protein 2	Control PARP activity tankyrase
<b>PinX1</b> PIN2-interacting protein 1	Inhibition of telomerase activity
<b>hRap1</b> Human repressor activator protein 1	Negative regulator TL (exact function unknown). Component of the DNA repair response system
<b>Pot1</b> Protection of telomeres protein 1	Binds to single strand of telomeric DNA, involved in telomere-length maintenance and telomere protection

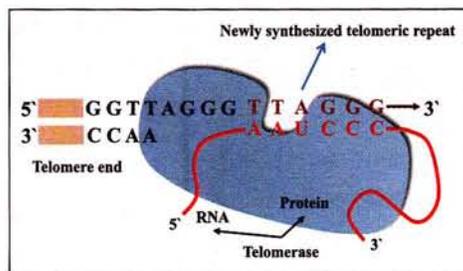
**Table 1.**  
**Telomere-associated proteins and their functions.**

is restricted to germ-line cells and cancer cells. It took almost a decade after the demonstration of telomerase function for the protein responsible for this activity to be identified. The catalytic subunit of telomerase was initially purified from *Euplotes*, a ciliated protozoan, by Thomas R Cech's group. Sequence analysis showed that it contained a reverse transcriptase (RT) motif and was homologous to a protein identified by Vicki Lundblad's group in a genetic screen in yeast that was required for yeast telomere maintenance. Thereafter, based on the conserved sequence in the RT-domain, the cDNA encoding the human homologue of the catalytic subunit of telomerase (hTERT) was identified almost simultaneously by the groups of Thomas R Cech and Robert A Weinberg.

The catalytic subunit of telomerase protein is shaped similar to a hand and its 'fingers' and the 'thumb' pull the hTR and telomeric



The catalytic subunit of telomerase protein is shaped similar to a hand and its 'fingers' and the 'thumb' pull the hTR and telomeric DNA into the active site (the 'palm'). Telomerase elongates the G-rich 3' overhang at the end of telomeres by a reiterative reverse transcription mechanism. Elongation process starts with telomerase recognizing the 3' overhang of the telomere. Then the hTR template base-pairs with the telomeric repeat sequence to initiate elongation of the 3' DNA end. The RNA template has nucleotides that match the TTAGGG repeat sequence such that only one repeat of the sequence can be added in a single elongation (*Figure 7*). Telomerase continues to synthesize telomeric repeats on the same DNA strand by unwinding the DNA–RNA hybrid, holding the DNA end while the RNA slides down 6 bases and allows the next alignment and base-pairing. Telomerase continues elongation of the telomere sequence adding DNA in 6-base units. This 6-base-pair addition by telomerase is utilized for detecting its activity in a ladder pattern by TRAP assay (Telomeric Repeat Amplification Protocol assay) (*Box 2*) [7]. Studies using this sensitive assay have shown that telomerase activity is restricted to cancer cells, adult germ-line cells and embryonic stem cells, while it is undetected in normal human somatic cells.

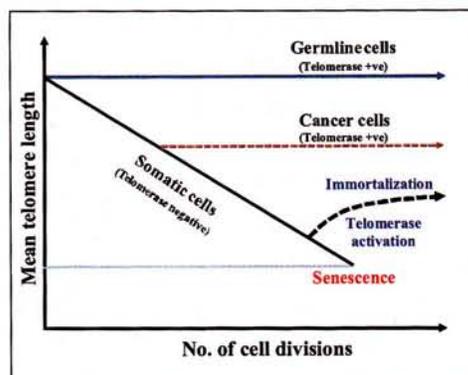


**Figure 7. Addition of nucleotides at telomeres by telomerase.**

### Senescence, Aging and Cancer

Since most somatic cells do not show detectable levels of telomerase activity, they conform to the Hayflick limit and undergo senescence after a limited number of divisions *in vitro*. Senescent cells are characterized by a large, flat morphology, a high frequency of nuclear abnormalities and a positive staining  $\beta$ -galactosidase activity at pH 6.0. The telomere erosion hypothesis predicted that with successive cell divisions, the telomeres get shortened and critically short telomeres lead to senescence (*Figure 8*). This hypothesis gained popularity when experiments demonstrated that cells of increased passage numbers had shorter

**Figure 8. Relation between telomere length, telomerase and cell division.**



Cells from older donors had shorter telomere length and became senescent after fewer population doublings in culture compared to younger donors, suggesting that senescence at the cellular level could be correlated with organismal aging.

In contrast to normal somatic cells, germ cells, some adult stem cells and most cancer cells show high levels of telomerase activity, emphasizing the requirement of telomere stabilization for their prolonged proliferation.

fewer population doublings in culture compared to younger donors, suggesting that senescence at the cellular level could be correlated with organismal aging. Indeed age-related diseases and premature ageing syndromes are characterized by short telomeres. If telomere shortening is the key determinant of cellular life span, then preventing this shortening should lead to immortality. Experiments in the early 90's demonstrated that re-constitution of telomerase activity in somatic cells such as human diploid fibroblasts extended and maintained their telomere length and rendered them immortal, i.e., grow beyond the Hayflick limit of 60–80 population doubling (Figure 8). More recently, over-expression of telomerase was shown to increase the longevity of *C. elegans*. Together, these experiments demonstrate a crucial role for telomere maintenance in cellular and organismal lifespan.

In contrast to normal somatic cells, germ cells, some adult stem cells and most cancer cells show high levels of telomerase activity, emphasizing the requirement of telomere stabilization for their prolonged proliferation. For decades scientists had failed to transform normal human cells into cancerous cells *in vitro*. With the discovery of telomerase, introduction of a combination of genetic elements along with hTERT sufficed to convert normal human cells into cancerous cells [8]. On the other hand, inhibition of telomerase in human cancer cells halts their growth, induces apoptosis and disrupts their ability to form tumors, clearly demonstrating that telomerase activation is an essential component of carcinogenesis. In addition to its role in extending and maintaining telomeres, several non-telomeric functions of telomerase that contribute to carcinogenesis have been demonstrated in recent years. All these observations have made telomerase a very attractive target for cancer therapeutics.

Taken together, these observations reveal that telomere length and telomerase activity are important factors in the pathobiology of several human diseases. Thus, the early discoveries of telomeres and telomerase structure and function by Greider, Blackburn and Szostak have unveiled a path to understanding the molecular bases underlying stem cell biology, ageing, cancer and





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other related human diseases, and continue to be an intensively studied topic across the world.

### Suggested Reading

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