

TELOMERES AND TELOMERASE: THE MEANS TO THE END

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by

ELIZABETH H. BLACKBURN

Department of Biochemistry and Biophysics, The University of California
San Francisco, San Francisco, CA 94158, U.S.A.

INTRODUCTION

DNA carries coding and noncoding sequences. Noncoding DNA both regulates and ensures the continued inheritance of DNA's coding information. In eukaryotes, by protecting the chromosome ends and thereby the chromosomes themselves, telomeric DNA is a class of noncoding DNA that ensures the stable inheritance of the genetic material. Research begun in the 1930s on the cytogenetics of telomeres was followed by a molecular understanding of telomeric DNA and its maintenance, which began in the 1970s and continues apace today. This fundamental, question-driven basic research has led into realms of human health and disease that have turned out to inform medicine in new ways.

BEGINNING THE ENDS

"You corn kernels, ... may you succeed, may you be accurate."

Popul Vuh

Tracing the beginnings of the interwoven stories of science can be arbitrary, as beginnings are so often lost in the mists of time. For me, arguably the story of telomeres and telomerase began thousands of years ago, in the cornfields of the Maya Highlands of Central America. Today, under the brilliant, shifting sunlight of the Central American highlands, lush corn plants cover every inch of sloping land wherever they can gain a foothold. There, over millennia, agricultural breeding generated corn (maize) crops from the ancestral plant teocinte. Estimates place the early cultivation of corn in the Central American highlands to around 7,000 years ago, and while early maize cobs dated from then were tiny, over millennia they progressively got bigger and bigger. Agricultural breeding is a process of consciously selecting the "best" plants. It was known that "like begets like", so that if one used the kernels from the biggest ears of corn in the planting for next year, a better crop would result. Intensely cultivated areas were carved out of the Central American rainforests and devoted to the production of corn. Maize came to occupy a central position in the agriculture and culture of the ancient Maya,

and the Mayans had a maize goddess. Their ancient Council book, the Popul Vuh, includes many references to maize. The Popul Vuh even evokes genetic principles: “You corn kernels,... ...may you succeed, may you be accurate” (Popul Vuh).



Figure 1. A form of the Mayan corn god.

As maize became important for human food worldwide, modern agricultural research on maize breeding continued the corn breeding begun thousands of years ago in the Central American highlands.

THE TELOMERE CONCEPT

“This is the beginning of the end.”

Charles Maurice de Talleyrand 1754–1838 (announcing Napoleon’s defeat at Borodino).

Perhaps another, more modern beginning to the story of telomere research is the discovery of X-rays by Roentgen. Hermann Muller, working on the fruit fly *Drosophila*, showed that X-rays could be used to produce mutations and chromosome fragmentation. By the end of the 1920s it was understood that the hereditary material was in chromosomes: Mendel’s work, begun on heritable traits, had been integrated with findings showing that the inheritance

patterns of genetic traits (or genes) corresponded with the regular movements of chromosomes in cells in meiosis and mitosis. By the 1930s, in the United States maize breeding research using such genetic principles came to be undertaken under government sponsorship, in agricultural research stations. And in one such station, the Missouri Agricultural Research Station, the geneticist and cytologist Barbara McClintock worked with maize, using the methods she had developed for examination of individual chromosomes. Genes were arrayed along chromosomes, as Muller's work showed. But in the 1930s there was no particular interest in what was at the ends of those arrays of genes, until it was noticed that the ends had some distinct properties (for a brief review, see [1]) In the early 1930s McClintock concluded that "the natural ends" of chromosomes (McClintock's 1931 phrase; [2]) were functionally different from experimentally-induced or accidental chromosomal breaks. A "stickiness" of broken ends of chromosomes (causing chromosomal fusions) was one of their defining features, while in contrast telomeres, the natural ends of chromosomes, had no such stickiness. This recognition arose from McClintock's research on broken ends of chromosomes and their behavior [3]. Independently, Muller reached the same conclusion from his fruit fly work, and in 1938 named these ends "telomeres". [4] (Reviewed in [1]).

DIVING INTO POND WATER

"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

Sir Winston Churchill, Speech in November 1942

On reading the insightful early cytogenetic work of McClintock and Muller from the 1930s and 1940s, it is sometimes hard to remember that their deductions of the fundamental cytogenetic properties of the natural ends of chromosomes preceded any knowledge that the genetic material is DNA. The molecular mechanisms underlying the telomeric properties were completely unknown when, in the mid-1970s, I first began research using DNA purified from the ciliated protozoan *Tetrahymena*, as described below. From these molecular analyses emerged the nature of the specialized DNA-protein complex that comprises the telomere, distinguishing it from an accidental DNA break.

First, the sequence and structural features of telomeric DNA had to be understood. By the early to mid-1970s, viral and bacteriophage DNAs, and in some cases their ends, had been studied both biochemically and genetically. But what was the end of a cellular DNA in a eukaryotic nucleus – a chromosomal end – like? What was most daunting to a molecular biologist interested in that question in the 1970s, at the time just before the advent of DNA cloning methodologies, was the sheer length of typical chromosomal DNAs.

By the early 1970s Kavanoff and Zimm had carefully isolated chromosomal DNA in as intact a form as possible from fruit fly cells. [5] The kind of

molecular weight range they deduced corresponded to long DNA molecules extending from one end of the chromosomes to the other. Thus the typically long chromosomal DNAs from a cellular nucleus were thousands of times longer than phage DNAs. This presented an enormous technical hurdle with respect to analyzing their telomeric regions. Answering the question of the molecular nature of telomeres meant going into pond water.

The specific pond water denizen in question was a single-celled ciliated protozoan, *Tetrahymena thermophila*. In the early 1970s Joe Gall, at Yale University, had discovered that *Tetrahymena* harbors a class of abundant, homogenous, short, linear chromosomes (“minichromosomes”). These were the key to my being able to analyze telomeric DNA directly. I first encountered *Tetrahymena* when I joined Joe Gall’s lab as a postdoctoral fellow at Yale. Although single-celled, a ciliated protozoan such as *Tetrahymena thermophila* contains two different types of nucleus. As Grell describes it [6] “the majority of ciliates... have generative nuclei capable of unlimited reproduction as well as somatic nuclei which perish sooner or later to be re-formed by descendants of the generative nuclei.” Thus *Tetrahymena* economically combines both “soma” and “germline” into one cell. The abundant “minichromosomes” that Joe Gall discovered resided in the somatic nucleus. These linear DNA molecules bore the genes encoding ribosomal RNAs (rDNA), their high abundance ensuring sufficient expression for the large *Tetrahymena* cell. Kathleen Karrer, then a graduate student in Joe Gall’s lab, had just discovered that the rDNA molecules consisted of two equal halves in a palindromic arrangement. This made them even more attractive: each telomeric end region would be the same as the other end region! The abundance and relative shortness (only 20,000 base pairs) of these molecules would, I reasoned, make it feasible to apply methods on them like those that had been used by Ray Wu and colleagues, for example, to sequence nucleotides at the ends of bacteriophage DNA in the early 1970s.

It was with these rDNA molecules that, very soon after arriving at Yale in early 1975, I started to use methods for determining the DNA sequences at the rDNA ends. I describe my experiments that succeeded in piecing together the telomeric sequence in more detail in the autobiography in this volume. Briefly, initially, upon fractionation of depurination products of radiolabeled rDNA end regions, the strong CCCC sequence spot seen was particularly informative (Figure 1).

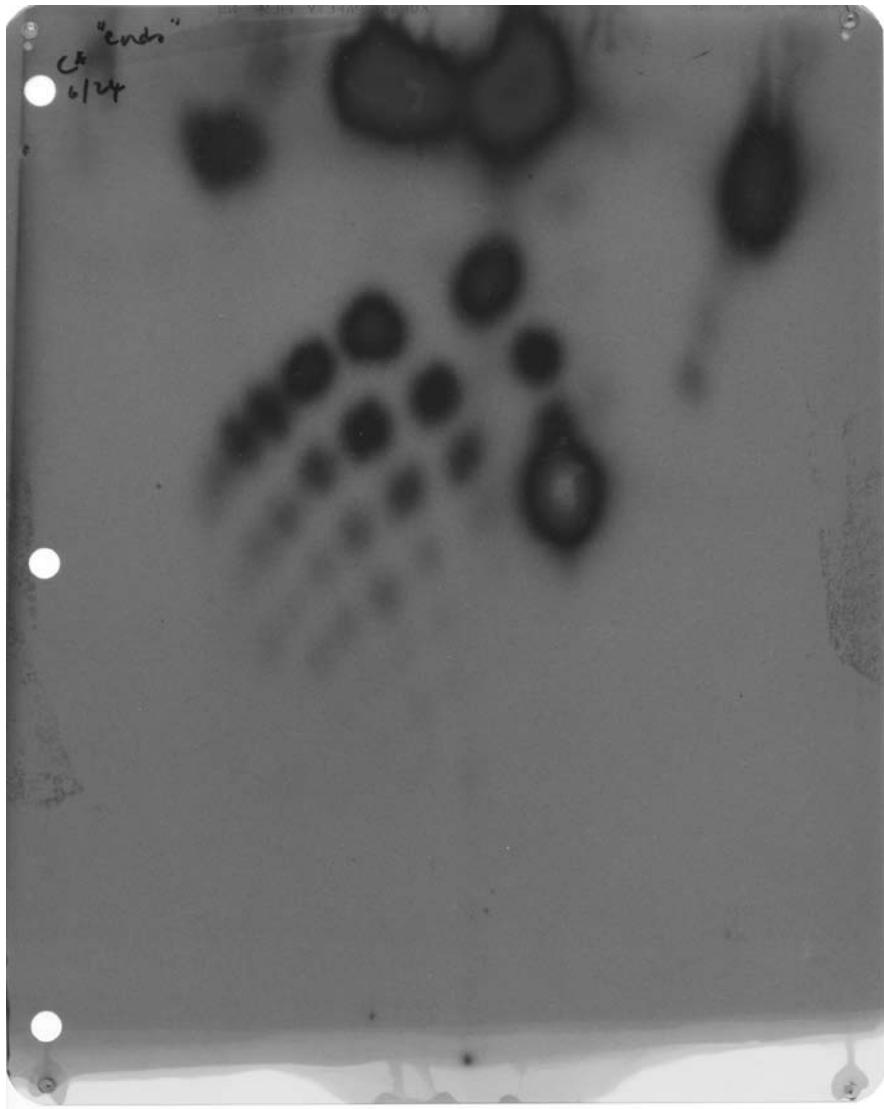


Figure 2. First autoradiogram showing a prominent CCCC sequence (the bottom right spot).

By August 1976 I was confident that the *Tetrahymena* rDNA molecules ended in (CCCAA)n sequence, and I was looking to see whether this same repeated sequence was also present in the other (much longer) chromosomal DNAs of *Tetrahymena*, and exploring the molecular structures of the DNA end regions. The results describing tandemly repeated CCCAA sequences at the rDNA ends were published in 1978. [7] One experiment done in 1979, radiolabeling the rDNA using just ^{32}P -labeled dCTP, and unlabeled dATP, and separating the products by denaturing gel electrophoresis, showed a

beautiful ladder of tiger stripes extending up the gel. The size of every band in this regular ladder was 6 bases more than the band below it – a strikingly visual confirmation of the repeated hexameric sequence I had deduced! This arrestingly characteristic pattern of bands was the first example of the pattern that would later become important for our discovery of telomerase enzyme activity, as described in the 2009 Nobel Lecture by my co-awardee, Carol Greider.

In my early work, our molecular views of telomeres were first focused on the DNA. This was not only because DNA was so central to the problem of incomplete replication of linear DNAs, as had been recognized by the early 1970s (reviewed in Blackburn and Szostak. [8], but also for several years DNA was the only component of the telomeres that was identified. By 1980, DNA sequences were known for the ends of a few different eukaryotic nuclear DNAs. By then we had shown that the DNA ends of *Tetrahymena* macronuclear DNAs in general, not only the rDNA molecules, consisted of long arrays of the same simple 6-nt CCCCAA (C_4A_2) repeats [9]. Others showed that the ciliate *Oxytricha* and its relatives had very short tracts of 8-nucleotide (C_4A_4) repeats at their macronuclear DNA ends, and that the high copy-number linear rDNA minichromosomes in two different slime molds similarly had tracts of simple repeat sequences, CCCTAA and $C_{1-8}T$ respectively (Reviewed in [10, 11] [12]) at their DNA ends. These all resembled the sequence I had found for the *Tetrahymena* rDNA molecules. But how did this emerging common DNA sequence arrangement at the ends of the nuclear DNA molecules inform us about the special properties of telomeres? One approach was to see what proteins might be on the telomeric DNA. I describe my early unsuccessful efforts to identify telomeric proteins in my autobiography in this volume.

Now we know that the essential telomeric sequences are surprisingly similar among phylogenetically widely divergent eukaryotes. Each end of a chromosome consists of a block of very simple telomeric sequences which are tandemly repeated over and over again, all the way to the molecular end of the chromosomal DNA. All of the chromosomes in a given organism have the same species-specific telomeric repeat sequence. However the same telomeric repeat sequence crops up in very diverse eukaryotes. For example, human telomeres consist of AGGGTT repeats, tandemly repeated for thousands of nucleotides at the ends of all of our chromosomes. The same repeated AGGGTT sequence is the telomeric sequence of the mold, *Neurospora*, the slime mold, *Physarum*, and the trypanosome protozoan parasites. (This makes telomeric DNA sequences possibly the world's worst sequences for deducing phylogenetic relationships!) Telomeric DNA generally has a strand composition asymmetry, resulting in a G-rich and a C-rich strand. It is the G rich strand that is always oriented in the 5' to 3' direction toward the end of the chromosome. This overall structural conservation of telomeres suggests that this general arrangement and composition of DNA strands is of fundamental importance for telomere function.

THE LINES OF EVIDENCE THAT LED TO THE CONCEPT THAT TELOMERASE ACTIVITY EXISTED

That telomeric DNA had certain molecular behaviors indicative of dynamic properties *in vivo* had emerged by the early 1980s. Four main lines of such molecular evidence were instrumental in spurring me to hunt for a new type of enzymatic activity that might synthesize telomeric DNA and elongate telomeres. This evidence took the form of molecular observations on telomeric DNA not readily explainable by any of the knowledge then about DNA replication or recombination.

First, the telomeric CCCCAA repeat tracts (which we eventually ended up referring to by their sequence on the complementary DNA strand, GGGGTT repeats) in the ciliates *Tetrahymena* and *Glaucoma* were heterogeneous in length; that is, the DNA molecules in the population carried different numbers of repeats.[7, 13] Perfect DNA replication of parental DNA to make two daughter DNAs was not predicted to produce such heterogeneity.

Second, during development of the somatic macronucleus in different *Tetrahymena* strains, telomeric GGGGTT repeat tracts were found to become joined, by then-mysterious means, to various sequences in the rDNA minichromosomes; that is, new telomeres were forming on macronuclear chromosomes. Meng-Chao Yao, continuing work he had started as a Ph. D. student in Martin Gorovsky's laboratory and then as a postdoctoral fellow in Joe Gall's lab (at the same time I was there), had observed this first for *Tetrahymena* rDNA telomeres.[14] But a single TTGGGGTT sequence already present at this position in the precursor DNA sequence had made it conceivable that this sequence could itself somehow be a seed sequence for repeated unequal recombination events to generate multiple repeats, for example. However, then my lab at Berkeley made similar observations for other rDNAs and non-rDNA telomeres of the somatic nucleus, with the difference that in these cases the telomeric DNA sequences were found to be joined to sequences where there was no initial GGGGTT repeat at all. [15] Thus in 1982 I wrote about these observations: "...the sequences common to the macronuclear DNA termini must be acquired by these subchromosomal segments during their formation. Two types of routes can be envisaged: Telomeric sequences are transposed or recombined onto the developing macronuclear DNA termini, or the simple, repeating telomeric sequences are synthesized de novo onto these termini by specific synthetic machinery" [15]. Simultaneously, David Prescott's group in Colorado had made the observation that T₄G₄ repeats similarly appeared to become joined onto the ends of the short chromosomes of the macronucleus in a hypotrichous ciliate. [16]

Third, as described in detail in Jack Szostak's 2009 Nobel Lecture, we had discovered that yeast telomeric sequence DNA (irregular TG₁₋₃ repeats, which Janice Shampay, a graduate student in my lab at UC Berkeley, first sequenced as part of our collaboration with Jack) was added directly to the ends of *Tetrahymena* GGGGTT repeat telomeres maintained in yeast.

[17, 18] In this collaboration, we showed that a telomere from the ciliated protozoan *Tetrahymena*, consisting of GGGGTT repeats, was able to function as a telomere in the yeast *S. cerevisiae* in a particular sense: specifically, the *Tetrahymena* telomeric sequences, when put into a yeast cell on the ends of a linearized plasmid DNA molecule, could stabilize the plasmid, such that now it was maintained indefinitely as an extrachromosomal, linear DNA molecule through many rounds of replication, mitosis and even meiosis. But in addition, something very interesting always happened to the introduced foreign (*Tetrahymena*) telomere in yeast. We found that yeast telomeric repeats, which Janice Shampay sequenced and found to be TG₁₋₃ repeats, were added to the distal end of the foreign, GGGGTT-repeat telomere after it had been maintained in dividing yeast cells. Other observations made soon after, in the course of following up these findings, further highlighted the dynamism of telomeric DNA in cells. For example, by the early 1980's Janice Shampay had also observed that if we introduced such a high-copy-number plasmid into yeast (thereby adding of the order of 100 extra telomeres into these cells), the telomeres of the chromosomes themselves, whose average length had heretofore remained steady for 300 generations of previous mitotic passaging, now underwent a slow shortening over the ensuing cell divisions (EHB and Janice Shampay, unpublished results).

Fourth, Piet Borst and his collaborators published an intriguing observation in 1983. They were monitoring the inheritance of a gene of trypanosomes (which cause sleeping-sickness) encoding a variant surface antigen. These antigens play important roles in the parasite's ability to evade the host's immune response. The gene they had found was located on a telomeric restriction fragment. In the course of passaging the trypanosome cells, this gene's restriction fragment steadily became progressively longer, implying that the telomeric DNA tract was growing.[19]

Finally, from yet another independent direction, a cytogenetic observation by Barbara McClintock reinforced my nascent notion that some undiscovered kind of developmentally-controlled cellular enzymatic activity might act on telomeres. In essence, first in a conversation, and later in a 1983 letter, McClintock described how, long ago, she had identified a maize mutant that had lost the normal capacity of maize to heal broken chromosome ends that specifically exists very early in plant development – just after fertilization. In the letter McClintock wrote to me in 1983, she explains this (Figure 3):



Cold Spring Harbor Laboratory
P.O. Box 100, Cold Spring Harbor, New York 11724 (516) 465-8000

March 11, 1983

Dr. Elizabeth H. Blackburn - 2 -

Dr. Elizabeth H. Blackburn
Department of Molecular Biology
University of California
Berkeley, California 94720

Dear Dr. Blackburn:

Ky apologies for this delay in responding to your letter of February 18 requesting reprints or preprints of articles of mine dealing with telomeres. My studies of them relate to the behavior of newly broken ends of chromosomes. These studies commenced in the early 1930s and continued into the 1940s. Reprints of reports of these studies are long since exhausted. In those early days one did a thorough job before publishing. Thus, my articles are long and detailed--the type of article so one wants to read in this day of avalanching mini-reports. Enclosed is a red-marked list of publications relating to fusion of broken ends of chromatids (the chromatid type of breakage-fusion-bridge cycle) and of chromosomes (the chromosome type of breakage-fusion-bridge cycle). At least you will have received references to my studies of the behavior of broken ends of chromosomes, even though you may have no desire to examine these reports.

In brief the results are as follows. Ring chromosomes in maize undergo chromatid exchange. The rate of such exchange is proportional to the length of the DNA in the ring chromosome. This exchange results in a double-strand, dicentric ring chromosome. Separation of the centromeres at anaphase produces two bridges, side-by-side. Each bridge is ruptured. A broken end from each bridge enters a telophase nucleus. The two newly broken ends then fuse, reestablishing a ring chromosome whose size and content depend on the position of rupture that had occurred in each strand. These fusions are *phragmocysts*.

In my maize stocks *chromatid* fusions occur in gametophytic and endosperm tissues but not in sporophytic tissues. A chromosome ruptured at a mitotic anaphase will appear as a dicentric chromosome in the following gametophytic anaphase because of "fusions" during the

replication cycle that occur at the position of break during the previous anaphase. These anaphase bridges and breaks, with subsequent dicentric chromatid formations, continue in the gametophytic and endosperm mitoses. In the embryo, however, the broken end "heals". Because an anaphase bridge may be ruptured at many or any position between the two diverging centromeres, the capacity for "healing" must apply to many or any broken end. I have observed many chromosomes with a healed end. Healings took place all along the arm of a chromosome. Chromosomes having such a healed end have been propagated over many plant generations. Thus, the healing process provides a new telomere, as stable as any other.

During these studies I found a mutant whose effect was to eliminate the capacity of cells of the sporophyte to produce a telomere at a newly broken end. When a newly broken end was introduced into a zygote this end continued to undergo the chromatid type of breakage-fusion bridge cycle, and in all mitoses throughout plant development. It would appear that this mutant affects the production or the action of an enzyme required for formation of new telomeres. I have no published report of this observation. The evidence was obtained incidentally in the course of another study. For purposes of publication, the study would need to be expanded and this I did not do. It is evident, however, that in my basic genetic stock, telomere-forming repair enzymes are not produced or made available to cells of the gametophyte and endosperm but are produced in embryonic cells.

Thank you for including the topics to be discussed in your chapter on Centromeres and Telomeres. I look forward to reading it when it appears.

Sincerely,

Barbara McClintock
Barbara McClintock

Figure 3. McClintock letter to EHB 1983.

Finding a mutant implied that there is a gene associated with the ability to heal – a gene that could be mutated to nonfunctionality. I was struck by the implication that in zygotes, a fully functional telomere (“healed end” in McClintock’s terminology) was generated from a broken chromosome end not just by chance but rather, by an active, developmentally controlled process; a process, furthermore, occurring just after fertilization – the developmental stage equivalent to when ciliate chromosomes become broken (albeit deliberately in their case) and telomeric DNA is efficiently added to the freshly produced DNA ends.

This remarkable information was another one of the reasons that I decided to look for telomerase. The capacity of ciliates to form de novo telomeres just after fertilization (equivalent to the zygote) was just too striking a parallel to ignore.

TETRAHYMENA CELLS BY A BIOCHEMICAL APPROACH

“If your knees aren’t green by the end of the day, you ought to seriously re-examine your life.”

– Bill Watterson (American Author of the comic strip Calvin & Hobbes, b. 1958)

Tetrahymena cells provided an attractive system to use to hunt for this putative telomeric DNA-adding enzymatic activity. My choice of approach was to prepare extracts from *Tetrahymena* cells. As Joe Gall had pointed out when I proposed sequencing DNA end regions, they could be grown in large quantities relatively inexpensively. Furthermore, their developmental time

course could be synchronized, thanks to the efforts of several laboratories, notably those of David Nanney, Peter Bruns, Ed Orias, Sally Allen and their colleagues. Hence, one could make cellular extracts from a large population of cells all undergoing macronuclear development and, concomitantly, I hypothesized, the putative telomere addition reactions. This, therefore, seemed to me likely to be a developmental stage when any such activity would be in high demand by the cell and therefore, I reasoned, would allow the best chance of its being detectable. The big question was the choice of substrates to use. What DNA would be best to use to prime any telomeric DNA addition, and what nucleotide building-block precursors would be required? Would both strands of the telomeric DNA have to be synthesized together in a coupled reaction, or perhaps even in a reaction that had to be coupled to the production of the freshly-cut DNA ends? Would it be preferable to provide DNAs that resembled the freshly-cut ends in the developing macronucleus, or pre-existing telomeric repeat tracts that could be further elongated: both reactions would be expected to be performed in *Tetrahymena* cells at this stage. To make sure I did not miss any of these possibilities, I added a mixture of all four deoxynucleoside triphosphates and all four ribonucleoside triphosphates, an energy-generating (ATP-generating) enzyme system, and a mixture of cloned DNA fragments, purified from bacterial cells, that would present to any enzymes in the *Tetrahymena* extracts both telomeric and nontelomeric DNA termini. I prepared cell extracts from cells at this developmental stage, adapting a method that my graduate student Peter Challoner had in turn adapted from one used by Tom Cech and collaborators to examine rDNA gene expression (which led Tom Cech to the discovery of self-splicing RNA). Peter had found that incubating such extracts (for a different experimental goal) allowed him to detect changes in the DNA restriction fragments that he had added to his extracts. The changes had even hinted at some form of alteration specific to the telomeric DNA ends. As described elsewhere (Appendix, [1]) in early 1984 I was able to see increasing amounts of telomeric GGGTT-hybridizing repeat sequences were somehow generated during the course of the reactions. The hunt was on!

THE DISCOVERY OF TELOMERASE

“... to make an end is to make a beginning.”
– T.S. Eliot 1888–1965, Four Quartets: “Little Gidding”

The next immediate need was to greatly simplify and refine the reaction conditions, in order to unravel what was actually occurring during the reactions that were being carried out by enzymes apparently present in the *Tetrahymena* cell extracts. In 1984 Carol Greider joined my lab at UC Berkeley as a Ph.D. student and was immediately interested in doing just this. This work, which led us to discover telomerase activity, is described in detail in Carol Greider’s 2009 Nobel Lecture in this volume, so I summarize only briefly some points here.

July 15, 1985

Dr. Benjamin Lewin, Editor
Cell Editorial Offices
292 Main Street
Cambridge, MA 02142

Dear Ben,

Please find enclosed a manuscript entitled "Identification of a specific telomere terminal transferase activity in *Tetrahymena* extracts" by Carol W. Greider and Elizabeth H. Blackburn, which we are submitting for publication in *Cell*. We report here the discovery of a novel telomere elongation activity which we believe forms the basis of telomere replication and stabilization *in vivo*, so we think it should be of general interest to *Cell* readers.

Thank you for your consideration.

Sincerely yours,

Elizabeth Blackburn

Carol Greider

EB/CG:cv

Figure 4. Letter to Editor of *Cell*, 1985.

We discovered that short fragments of DNA, synthesized chemically as DNA oligonucleotides and therefore available in high concentrations, would get telomeric GGGGTT repeats added to their 3' ends when incubated with *Tetrahymena* extracts. This enzyme reaction was more efficient when the extracts were made from cells at the developmental stage when new telomeres are added during macronuclear development. Fortunately, the reaction, at least in the test tube, was not obligatorily or mechanistically coupled to synthesis of the complementary DNA strand or to DNA cleavage. [20]

Ribonuclease treatment abolished this telomeric DNA repeat addition capability of the extract. Hence the enzyme activity needed RNA. Protease treatment also destroyed the enzyme reaction, implicating required protein component(s) as well as RNA.[21]

The essential RNA component of telomerase was identified and found

to contain a sequence, 5' CAACCCCAA 3'. This sequence is complementary to one and a half repeats of the 6 nucleotide repeat sequence that was synthesized *in vitro*. Starting with this powerful hint, we found that all the properties of the synthesis reaction *in vitro*, including its particular DNA and nucleoside triphosphate precursor requirements, added up to a coherent model by which telomere synthesis by telomerase (as we eventually named the enzymatic activity) is templated by repeated rounds of copying of this short template sequence in the telomerase RNA. The synthesis is aided by alignment of the 3' end of the DNA primer on the template, thereby positioning the primer for addition of the next templated repeat.[22]

We thus had discovered that *Tetrahymena* telomerase added tandem repeats of the *Tetrahymena* telomeric DNA sequence, TTGGGG, onto the 3' end of a variety of G-rich telomeric DNA sequence oligonucleotide primers, independently of an exogenously added nucleic acid template. The cellular activity that carried out this reaction was both RNase- and protease-sensitive. Similar experiments were then derived for telomerase activities from the ciliates *Euplotes* and *Oxytricha*, – by Dorothy Shippen-Lenz in my lab and by Alan Zahler in David Prescott's lab, respectively, and from human cells by Gregg Morin in Joan Steitz's lab at Yale. Each telomerase synthesized its own species-specific sequence – GGGGTTTT repeats (hypotrichous ciliates) or AGGGTT repeats (human cells). These other telomerase activities were very like the *Tetrahymena* telomerase in their primer recognition and other characteristics, including ribonuclease-sensitivity, arguing for the generality of this enzyme activity among eukaryotes. When Dorothy Shippen identified and sequenced the RNA moiety of the telomerase of the ciliate *Euplotes*, it gratifyingly contained the sequence 5' CAAAACCCAAAA 3'. Experiments indicated that this sequence was indeed the templating domain for synthesis of GGGGTTTT repeats, the telomeric sequence of *Euplotes*. Together, these findings established telomerase as a widespread, reverse transcriptase, unusual in being of cellular origin and in carrying its own internal RNA template for repeated DNA synthesis.

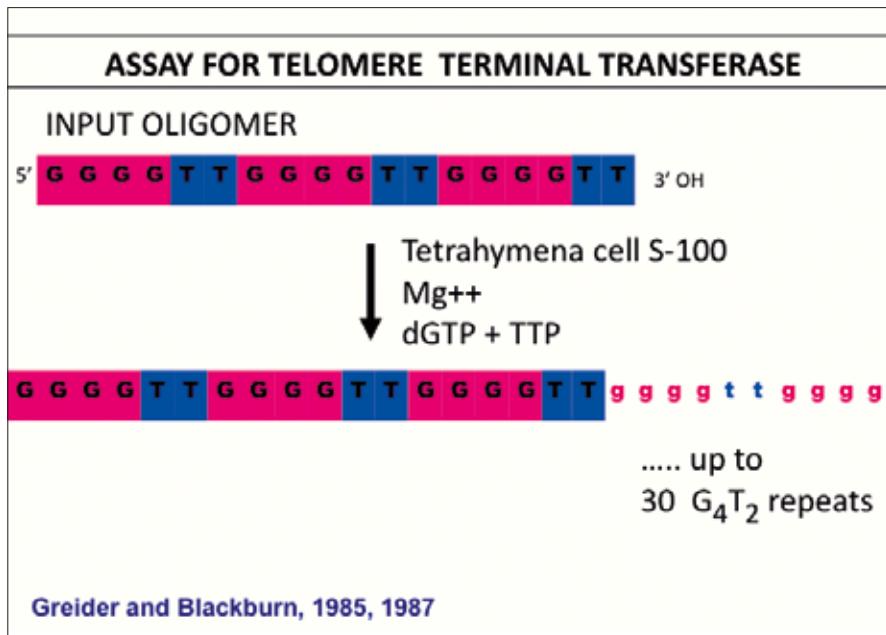


Figure 5. The original telomerase assay. Greider and Blackburn, 1985, 1987).

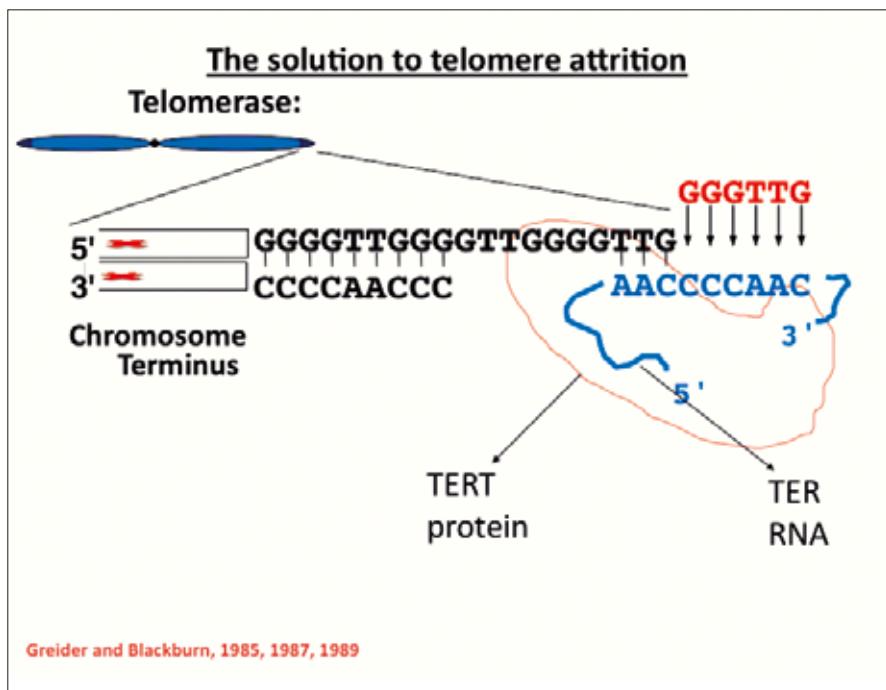


Figure 6. A model for the action of the *Tetrahymena* telomerase. Greider and Blackburn, 1985, 1987, 1989).

DEMONSTRATION OF THE REVERSE TRANSCRIPTASE ACTION OF TELOMERASE *IN VIVO*

“They didn’t have to walk around to see what was under the sky; they just stayed where they were. [And] as they looked, their knowledge became intense.”

— POPUL VUH, P. 165.

We now had good evidence that the telomerase enzyme could synthesize the G-rich strand of telomeric DNA *in vitro*. Proving that the 5' CAACCCCAA 3' sequence in the *Tetrahymena* telomerase RNA gene was the template for telomere synthesis *in vivo* required site-directed mutagenesis of this sequence in the telomerase RNA gene (which we called the TER gene). Again, *Tetrahymena* provided the first key to being able to do these experiments. Its telomerase RNA gene had been recently cloned by Carol Greider, and we had devised a system in my lab for overexpression of such mutated genes in *Tetrahymena* cells. We inserted the engineered telomerase RNA genes into a self-replicating vector devised by Guo-Liang Yu, a graduate student in my lab. Guo-Liang then introduced them into *Tetrahymena* cells by microinjection of the DNA molecules. He analyzed and sequenced the telomeres in the cells expressing the mutated telomerase RNAs. These experiments, done with help from Guo-Liang's labmates Laura Attardi and John Bradley, established the *in vivo* role of telomerase in three ways. As predicted from the sequence of the telomerase RNA and from the *in vitro* experiments described in Carol Greider's Nobel Lecture, altered telomere repeats specified by the mutant gene appeared in the telomeres that he cloned out of the transformant cells. This proved that telomerase was the cellular reverse transcriptase enzyme that synthesizes telomeres in cells by copying its own internal RNA template within the TER moiety of the enzyme complex. The cells rapidly showed abnormal nuclei indicative of failure to segregate their DNA properly. This indicated that the correct DNA sequence was necessary for proper nuclear behavior [23].

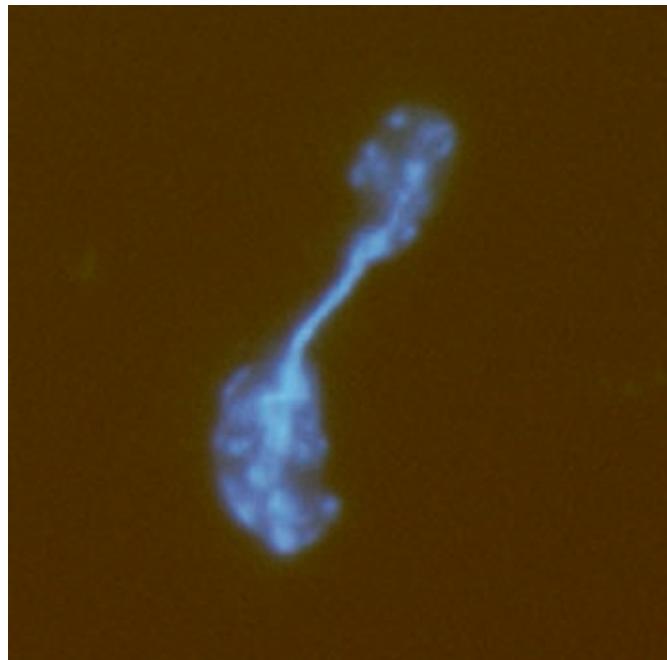


Figure 7. A *Tetrahymena* cell expressing a telomerase RNA with mutated template attempts to divide.

DEMONSTRATION OF THE NEED FOR TELOMERASE FOR CELL GROWTH

*“Like as the waves make towards the pebbled shore,
So do our minutes hasten to their end.”*

— WILLIAM SHAKESPEARE, 1564–1616, SONNET 60.

In addition to proving the templating role of telomerase RNA in *Tetrahymena*, we obtained a bonus result from these experiments. *Tetrahymena* cells are normally effectively immortal. With one particular template mutation, Guo-Liang found none of the predicted sequence DNA was added onto telomeric ends. Instead, the cells continued to grow for only about 20 to 25 more cell divisions. During that time their telomeres progressively shortened. The cells then ceased to divide. This result was the first demonstration that interference with normal telomerase function itself could limit cellular lifespan. It established that continuing action of telomerase was necessary for replicative immortality of cells [23].

Tetrahymena thermophila

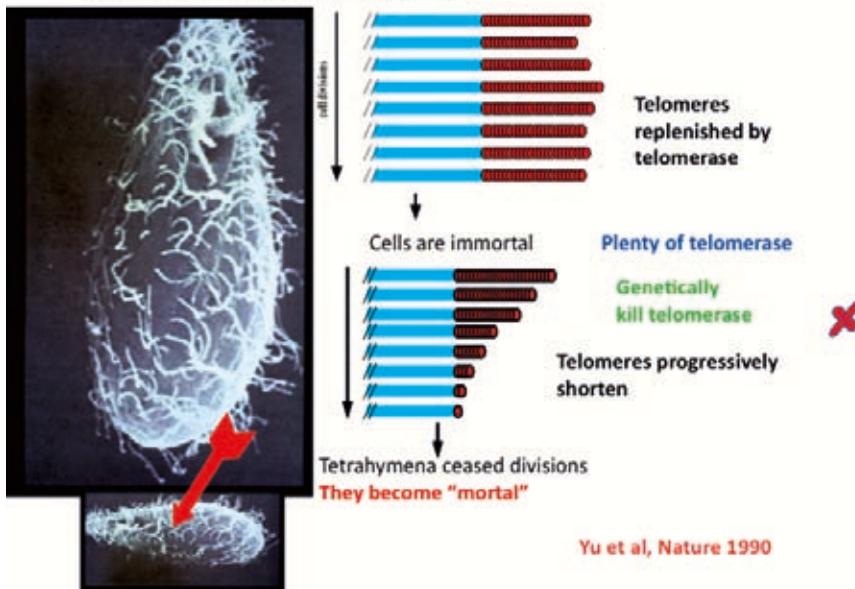


Figure 8. *Tetrahymena* cells' dependence on telomerase. (Yu et al., *Nature*, 1990).

The action of telomerase thus could explain how replication of the 5' ends of the chromosomal DNA can be completed, without the loss of terminal sequences that would result from normal semi-conservative DNA replication mechanisms: continuous addition of telomeric DNA to the chromosomal ends by telomerase could counterbalance this predicted terminal DNA attrition.

TELOMERES AS PROTEIN-DNA COMPLEXES

“Having well polished the whole bow, he added a golden tip.”

– Homer (“Smyrnae of Chios”), *The Iliad* (bk. IV, III).

As recounted in my autobiography in this volume, soon after identifying the telomeric sequence, I found that in *Tetrahymena* chromatin, telomeric DNA tracts were protected by bound protein(s), distinct from nucleosomes. I tried to identify the proteins on *Tetrahymena* telomeres, but did not succeed in this. It was others' work, initially using yeast molecular genetic approaches, that unlocked the door to telomeric proteins. Now an extensive list of proteins associated with telomeres, from various eukaryotes, is known. Many of these have been characterized to varying extents with respect to biochemistry, structure, occupancy levels on telomeres and circumstances that lead to measurable changes in these occupancy levels. Many functions are deduced, often by looking at the consequences to cells of mutating or deleting the protein. But despite the extensive work that has built up the current molecular knowledge of the telomeric DNA-protein complex, the

actual picture of a telomere is in some ways still a rather ghostly and partial image. This is almost certainly because telomeres are highly dynamic.

TELOMERES AS A DYNAMIC HOMEOSTATIC SYSTEM

“Stability is not immobility.”

– Klemens von Metternich, Austrian statesman, 1773–1859.

During the 1990s, the view of a telomere that emerged was that of a self-regulating entity, normally resilient to change and buffered from it by a variety of molecular mechanisms. Mike McEachern, a postdoctoral fellow then in my laboratory, proposed a model for telomere dynamics based on his experiments with telomeres in *Kluyveromyces lactis*, a budding yeast. The essence of the model is that, first, the rate of shortening of a telomere in the absence of telomerase stays constant as that telomere shortens. But the probability of lengthening it by telomerase actually changes depending on the telomere length – the shorter the telomere, the more likely it is to be lengthened by telomerase action. Mike deduced this largely from a series of experiments in which he altered the telomerase RNA template sequence to direct the synthesis of various repeats. Anat Krauskopf, then concurrently a postdoctoral fellow with Mike in my lab, extended these findings: Telomere length regulation became altered in a way that tracked with altered binding of a yeast telomeric protein to the mutated telomeric sequence. Together, Mike’s and Anat’s experiments showed that a major contributor to such negative regulation of telomerase action on telomeres is the telomere protein-DNA complex structure itself. Thus the telomere itself was a like a gatekeeper, regulating access of telomerase onto the telomere, even in the presence of excess telomerase in the cells. Over subsequent years much more has been learned about the details of the proteins involved, but this general model has stood the test of time.

A general and important corollary concept is that telomeres can exist in two states: capped or uncapped. Capped telomeres signal the cells to keep on proliferating, all other things being well. But uncapped telomeres in the cell signal the cell; if uncapping is persistent, it signals the cell to arrest its divisions. Mike McEachern and Anat Krauskopf showed that one of the most striking properties of a telomere is how resilient it can be to molecular insults of a variety of types, and then, like the last straw, just one more molecular change is sufficient for the telomere to collapse catastrophically into disaster. Thus it emerged that cells have evolved elaborate and overlapping, redundant or mutually reinforcing mechanisms to ensure that their telomeres stay functional. [24]

Currently, the combined picture from the results from many researchers is that the telomere in a cell is a highly dynamic structure. Rather than being a rock-stable complex, it is perhaps reminiscent of a swarm of bees: the size and shape of the swarm overall appears the same, but in reality its composition is constantly changing as the bees (the telomeric proteins) of the swarm constantly come off it and are replaced by other bees.

SIMILAR MOLECULAR MACHINERIES: DIFFERENT LIFE HISTORIES

"Have regard to the end." [Lat., Finem respice (or Respice finem).]

– Chilo of Sparta (Chilon).

The structure and the function of telomeres are highly evolutionarily conserved among eukaryotes. This conservation underlies why in the early 1980s Jack Szostak and I were able to successfully propagate *Tetrahymena* telomeres in the distantly related organism baker's yeast. As described above, and in detail in Jack Szostak's 2009 Nobel Lecture, we found that yeast telomeric sequences were added to the introduced *Tetrahymena* telomeric ends on a plasmid, thereby stabilizing the plasmid and allowing it to replicate indefinitely, in linear form, as an extrachromosomal plasmid. Similar conservation applies to the telomerase mechanism for telomere maintenance: throughout the eukaryotes telomerase, a specialized ribonucleoprotein reverse transcriptase, is used to maintain the ends of eukaryotic chromosomes, with relatively rare exceptions. Telomerase RNA and core protein of telomerase, TERT, each retain well-recognizable conserved features in even the most distantly related eukaryotes.

In the face of this widespread conservation of telomeres and telomerase, extending down to the deep roots of eukaryotic evolution, a fascinating finding is the great variety of telomere maintenance stories that play out during the lives of different eukaryotes. Among mammals alone, even under favorable living conditions species clearly differ in their maximal possible lifespans, implying that maximum lifespan has considerable genetic determination. Humans can have a life expectancy of about eighty years, and laboratory mice about two years. Thus, it is reasonable to contemplate the possibility that the rate-limiting steps causing aging and eventual death may differ between these two species, despite the common underlying cellular and molecular mechanisms they share. Even within the mammals, the qualitative and quantitative contributions of telomere maintenance to cellular proliferative lifespans seem to differ widely [25]. And, extending further out from mammals to invertebrates, despite much conservation of fundamental molecular and cellular mechanisms, it is possible that those mechanisms that contribute to their aging and death from old age may be divergent from those that are quantitatively important or rate-limiting for aging and lifespan in humans. All of these considerations have raised the question of whether telomere maintenance is a quantitatively important determinant of normal human aging and lifespan.

TELOMERASE IN HUMAN HEALTH AND DISEASE

a) Telomerase in cancer cells

“We ought to consider the end in everything.” [Fr., *En toute chose il faut considerer la fin.*]

– Jean de la Fontaine, Fables (III, 5).

One special and notable context in which telomerase plays a prominent role in humans is in human cancer cells. Hyperactive telomerase in the cancer cells is a prominent characteristic of the great majority of most types of malignant human tumors. In this setting of the cancer cell – which, importantly, has undergone multiple other genetic and epigenetic changes in its progression to tumorigenicity – telomerase plays cancer-promoting roles. Most clearly, it promotes cellular immortality by providing cancer tell telomeres with the means for continuous replenishment. The high level of telomerase that characterizes human cancer cells thus is a rational target for anti-cancer therapies.

b) Telomere maintenance and human life histories

“The end crowns all,
And that old common arbitrator, Time,
Will one day end it.”

– William Shakespeare 1564–1616, The History of Troilus and Cressida (Hector act IV, v.).

As described above, abrogating telomerase in otherwise effectively “immortal” single-celled species causes progressive telomere shortening over several cell generations followed by cessation of cell division (“senescence”). This naturally led to the question of whether the same progressive process operates to cause human aging and limit human lifespan. Rare genetic mutations in telomerase component genes leading to reduced telomerase levels and telomere shortening in humans clearly have adverse disease-causing effects and can prevent the affected individual from attaining an old age. Yet until recently, for the vast majority of people, who by definition are not “mutants”, the contributions of insufficient telomere maintenance to aging and lifespan limitation was less clear. What does one observe in the human population in general?

Large amounts of epidemiological molecular data on humans and their *in vivo* telomere maintenance have now accumulated. From our present knowledge of telomeres, the picture that has emerged is that telomere maintenance is linked to human aging and diseases of aging. First, telomere shortness in white blood cells is linked to a large and impressive list of the major diseases of aging: in multiple cohorts, often involving hundreds to thousands of individuals, short telomere length has been found to be associated with risk of, and incidence of, cardiovascular disease, stroke, vascular dementia, osteoporosis and obesity and risks for diabetes and certain cancers. Longer mean white blood cell telomere length is not consistently linked to longer

lifespan, but longer telomere length has been linked to more years of healthy life, in a cohort of people in their seventies. [26]

Second, telomerase activity is not only present in many normal human somatic cells but also, importantly, quantifiable ([27–30]) in adult (including elderly) humans; even in resting white blood cells, as well as in stem and proliferating progenitor cell types, telomerase is active. This means that telomere shortening in normal cell populations has the possibility of being counterbalanced, or even reversed, throughout life. While cross sectional studies show a slow loss of telomeric length across populations of humans in general, the datapoints are noticeably scattered: it is not uncommon for an 80-year old's telomeres to be as long as those in a 30-year old. What might account for such scatter? In fact, lengthening of telomeres in white blood cell populations is now found to be much more common than expected from the previous models of inexorable telomere shortening throughout life. However such models had been based almost solely on cross sectional studies and on the presumption of lack of telomerase in the normal cells of adult humans.

What determines and regulates the variation in long-term telomere maintenance in people? While genetic influences have been detected, non-genetic factors are also coming to the fore as significant influences on telomere length maintenance in human white blood cells. In summary, in humans, telomere maintenance status results from the integrated influences of many factors, genetic and non-genetic. The non-genetic influences include modifiable factors; notably, psychological stress, behavioral and even nutritional factors.

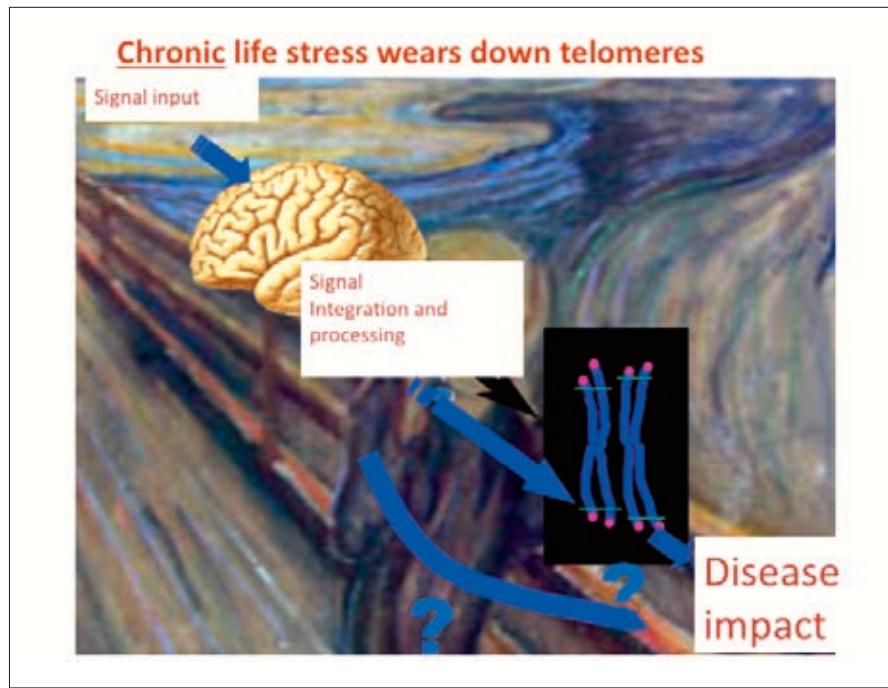


Figure 9. Input of stress on telomeres and its disease impact.

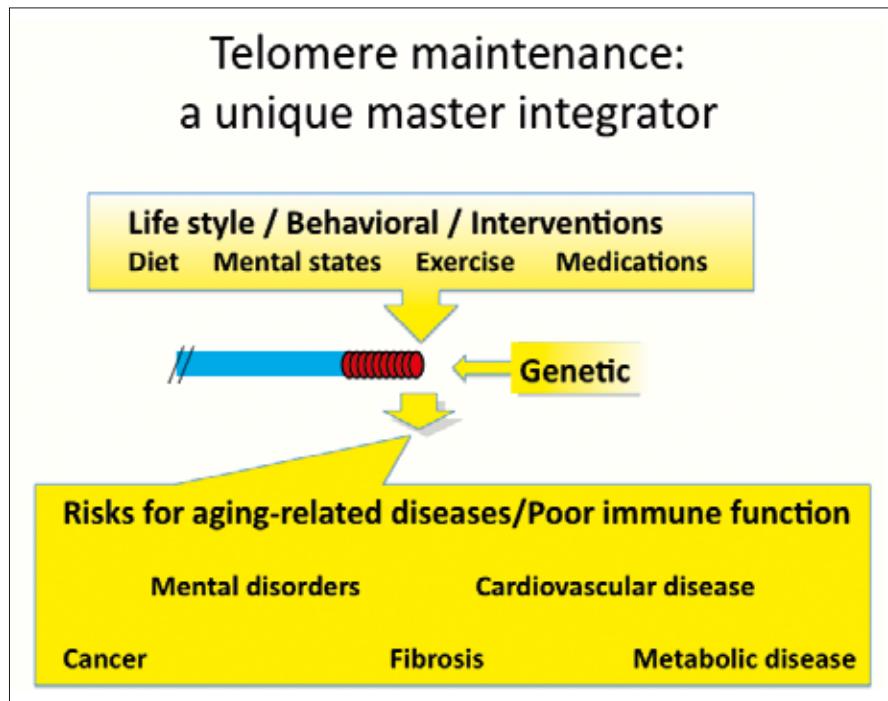


Figure 10. The telomere as an integrator of many factors.

The major conditions and diseases or disease risks occurring with human aging have now been associated with shortness of blood cell telomeres: prominently, cardiovascular disease, cancers, diabetes and impaired immune system function in various forms. Thus, telomere shortness is not specifically associated with any one disease. Rather, this seeming non-specificity of telomere maintenance may instead be more usefully considered as reflecting – perhaps causing – aging more fundamentally. Telomere maintenance status may be a truer integrative measure of actual “biological age” than chronological age. Furthermore, human life conditions impact on telomere maintenance in humans. Perhaps telomere monitoring will become as common as regular weighing as an integrative indicator of health. Certainly, these findings and implications are taking the field of telomere and telomerase biology into realms far from the single-celled pond microorganisms in which I began this work.

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Portrait photo of Professor Blackburn by photographer Ulla Montan.