Title: ASSAY FOR TELOMERE LENGTH REGULATORS

Abstract: The present invention provides an assay that identifies genes required for telomerase-dependent telomere elongation by measuring the de novo telomere addition at a single chromosome.
ASSAY FOR TELOMERE LENGTH REGULATORS

RELATED APPLICATION DATA


STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made in part with government support under Grant No. R37AG009383 awarded by the National Institutes of Health. The United States government has certain rights in this invention.

INCORPORATION OF SEQUENCE LISTING

[0003] The material in the accompanying sequence listing is hereby incorporated by reference into this application. The accompanying sequence listing text file, name JHU3790_IFO_Sequence_Listing, was created on ________, and is __ kb. The file can be assessed using Microsoft Word on a computer that uses Windows OS.

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

[0004] The invention relates generally to chromosome maintenance and cell viability, and more specifically to an assay for identifying telomere length regulators.

BACKGROUND INFORMATION

[0005] Telomeres are genetic elements located at the ends of all eukaryotic chromosomes that preserve genome stability and cell viability by preventing aberrant recombination and degradation of DNA. In humans, the telomeric sequence is composed of 10-20 kilobases of TTAGGG repeats. There is increasing evidence that gradual loss of telomeric repeat sequences may be a timing ("clock") mechanism limiting the number of cellular divisions in normal somatic cells. In contrast, immortal cells can maintain a stable telomere length by telomere addition by telomerase, a ribonucleoprotein enzyme that is able to add TTAGGG repeats to the ends of chromosomes.

[0006] Telomere length is maintained during cell division through the action of telomerase, which is a unique reverse transcriptase that elongates telomeric DNA. Telomerase is relatively abundant in germline, stem cells and embryonic tissues, inflammatory cells, proliferative cells of renewal tissues, as well as cancer cells. In contrast, telomerase activity is difficult to detect in normal somatic human tissues. The correlation of
telomerase activity and cellular replication has prompted the association of telomerase and cancer. Telomerase activity has been found in approximately 85% of human cancers. Thus, it has been proposed that up-regulation or re-expression of telomerase may be a critical event responsible for continuous tumor cell growth.

[0007] The telomerase enzyme is made up of an essential core as well as several accessory proteins. The core telomerase consists of the RNA component (Telomerase RNA, TR) and the catalytic subunit (Telomerase Reverse Transcriptase, TERT). The structure of the RNA component is conserved from ciliates to humans, while the sequence is not. In the ciliate Tetrahymena the RNA is 150-200 nucleotides (nt) in length while in mammalian cells, the RNA component is significantly larger, 390-450 nt. The catalytic TElomerase Reverse Transcriptase (TERT) component, first identified in the ciliate Euplotes, has homologues in yeast (EST 2), human (hTERT), and mouse (mTERT) and most other eukaryotes. TERT contains sequence motifs similar to reverse transcriptase, and mutations of essential aspartate residues that are conserved in the catalytic triad of reverse transcriptases eliminate telomerase activity. Minimal telomerase activity can be reconstituted in an in vitro transcription/translation extract using TERT and TR components, indicating that these are sufficient for catalysis.

[0008] Given the association of telomerase activity with cancer and age related degenerative diseases, telomerase activity is important in clinical settings. Several analytical procedures for the quantification of telomerase activity have been reported. Methods for detecting telomerase activity, as well as for identifying compounds that regulate or affect telomerase activity, have been described. See PCT Pat. App. Pub. No. 93/23572 and U.S. Pat. Nos. 5,629,154, 5,648,215, 5,645,986, 5,695,932 and 5,489,508. Each of the foregoing patent publications is incorporated herein by reference.

[0009] The most frequently utilized assay is Telomeric Repeat Amplification Protocol (TRAP), which is a two stage PCR-based assay. In the first stage, telomerase adds 5'-TTAGGG-3' repeats to the end of a synthetic primer. In the second stage, the extended oligonucleotide products are amplified using a reverse primer. When visualized by autoradiography, a positive test by TRAP shows a ladder of bands. The band volume can then be quantified. TRAP is time consuming, labor intensive, PCR-dependent and susceptible to inhibition by extracts of clinical samples. Furthermore, it is difficult to quantify telomerase activity because of logarithmic amplification of telomerase products in the PCR amplification step, which is subject to false positive results. The susceptibility of
the TRAP assay to Taq-polymerase inhibitors can result in the production of false negative results.

[0010] A similar telomerase assay that replaced the electrophoretic step of the TRAP assay with an ELISA detection system has been developed. This system is also PCR-dependent although the ELISA detection method appears to offer no clear advantage over the traditional TRAP. In an effort to eliminate technical issues associated with TRAP, in situ hybridization assays for the quantification of human Telomerase (hTR) RNA and human Telomerase Reverse Transcriptase (hTERT) mRNA were developed. However, hTR and hTERT expression does not necessarily equate to telomerase activity.

[0011] Another telomerase assay is disclosed in PCT/IL0 1/00808 (WO 02/20838). This assay uses rotating quinone-functionalized magnetic beads to generate H2O2 within the assay. The endogenous production of H2O2 putatively overcomes the problem of luminol being sparingly soluble in aqueous buffer solutions. However, the rotating magnetic beads reduces the ability to develop high throughput screening protocols and may impact on the sensitivity depending on the length of oligonucleotide primer employed.

[0012] Researchers have stated that the amount and length of telomeric DNA in human fibroblasts decreases as a function of serial passage during aging in vitro, and possibly in vivo. It was later shown that this telomere shortening causes cellular senescence. They also state that tumor cells are characterized by shortened telomeres and increased frequency of aneuploidy, including telomeric associations. Since the loss of telomeric DNA ultimately causes cell-cycle arrest in normal cells, the final steps in this process may block growth in immortalized cells. Whereas normal cells with relatively long telomeres and a senescent phenotype may contain little or no telomerase activity, tumor cells with short telomeres may have significant telomerase activity. Telomerase may therefore be an effective target for anti-tumor drugs. There are a number of possible mechanisms for loss of telomeric DNA during ageing, including incomplete replication, degradation of termini (specific or nonspecific), and unequal recombination coupled to selection of cells with shorter telomeres.

[0013] Long-term cell viability is critically dependent on maintenance of telomere length. In humans, syndromes of telomere shortening cause age-related degenerative diseases that are often fatal. At the cellular level, the loss of tissue renewal that contributes to these diseases is caused by short telomeres inducing apoptosis or cellular senescence. On the other hand, cancer cells avoid cell death by increasing or maintaining telomere lengths. Telomere shortening occurs during normal cell division because DNA replication fails to
copy the very end of the chromosome. Telomerase adds telomere repeats onto chromosome ends to balance the shortening that occurs due to replication. The delicate balance of shortening and lengthening is regulated by an intricate series of feedback mechanisms to establish a robust telomere length equilibrium.

[0014] Telomere length maintenance is essential for cell viability. Telomere shortening that occurs during cell division is balanced by telomerase, which adds telomere repeats onto chromosome ends. The delicate balance of shortening and lengthening is regulated by an intricate series of feedback mechanisms that establish a dynamic telomere length equilibrium. In humans, syndromes of telomere shortening cause age-related degenerative diseases including dyskeratosis congenita, pulmonary fibrosis, aplastic anemia and others. Elucidating the molecular interactions that regulate telomere elongation is essential to understand telomere function and how it is disrupted in disease.

[0015] In both yeast and human cells, short telomeres induce either senescence or apoptosis through activation of the DNA damage response. Similarly in telomerase negative mTR−/− mice, or in Telomerase heterozygous mice mTR+/−, after four to five generations of interbreeding, cells with short telomeres undergo apoptosis or cellular senescence. The short telomeres are the cause of this apoptosis or cellular senescence, since it occurs even when some telomerase is present. This cell loss can either contribute to age-related disease or can limit the tumor growth in vivo.

[0016] The ATM and ATR kinase-dependent DNA damage response pathways are activated in primary human cells when telomeres are critically short. Induction of telomere dysfunction through a different mechanism, the removal of shelterin components, also activates ATM or ATR-dependent signaling. Which pathway is activated is dependent on which shelterin component is removed. Deletion of TRFI activates the ATM pathway while removal of POT 1 primarily activates the ATR pathway.

[0017] The role of ATM in regulating telomere elongation in mammalian cells has been more controversial than in yeast. In human cells, a prominent, early paper suggested that ATM plays no role in human telomere maintenance. However other reports suggested cells might have shorter telomeres in the absence of ATM. The different methods for measuring telomeres and the small number of samples analyzed left this unresolved. Mouse studies on ATM and telomere elongation have also failed to find a definitive role for ATM. To detect telomere shortening in the absence of telomerase, it requires four to six generations of interbreeding telomerase null mice. Two groups, including our own, showed that first generation ATM null mice do not have short telomeres. Progressive breeding of ATM+/-
heterozygotes did not show telomere shortening. However, to detect telomere shortening in telomerase null mice, four to six generations of progressive breeding is required. Since ATM−/− mice are sterile, it is not possible to interbreed them to examine telomere length over many generations. Thus, the failure to see short telomeres in these mice might be simply due to the limitations of breeding. In addition, as discussed below, ATM and ATR play partially overlapping roles in several species, thus to see major changes in telomere length in mice may require reduction in both pathways.

[0018] The ATM protein kinase is a central regulator of the cellular response to DNA damage and the response to telomere dysfunction. After recognition of damage, ATM signals cell cycle arrest and induction of repair pathways. Ataxia telangiectasia (AT) patients, who lack ATM function, have immune system defects, neurological impairment, are cancer prone and radiosensitive. A role for ATM in telomere length maintenance was suggested when the ATM gene was cloned and shown to be the homolog of the yeast Tell gene. In yeast, loss of TellATM function leads to short telomeres. However, there have been conflicting results regarding the role of ATM in regulating telomere elongation in mammalian cells. In human cells, a prominent, early paper suggested that ATM plays no role in human telomere maintenance. However, other reports suggested cells might have shorter telomeres in the absence of ATM. Modification of human TRF1 protein by both ATM and tankyrase regulates binding of TRF1 to the telomere; however, this regulation of TRF1 is not conserved in mice.

[0019] At the cellular level, the loss of tissue renewal is caused by short telomeres that activates a DNA damage response, resulting in apoptosis or senescence. Critically short telomeres activate the ATM and ATR kinase-dependent pathways in primary human cells, leading to senescence. In addition, induction of telomere dysfunction through the removal of shelterin components also activates ATM or ATR dependent signaling and cell cycle arrest. Cancer cells avoid cell death through increased telomerase expression or other mechanisms that maintain telomere length.

[0020] While there has been significant progress and ongoing studies to understand the role of ATM and ATR in telomere dysfunction, less is known about the role of these kinases in normal telomere elongation, when telomeres are not critically short. A role for ATM in telomere length maintenance was first evident when the ATM gene was cloned and shown to be the homolog of the Tell gene in yeast. Loss of TellATM function leads to short telomeres. Interestingly, while deletion of the related kinase Mecl ATR does not itself cause telomeres shortening, the double mutant of TellATM Mecl ATR shows further shortening not
seen in Tell\textsuperscript{ATM} mutant alone. This implies that Mecl\textsuperscript{ATR} may partially compensate for the loss of Tell\textsuperscript{ATM}.

[0021] Given the conserved role of ATM in telomere length regulation in \textit{S. cerevisiae}, \textit{S. pombe}, and \textit{Arabidopsis}, the role of ATM kinase in telomere elongation in mammalian cells was revisited. To avoid the issues of breeding ATM\textsuperscript{-} mice and missing small effects of telomere length changes on long telomeres, an \textit{in vivo} telomere elongation assay was developed where telomerase repeat addition can be monitored over 48 hours. The assay is called ADDIT (Addition of \textit{de novo} initiated telomeres) and measures telomere addition at a single chromosome end.

**SUMMARY OF THE INVENTION**

[0022] The present invention is based on the seminal discovery of an assay that can identify genes required for telomerase-dependent telomere elongation by measuring the \textit{de novo} telomere addition at a single chromosome. Here, the successful development of a \textit{de novo} telomere elongation assay in mouse cells is reported. The assay is used to demonstrate that ATM kinase pathway regulates the elongation of telomeres by telomerase. This highlights the conserved nature of the pathways that regulate telomere length across species and suggests novel approaches to manipulating telomere length. An illustrative schematic of the screening assay of the invention is provided in the Examples.

[0023] The present invention relates to a method of identifying a regulator of telomere length which includes a) culturing a mammalian cell comprising a modified chromosome containing an internal telomere seed sequence and an endonuclease cleavage site downstream of the telomere seed sequence, wherein the cell conditionally expresses an endonuclease that cleaves and exposes the telomere seed sequence; b) contacting the cell of (a) with an agent that modulates expression of a selected gene or pathway in the cell; and c) measuring \textit{de novo} telomere addition to the seed sequence in the presence and absence of the agent, wherein addition of telomere sequence in the presence of the agent, but not in the absence of the agent, and the degree of addition, is indicative of identification of the agent as being a regulator of telomere length, thereby identifying a regulator of telomere length.

[0024] In one aspect, the measuring of c), above, is by a technique including PCR, such as a modified single telomere length analysis (STELA) or by PCR followed by nucleotide sequencing. In one aspect, the agent that stimulates cleavage of the chromosome at the cleavage site is a tetracycline such as doxycycline. In another aspect, the agent that affects expression of a selected gene or pathway in the cell is selected from a small molecule, a peptide, a nucleic acid molecule, or a protein. For example, the nucleic acid molecule is an
antisense or a siRNA molecule. One of the advantages of the present invention is that the screening can be performed after one cell cycle, e.g., 48 hours.

[0025] In some embodiments, the pathway is a kinase pathway, for example, the pathway is the ataxia telangiectasia mutated (ATM) kinase pathway or a cyclin dependent kinase pathway.

[0026] In another aspect, the invention provides an isolated mammalian cell line characterized by genome including a modified chromosome containing a telomere seed sequence and an endonuclease cleavage site downstream of the telomere seed sequence, wherein conditional cleavage of at the cleavage site will allow de novo elongation of the seed sequence. The cells are preferably mammalian cells, including murine or human cells. In an illustrative example provided herein the modified chromosome is mouse chromosome 4.

[0027] In yet another aspect, the invention provides a kit which includes cells of the mammalian cell line of the invention along with reagents for culturing the cells. The kit may further include reagents for measuring de novo telomere addition.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0028] Figures 1A-1B are pictorial representations relating to generation of a cell line to assay de novo telomere addition.

[0029] Figure 2 is a pictorial representation showing that modified STELA PCR products indicate de novo telomere addition in mTR+ cells.

[0030] Figures 3A-3C are pictorial and graphical representations showing that de novo telomere addition occurs only in telomerase-positive cells. Figure 3C includes SEQ ID NOs: 1-10 (ordered from top to bottom).

[0031] Figures 4A-4C are pictorial and graphical representations showing that de novo telomere addition is absent in siTERT-treated cells.

[0032] Figures 5A-5B are pictorial representations showing classification of de novo telomere addition. Figure 5A includes SEQ ID NOs: 11-16 and Figure 2B includes SEQ ID NOs: 17-34 (ordered from top to bottom).

[0033] Figures 6A-6B are pictorial and graphical representations showing de novo telomere addition occurs as early as 24 hrs after seed sequence exposure.

[0034] Figure 7 is a flow diagram depicting the pipeline of PacBio® sequence read analysis.
[0035] Figure 8 are pictorial and graphical representations showing that inhibition of ATM blocks \textit{de novo} telomere addition. Figure 8C includes SEQ ID NOs: 35-51 (ordered from top to bottom).

[0036] Figure 9 is a graphical representation showing that telomere lengths shorten in the presence of ATM inhibitor KU55933.

[0037] Figures 10A-10C are pictorial and graphical representations showing that \textit{de novo} telomere addition occurs in G2/M phase.

[0038] Figure 11 is a graphical representation showing that inhibition of Cdk5 prevents \textit{de novo} telomere addition.

[0039] Figure 12A and 12B are graphical representations showing that Cdk1 is required for \textit{de novo} telomere addition.

\textbf{DETAILED DESCRIPTION OF THE INVENTION}

[0040] The present invention is based on discovery of an assay that can identify genes required for telomerase-dependent telomere elongation by measuring the \textit{de novo} telomere addition at a single chromosome over just one cell cycle.

[0041] Before the present compositions and methods are further described, it is to be understood that this invention is not limited to particular compositions, methods, and experimental conditions described, as such compositions, methods, and conditions may vary. It is also to be understood that the terminology used herein is for purposes of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only in the appended claims.

[0042] As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0043] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods and materials are now described.

[0044] The present invention describes a new assay, named ADDIT, that can identify genes required for telomerase-dependent telomere elongation by measuring the \textit{de novo} telomere addition at a single chromosome. Using this assay, the inventors show that the
ATM kinase pathway and the cyclin dependent kinase pathway utilized in the cell cycle are required for telomerase-mediated telomere addition. Activation of ATM and cyclin dependent kinase 1 was shown to cause telomere elongation. Using an independent approach, they also found that inhibition of ATM kinase activity prevented bulk telomere elongation by telomerase and activation of ATM caused telomere elongation in cell culture experiments assayed by Southern blot.

[0045] The mechanism of telomere length maintenance involves many inter-dependent regulatory pathways that act together to establish length homeostasis. This process involves the interaction of telomere binding proteins such as shelterin with telomerase to regulate elongation. In addition to dedicate telomere binding proteins, DNA damage proteins including MRN complex and Ku are conserved players in length maintenance from yeast to mammals. Protein modification also plays a key role in regulating telomere length. It is demonstrated that one of the key regulators of telomere length, ATM kinase pathway, is also conserved from yeast to mammalian system. It is well documented that ATM is required for normal telomere maintenance in different yeast species including \textit{S. pombe} in which ATM mediates the telomerase recruitment. ATM homologue in Arabidopsis also plays a role regulating telomere lengths by promoting elongation of short telomeres. Drosophila telomeres do not use telomerase for telomere length maintenance, yet remarkably the role of ATM is conserved in telomere function. It suggests that even when telomerase was lost from this evolutionary branch of Diptera, the process that regulate telomere length was still retained.

[0046] ATR may compensate for loss of ATM.

[0047] Data from both the ADDIT assay and Southern analysis suggests ATM kinase is required for telomere length maintenance. When ATM was inhibited by siRNA different results were seen in ADDIT assay and the longer term bulk culture experiment: telomere addition was completely blocked in ADDIT assay but not when cells were grown continuously in culture. This difference may be due to the transient siATM knockdown that does not sufficiently block ATM in longer-term culture conditions. Another possibility is that ATR kinase may compensate for ATM loss in the longer-term culture experiments. Previous studies in \textit{S. cerevisiae} suggest ATR plays a minor, yet critical role in telomere maintenance. Cells lacking Tell\textsuperscript{ATM} are completely defective in telomere extension within the first few hours after the creation of short telomere. The bulk telomere lengths of tell\textsuperscript{TM} cells are short yet stable. Progressive telomere shortening is only seen when both Tell\textsuperscript{ATM} and Mecl\textsuperscript{ATM} are deleted, suggesting Mecl\textsuperscript{ATR} may play some role in telomere
elongation when Tell\textsuperscript{ATM} is missing. Similar to \textit{S. cerevisiae}, the shortest telomere phenotype of \textit{S. pombe} was seen in the Rad3\textsuperscript{ATR}/Tell \textsuperscript{ATM} double mutants. Interestingly, Rad3\textsuperscript{ATR} mutant cells have shorter telomere lengths compared to Tell \textsuperscript{ATM}, indicating Rad3\textsuperscript{ATR} play more critical role in telomere length regulation than does \textit{S. pombe} Tell \textsuperscript{ATM}.

Previous studies in mice indicated that ATM is not required for specific rescue and elongation of the shortest telomeres. Briefly, the ATM\textsuperscript{+/−} mice were crossed to mTR\textsuperscript{−/−} G5 late generation mice with short telomeres. In the F1 mice that resulted, shortest signal free ends were rescued in both ATM\textsuperscript{+/+} and ATM\textsuperscript{−/−} offspring, suggesting ATM is not essential for elongation of the shortest telomeres. This elongation, however, may be due to ATR compensating for the loss of ATM. The role of the ATR kinase in these pathways has not been examined as ATR null mice are lethal. Ataxia telangiectasia (AT) patients with mutations in the ATM gene have shorter telomeres compared to their age-matched controls, but not as short as other patients with telomerase mutations. This observation suggests ATR may also compensate for the loss of ATM function in telomere length maintenance in human. Dissecting the mechanism of telomere shortening in AT patients may have implications for individualized treatment plans. It is important to understand whether short telomeres can directly contribute to a more severe AT clinical phenotype and if telomere could be a potential therapeutic target.

Previous findings in \textit{S. cerevisiae} imply that the primary function of ATM in telomere maintenance is by modulating the access of telomerase to its substrate, telomere, rather than by altering the enzyme activity level of telomerase. The kinase activity of ATM is required for telomere maintenance as kinase dead mutant show short telomeres. While specific ATM substrates that affect telomere length have been characterized in \textit{S. pombe}, the key substrates in \textit{S. cerevisiae} are still controversial. In \textit{S. pombe}, Tell \textsuperscript{ATM} and/or Rad3\textsuperscript{ATR} phosphorylate a shelterin component Ccq1 that then interacts with telomerase subunit Estl to mediate telomerase recruitment. In \textit{S. cerevisiae}, while Tell \textsuperscript{ATM} can phosphorylate the single-strand telomere binding protein Cdc13, this phosphorylation apparently is not responsible for recruitment of Estl. While the precise functional homologues of Ccq1 and Estl in mammalian cell are not fully established likely due to sequence divergence and/or convergent evolution, shelterin components are still excellent candidates for ATM substrates in mammals given the conservation of length maintenance mechanisms across phyla. Previous studies in human cells suggest ATM phosphorylation of TRF1 can alter TRF1 association with telomeres, which can affect length regulation as well.
as end protection. Further, identification of ATM kinase target(s) will help to understand how ATM regulates telomerase recruitment and telomere elongation in mammalian cells.

0051 Most of the key players and requirements for telomere length regulation have been investigated in the model organism *S.cerevisiae*, including cyclin-dependent kinase (Cdk). Cdks are serine/threonine kinases responsible for various cellular processes such as cell cycle progression and transcription. In both *S.cerevisiae* and *S.pombe*, a single Cdk, Cdk1, is responsible for regulating cell cycle transitions. In addition to its critical role in cell cycle regulation, a previous study from our lab showed that Cdk1 is required for telomere elongation by regulating the generation of the 3' overhang. In higher eukaryotes, there are a growing number of Cdk family members identified. Functional characterization of these Cdks elucidated specific roles in different cellular processes such as cell cycle regulation, transcription and others, but the role in telomere length regulation is not well elucidated.

0052 The examples herein illustrate that telomerase-mediated telomere addition is regulated in a cell-cycle dependent manner in mammalian cells, and that mammalian Cdks, including Cdk1, are required for telomere elongation *in vivo*.

0053 The essential role of Cdk1 in telomere addition.

0054 Previous studies have shown that many of the key regulators required for telomere length maintenance are identical between the natural telomeres and *de novo* telomere ends. The yeast Cdk1 activity is also required to generate the 3' G-rich overhang, which is important for telomere length maintenance, at both *de novo* telomere ends as well as the natural telomeres. It is possible that Cdk1 activates a nuclease(s) that is involved in single-strand resection, such as Mrel 1 that is involved in resection at double-strand DNA break sites. In yeast, Cdk1 phosphorylates the nuclease Dna2, which is involved in generating the 3' overhang at telomeres. It will be interesting to find whether mammalian Cdk1 is also responsible for generating the 3' overhang by activating a nuclease(s) responsible for 5' strand resection.

0055 In addition, Cdk1 appears to be involved in regulating telomerase recruitment. The results from ADDIT assay shown the examples demonstrated that Cdk1 activity is required for *de novo* telomere addition in mammalian cells, indicating the conserved role of Cdk1 in telomere length regulation in higher eukaryotes.

0056 ADDIT assay may allow dissection of new pathways of telomere length regulation.

0057 The ADDIT assay described here will allow rapid dissection of the ATM pathway of telomere length regulation in addition to identification of new regulators of
telomere length. The very brief time of 48hrs required for the ADDIT assay allows the identification of essential genes that are difficult to probe for roles in telomere length regulation with the conventional method of long-term cell growth. The ADDIT assay was designed in mouse CASTEiJ cells that have telomere length and distribution very similar to humans, in contrast to standard laboratory strains. This allows functional probing of telomeres in a setting similar to human telomere length regulation. The assay will provide insights into telomere length homeostasis and may possibly identify potential targets for future therapeutics.

[0058] As such, the present provides to a method of identifying a regulator of telomere length which includes a) culturing a mammalian cell comprising a modified chromosome containing an internal telomere seed sequence and an endonuclease cleavage site downstream of the telomere seed sequence, wherein the cell conditionally expresses an endonuclease that cleaves and exposes the telomere seed sequence; b) contacting the cell of (a) with an agent that modulates expression of a selected gene or pathway in the cell; and c) measuring de novo telomere addition to the seed sequence in the presence and absence of the agent, wherein addition of telomere sequence in the presence of the agent, but not in the absence of the agent, and the degree of addition, is indicative of identification of the agent as being a regulator of telomere length, thereby identifying a regulator of telomere length.

[0059] In one aspect, the measuring of c), above, is by a technique including PCR, such as a modified single telomere length analysis (STELA) or by PCR followed by nucleotide sequencing. STELA was developed in 2003 by Duncan Baird. This technique may be incorporated as a part of the ADDIT assay. This technique allows investigations that can target specific telomere ends, which is not possible with TRF analysis described below.

[0060] Several techniques may be employed to assess average telomere length in eukaryotic cells. The most widely used method is the Terminal Restriction Fragment (TRF) Southern blot, which involves hybridization of a radioactive \(^{32}\)P-(TTAGGG)\(n\) oligonucleotide probe to restriction enzyme digested genomic DNA embedded on a nylon membrane and subsequently exposed to autoradiographic film or phosphoimager screen. Another histochemical method, termed Q-FISH, involves fluorescent in situ hybridization (FISH).

[0061] The present invention describes agents, such as chemical compounds or nucleic acid molecules, and the assay used for their identification, that modulate de novo telomere addition by affecting a gene or pathway implicated in telomere extension via telomerase,
such as a kinase pathway, for example, the ATM kinase pathway or a cyclin dependent kinase pathway active in cell cycling.

[0062] As used herein, an agent identified as a regulator of telomere length acts to increase extension of telomeres. The agent may interact directly with a gene promoter to effectuate an increase or decrease in transcription or the agent may interact in a number of other ways to indirectly increase telomere addition. For example, the agent may activate a particular signal transduction pathway leading to increased or decreased transcription of a gene. Alternatively, the agent may act to suppress repressors of transcription by direct binding to the transcriptional repressor thus blocking binding of the repressor to a promoter. Alternatively, the agent may act indirectly to suppress transcriptional repressors or increase transcription.

[0063] Agents to be screened encompass numerous chemical classes, though typically they are chemical compounds, such as an organic molecule, and often oligonucleotides or small organic compounds (i.e., small molecules) having a molecular weight of more than 100 Daltons and less than about 2,500 Daltons. Test agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The test agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Test agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

[0064] Agents may be obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification to produce structural analogs.

[0065] In one aspect, an agent for use in with the present invention is a polynucleotide, such as an antisense oligonucleotide or RNA molecule. In various aspects, the agent may
be a polynucleotide, such as an antisense oligonucleotide or RNA molecule, such as microRNA, dsRNA, siRNA, stRNA, and shRNA.

[0066] MicroRNAs (miRNA) are single-stranded RNA molecules, which regulate gene expression. miRNAs are encoded by genes from whose DNA they are transcribed but miRNAs are not translated into protein; instead each primary transcript (a pri-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional miRNA. Mature miRNA molecules are either fully or partially complementary to one or more messenger RNA (mRNA) molecules, and their main function is to down-regulate gene expression. MicroRNAs can be encoded by independent genes, but also be processed (via the enzyme Dicer) from a variety of different RNA species, including introns, 3' UTRs of mRNAs, long noncoding RNAs, snoRNAs and transposons. As used herein, microRNAs also include "mimic" microRNAs which are intended to mean a microRNA exogenously introduced into a cell that have the same or substantially the same function as their endogenous counterpart. Thus, while one of skill in the art would understand that an agent may be an exogenously introduced RNA, an agent also includes a compound or the like that increase or decrease expression of microRNA in the cell.

[0067] The terms "small interfering RNA" and "siRNA" also are used herein to refer to short interfering RNA or silencing RNA, which are a class of short double-stranded RNA molecules that play a variety of biological roles. Most notably, siRNA is involved in the RNA interference (RNAi) pathway where the siRNA interferes with the expression of a specific gene. In addition to their role in the RNAi pathway, siRNAs also act in RNAi-related pathways (e.g., as an antiviral mechanism or in shaping the chromatin structure of a genome).

[0068] Polynucleotides of the present invention, such as antisense oligonucleotides and RNA molecules may be of any suitable length. For example, one of skill in the art would understand what lengths are suitable for antisense oligonucleotides or RNA molecule to be used to regulate gene expression. Such molecules are typically from about 5 to 100, 5 to 50, 5 to 45, 5 to 40, 5 to 35, 5 to 30, 5 to 25, 5 to 20, or 10 to 20 nucleotides in length. For example the molecule may be about 5, 10, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 40, 45 or 50 nucleotides in length. Such polynucleotides may include from at least about 15 to more than about 120 nucleotides, including at least about 16 nucleotides, at least about 17 nucleotides, at least about 18 nucleotides, at least about 19 nucleotides, at least about 20 nucleotides, at least about 21 nucleotides, at least about 22 nucleotides, at least about 23 nucleotides, at least about 24 nucleotides, at least about 25
nucleotides, at least about 26 nucleotides, at least about 27 nucleotides, at least about 28 nucleotides, at least about 29 nucleotides, at least about 30 nucleotides, at least about 35 nucleotides, at least about 40 nucleotides, at least about 45 nucleotides, at least about 50 nucleotides, at least about 55 nucleotides, at least about 60 nucleotides, at least about 65 nucleotides, at least about 70 nucleotides, at least about 75 nucleotides, at least about 80 nucleotides, at least about 85 nucleotides, at least about 90 nucleotides, at least about 95 nucleotides, at least about 100 nucleotides, at least about 110 nucleotides, at least about 120 nucleotides or greater than 120 nucleotides.

[0069] The term "polynucleotide" or "nucleotide sequence" or "nucleic acid molecule" is used broadly herein to mean a sequence of two or more deoxyribonucleotides or ribonucleotides that are linked together by a phosphodiester bond. As such, the terms include RNA and DNA, which can be a gene or a portion thereof, a cDNA, a synthetic polydeoxyribonucleic acid sequence, or the like, and can be single stranded or double stranded, as well as a DNA/RNA hybrid. Furthermore, the terms as used herein include naturally occurring nucleic acid molecules, which can be isolated from a cell, as well as synthetic polynucleotides, which can be prepared, for example, by methods of chemical synthesis or by enzymatic methods such as by the polymerase chain reaction (PCR). It should be recognized that the different terms are used only for convenience of discussion so as to distinguish, for example, different components of a composition.

[0070] In general, the nucleotides comprising a polynucleotide are naturally occurring deoxyribonucleotides, such as adenine, cytosine, guanine or thymine linked to 2'-deoxyribose, or ribonucleotides such as adenine, cytosine, guanine or uracil linked to ribose. Depending on the use, however, a polynucleotide also can contain nucleotide analogs, including non-naturally occurring synthetic nucleotides or modified naturally occurring nucleotides. Nucleotide analogs are well known in the art and commercially available, as are polynucleotides containing such nucleotide analogs. The covalent bond linking the nucleotides of a polynucleotide generally is a phosphodiester bond. However, depending on the purpose for which the polynucleotide is to be used, the covalent bond also can be any of numerous other bonds, including a thiodiester bond, a phosphorothioate bond, a peptide-like bond or any other bond known to those in the art as useful for linking nucleotides to produce synthetic polynucleotides.

[0071] A polynucleotide or oligonucleotide comprising naturally occurring nucleotides and phosphodiester bonds can be chemically synthesized or can be produced using recombinant DNA methods, using an appropriate polynucleotide as a template. In
comparison, a polynucleotide comprising nucleotide analogs or covalent bonds other than phosphodiester bonds generally will be chemically synthesized, although an enzyme such as T7 polymerase can incorporate certain types of nucleotide analogs into a polynucleotide and, therefore, can be used to produce such a polynucleotide recombinantly from an appropriate template.

[0072] In various embodiments antisense oligonucleotides or RNA molecules include oligonucleotides containing modifications. A variety of modification are known in the art and contemplated for use in the present invention. For example oligonucleotides containing modified backbones or non-natural internucleoside linkages are contemplated. As used herein, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

[0073] In various aspects modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3′-alkylene phosphonates, 5′-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3′-amino phosphoramide and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and borano-phosphates having normal 3′-5′ linkages, 2′-5′ linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3′ to 3′, 5′ to 5′ or 2′ to 2′ linkage. Certain oligonucleotides having inverted polarity comprise a single 3′ to 3′ linkage at the 3′-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

[0074] In various aspects modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and
methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

[0075] In various aspects, oligonucleotide mimetics, both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. In various aspects, oligonucleotides may include phosphorothioate backbones and oligonucleosides with heteroatom backbones. Modified oligonucleotides may also contain one or more substituted sugar moieties. In some embodiments oligonucleotides comprise one of the following at the 2’ position: OH; F; 0-, S-, or N-alkyl; 0-, S-, or N-alkenyl; 0-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted Ci to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are 0[(CH₂)nO]mCH₃, 0(CH₂)nOCH₃, 0(CH₂)nNH₂, 0(CH₂)nCH₃, 0(CH₂)nONH₂ and 0(CH₂)nON[(CH₂)nCH₃]₂, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2’ position: Ci to C₁₀ lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, CI, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Another modification includes 2’-methoxyethoxy (2’0CH₂CH₂0CH₃, also known as 2’-0-(2-methoxyethyl) or 2’-MOE).

[0076] In one embodiment, an agent features a chemically modified nucleic acid molecule that includes one or more chemical modifications described herein. Non-limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2’-deoxyribonucleotides, 2’-0-methyl ribonucleotides, 2’-deoxy-2’-fluoro ribonucleotides, "universal base" nucleotides, "acyclic" nucleotides, 5’-C-methyl nucleotides, and terminal glyceryl and/or inverted deoxy abasic residue incorporation. These chemical modifications are shown to preserve activity in cells while at the same time,
dramatically increasing the serum stability of these compounds. In one aspect, the chemically modified nucleotide used in the invention includes a 2'-deoxyribonucleotide, 2'-O-methyl ribonucleotide, 2'-fluoro ribonucleotide, 2'-amino ribonucleotide, 2'-0-amino ribonucleotide, 2'-C-allyl ribonucleotide, 2'-0-allyl ribonucleotide, 2'-methoxyethyl ribonucleotide, 5'-C-methyl ribonucleotide, or a combination thereof. In another aspect, the chemically modified oligonucleotide used in the invention includes a 2'-deoxyribonucleotide, 2'-0-methyl ribonucleotide, 2'-fluoro ribonucleotide, 2'-amino ribonucleotide, 2'-0-amino ribonucleotide, 2'-C-allyl ribonucleotide, 2'-0-allyl ribonucleotide, 2'-methoxyethyl ribonucleotide, 5'-C-methyl ribonucleotide, or a combination thereof.

[0077] In a non-limiting example, the introduction of chemically modified nucleotides into nucleic acid molecules provides a powerful tool in overcoming potential limitations of in vivo stability and bioavailability inherent to native RNA molecules that are delivered exogenously. For example, the use of chemically modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically modified nucleic acid molecules tend to have a longer half-life in serum. Furthermore, certain chemical modifications can improve the bioavailability of nucleic acid molecules by targeting particular cells or tissues and/or improving cellular uptake of the nucleic acid molecule. Therefore, even if the activity of a chemically modified nucleic acid molecule is reduced as compared to a native nucleic acid molecule, for example, when compared to a native unmodified nucleic acid molecule, the overall activity of the modified nucleic acid molecule can be greater than that of the native molecule due to improved stability and/or delivery of the molecule.

[0078] In related aspects, the present invention includes use of Locked Nucleic Acids (LNAs) to generate antisense nucleic acids having enhanced affinity and specificity for the target polynucleotide. LNAs are nucleic acid in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene (-CH₂-)ₙ group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2.

[0079] Other modifications include 2'-methoxy(2'-0-CH₃), 2'-aminopropoxy(2'-OCH₂CH₂CH₂NH₂), 2'-allyl (2'-CH-CH-CH₂), 2'-0-allyl (2'-0-CH-2'-CH-CH₂), 2'-fluoro (2'-F), 2'-amino, 2'-thio, 2'-Omethyl, 2'-methoxymethyl, 2'-propyl, and the like. The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on
the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

[0080] Oligonucleotides may also include nucleobase modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine, 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxy and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine (1H-pyrimido[5,4-b][1,4]benzoxazi-n-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrimido[3′,2′:4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases are known in the art. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds described herein. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and 0-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 C and are presently preferred base substitutions, even more particularly when combined with 2'-0'-methoxyethyl sugar modifications.

[0081] Another modification of the antisense oligonucleotides described herein involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance
the activity, cellular distribution or cellular uptake of the oligonucleotide. The antisense oligonucleotides can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthidine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes.

[0082] The method of the present invention employs use of vectors including a promoter and gene to be stably integrated into a genome. A "promoter" is a nucleic acid sequence that directs the binding of RNA polymerase and thereby promotes RNA synthesis. Promoter sequences include constitutive and inducible promoter sequences. In various aspects, the promoters can be naturally occurring promoters, hybrid promoters, or synthetic promoters. Hybrid promoters, which combine elements of more than one promoter, are also known in the art, and are useful in the present invention.

[0083] The vectors employed in the present invention may include a reporter gene/protein or reporter molecule to facilitate detecting the transcriptional activity of a gene, such as the telomerase gene. There are many genes and molecules that may be used in such a fashion. In various embodiments, the reporter protein may be luciferase (LUC), β-lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo, G418), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), β-galactosidase (β-gal), and xanthine guanine phophoribosyltransferase (XGPRT), an affinity or epitope tag, or a fluorescent protein. In exemplary embodiments, the reporter protein is GFP or eGFP. A number of additional fluorescent proteins are known in the art and suitable for use with the present invention, including but not limited to blue fluorescent proteins (e.g., EBFP, EBFP2, Azurite, mKalamal), cyan fluorescent proteins (e.g., ECFP, Cerulean, CyPet) and yellow fluorescent proteins (e.g., YFP, Citrine, Venus, YPet). The present invention may also employ affinity or epitope tags, such as poly-His, GST, HA, Flag, myc, CBP, CYD (covalent yet dissociable NorpD peptide), HPC (heavy chain of protein C) peptide tags, MBP, or other tag well known in the art.

[0084] A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA encoding a polypeptide if it is expressed as a preprotein
that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. The transcriptional and translational regulatory nucleic acid will generally be appropriate to the host cell used for expression, as will be appreciated by those in the art. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of host cells.

[0085] In another aspect, the invention provides an isolated mammalian cell line characterized by genome including a modified chromosome containing a telomere seed sequence and an endonuclease cleavage site downstream of the telomere seed sequence, wherein conditional cleavage of at the cleavage site will allow de novo elongation of the seed sequence. The cells are preferably mammalian cells, including murine or human cells. In an illustrative example provided herein the modified chromosome is mouse chromosome 4.

[0086] In yet another aspect, the invention provides a kit which includes cells of the mammalian cell line of the invention along with reagents for culturing the cells. The kit may further include reagents for measuring de novo telomere addition.

[0087] The following example is provided to further illustrate the advantages and features of the present invention, but are not intended to limit the scope of the invention. While they are typical of those that might be used, other procedures, methodologies, or techniques known to those skilled in the art may alternatively be used.

EXAMPLE 1

DEVELOPMENT OF MAMMALIAN DE NOVO TELOMERE ADDITION ASSAY

[0088] Long-term cell viability is critically dependent on maintenance of telomere length. In humans, syndromes of telomere shortening cause age-related degenerative diseases that are often fatal. At the cellular level, the loss of tissue renewal that contributes to these diseases is caused by cellular recognition of short telomeres inducing apoptosis or cellular senescence. On the other hand, cancer cells avoid cell death by increasing or maintaining telomere lengths.
[0089] Telomere shortening occurs during normal cell division because DNA replication fails to copy the very end of the chromosome. Telomerase adds telomere repeats onto chromosome ends to balance the shortening that occurs in every replication cycle. The delicate balance of shortening and lengthening is regulated by an intricate series of feedback mechanisms to establish a robust telomere length equilibrium. Elucidating the molecular interactions that regulate telomere elongation is essential to understand telomere function and how it is disrupted in disease.

[0090] To identify novel regulators of telomere elongation in a mammalian system, an in vivo telomere elongation assay was developed where telomerase repeat addition can be monitored over 48 hours. In this example, the successful development of a de novo telomere elongation assay in mouse cells is discussed that allows measurement of telomerase-dependent de novo telomere addition in one cell cycle.

[0091] RESULTS

[0092] Generation of cell line to assay de novo telomere addition.

[0093] To examine potential mammalian genes that regulate telomere length, an assay was developed that allows visualization of telomere addition in vivo, referred here as ADDIT (Addition of de novo initiated telomeres). In a chromosomally stable CASTEiJ mouse fibroblast cell line, chromosome 4 (chr4) was modified to generate an internal 480 bp telomere 'seed' sequence followed by a unique I-SceI endonuclease cut site (Figure 1A). The length of seed sequence was based on an early study showing that 400 bp of telomere repeats can act as functional telomeres. Using Southern blot analysis, two independent HYG-positive clones were identified, clone 1L and 1M, with a single chr4 allele correctly modified (data not shown).

[0094] To examine telomerase-dependent elongation, this cell line was engineered to conditionally express telomerase. The parental CASTEiJ mouse fibroblast cells were mTR− and a retrovirus containing mTR and the green fluorescence protein (GFP) that can be removed by FLP/FRT recombination was introduced. Telomerase activity was not found in the parental mTR− cells but was present in GFP-positive transduced cells (data not shown).

[0095] To cut the endogenous chromosome 4 at the engineered I-SceI site in vivo, a HA epitope tagged I-SceI endonuclease driven by a tetracycline-inducible promoter was stably integrated and single clones isolated. A clone SL13 that expressed HA-I-SceI only in the presence of doxycycline was identified (data not shown). To compare telomere addition in cells with and without telomerase, the cells were transfected with a construct expressing the flp recombinase, and flow cytometry was used to sort GFP positive (with mTR) and GFP
negative (without mTR) cells. mTR level was measured by quantitative RT-PCR and confirmed mTR was present in GFP-positive cells, but absent in GFP-negative cells (data not shown). These populations are referred to as mTR+ or mTR-, respectively.

Cleavage of chromosome 4 with I-Scel will expose a telomere seed and allow telomere addition by telomerase (data not shown). A time course of I-Scel induction showed that chromosome cutting occurred in vivo as early as 8 hours after doxycycline treatment (data not shown). As a control, the genomic DNA was digested in vitro with purified I-Scel endonuclease to compare the genomic cut site in vivo and in vitro on the Southern blot. Close to 10% I-Scel cutting after doxycycline induction was detected, which is similar to previously shown efficacy of I-Scel cleavage in vivo. While this efficiency of cutting is not quite as high as found in yeast, which has a smaller genome, it was concluded it might be sufficient to examine telomere elongation in mouse cells.

De novo telomere addition only in telomerase positive cells.

To compare telomere addition in cells with and without telomerase, Southern blot was performed with mTR- and mTR+ cells collected at several different time points after doxycycline induction.

To better visualize the telomere addition on the I-Scel cut telomere seed, the single telomere length analysis (STELA) (Baird et al., 2004, Hum Mol Genet 13, 1515-1524), a PCR-based approach that can measure telomere lengths from individual chromosome ends was modified. The STELA assay employs annealing and ligation of a linker, 'telorett', with TTAGGG and a unique 20-nucleotide sequence to the G-rich 3' telomere overhang followed by PCR of the telomere from a 'teltail' primer and primer in the HYG sequence on the engineered chromosome (Figure 2). The PCR product lengths will be proportional to the telomere lengths at the cleaved chr4 allele. As a control, to amplify the un-extended cut chromosome in vitro, a different linker, 'IScerette' was designed, which has a 4-nt homology to the 4-nt 3' overhang created by the I-Scel endonuclease at the cleavage site (Figure 2). Genomic DNA digested with I-Scel endonuclease in vitro and ligated with the 'IScerette' linker and PCR amplified, generated the predicted length of STELA PCR product indicating that the IScerette linker efficiently ligated to the cleaved DNA (data not shown). This in vitro IScerette PCR product serves as a marker for the base line length of the cut, unextended telomere seed sequence.

STELA PCR products from genomic DNA of telomerase-positive cells (mTR+) treated with doxycycline were longer than the control IScerette products (data not shown), suggesting there was new telomeric sequence addition onto the seed sequence. In contrast,
telomerase-negative cells (mTR-) treated with doxycycline resulted in STELA PCR product sizes only similar to and shorter than the base line, suggesting the longer products are dependent on telomerase activity.

[00101] Sequencing the de novo telomere addition products.

[00102] To further verify the longer STELA PCR products represent de novo telomere addition, the PCR products were sequenced with Pacific Biosciences (PacBio®) next generation sequencing technology. The PacBio® platform produces longer reads and is less GC bias compared to other next generation sequencing platforms. While errors were present in the sequence as expected, the telomere repeats were easily recognizable. All of the reads from the PacBio were filtered and only those that had unique HYG sequence followed by telomere sequence and the 'teltail' primer sequence were examined. This assures that only full length of STELA PCR products were analyzed. The reads were then aligned at the junction between the HYG sequence and the telomere repeats. Wild-type TTAGGG repeats were colored orange and variant telomere repeats were colored in darker orange (Figure 3A). Some of these variants arose due to errors in sequencing. An error rate similar to the published error rate of PacBio® sequencing, 10-15% was observed. The errors were uniformly distributed over the reference sequence, dominated by point insertions and deletions, as expected for PacBio® sequencing. There were three regions of divergent sequence in the original construct that are evident as darker orange stripes in the aligned reads (see for reference, in vitro IScelrette). These are small variation in TTAGGG sequence in the original SL13 clone and serve as useful internal reference points.

[00103] The PacBio® sequence reads from the mTR+ sample showed a heterogeneous population of telomere lengths and notably had a significant fraction of telomeric reads that contained the I-Scel recognition site followed by additional telomere sequences (Figures 3A and 3C). Telomerase has been shown to add telomere repeats onto primers (or sequences) that contain some non-telomeric sequence. The reads that are shorter than the reference sequence are presumably due to 5’ end resection occurring in vivo at telomeres or internal deletions during sequencing.

[00104] In both the mTR- and the IScerette samples, there were few reads that appeared slightly longer than the initial input of telomere seed sequence (Figure 3A). Careful examination of these sequences indicated that they did not contain telomere repeats that were added to the I-Scel site as seen in the mTR+ samples. This suggests that these longer products that are present in both in vitro and in vivo experiments occurred through slippage during STELA PCR and/or the PacBio® sequencing. Because of this slippage,
only those reads that contain telomere sequence past the I-Scel as *de novo* telomere elongation were defined.

[00105] Telomere elongation is regulated by two main processes of telomerase: 1) the processivity of the telomerase enzyme and 2) the recruitment of telomerase to the *de novo* end. Since the ADDIT assay measures addition of telomeres in only one cell cycle, the processivity of telomerase would regulate the length of *de novo* sequence added in one round of elongation. In contrast, the number of ends elongated will represent the probability of telomerase recruitment to an end in one cell cycle. The percentage of PacBio® reads with any *de novo* telomere sequence for each sample was measured, to address the probability of telomerase recruitment to the induced chr4 end. Notably, in the mTR+ cells, approximately 25% of the reads had telomere sequence after the I-Scel site indicative of *de novo* elongation (Fig 2.3B). The presence of this longer class is consistent with the heterogeneous smear longer than the base line in the STELA PCR products (data not shown). Telomere lengths from mTR- sample were also heterogeneous; however, no addition of repeats beyond the I-Scel site was seen (Figures 3A and B), suggesting *de novo* telomere addition did not occur. As expected, sequence reads from the *in vitro* IScerette control sample were less heterogeneous and matched the reference sequence although some insertions and deletions, likely due to sequencing errors, were seen (Figure 3A).

[00106] As an independent method to confirm that the *de novo* telomere addition is telomerase-dependent, cells were treated with siRNA against TERT (siTERT), the catalytic component of telomerase, to inhibit the telomerase activity and then performed the ADDIT assay. TERT RNA expression level was verified and was reduced more than 60% compared to the control by quantitative RT-PCR (Figure 4A). As expected, none of the PacBio® reads from siTERT-treated sample showed *de novo* telomere addition (Figures 4B and 4C). Altogether, these results confirm the *de novo* telomere addition observed by the ADDIT assay is telomerase-dependent.

[00107] *Classification of de novo telomere addition.*

[00108] During the elongation cycle, telomerase uses the template region of the mTR to add telomere repeats. The telomerase RNA has a primer-alignment region adjacent to the template sequence that specifies the nucleotides added by the active site (Figure 5A). The alignment region plays an important role in positioning the telomere substrate. For the mouse TR, there is a 2-nt alignment region, while the human RNA contains 5 nucleotides in the alignment region. Evaluation of the I-Scel cleavage site showed that it has some
sequence similarity to a telomeric sequence and can base pair with the mTR primer-alignment region (Figure 5A).

[00109] All of the reads in the mTR+ sample were classified into six different classes of telomere repeat addition (Figure 5B). Each of these classes can be distinguished by the degree of 3' end resection of the I-SceI site and unique positioning of the 3' end with primer-alignment and template sequence in mTR. In 76 of the 697 PacBio® reads analyzed (11%), new telomeric repeats were directly added after the I-SceI 3' overhang without any loss of nucleotides (Class 1, Figure 5B).

[00110] The most common class of telomere addition (Class 3, 44%) had the most complementarity between the mTR primer and template sequence (AGGG). In this class, 4-bp overhang was lost as the telomeric repeats were added beyond the 3' G-rich sequence. The next most common telomere addition (Class 5, 24%) occurred by telomerase recognizing the G-rich sequence located upstream of the initial cut site, which also results in formation of three G:C base pairs. Interestingly, in Class 2, the 3' end resection positions the de novo 3' end within the alignment region of mTR and resulted in the incorporation of a C at the junction with the telomere repeats that is not in the I-SceI site nor the telomere sequence. This occasional incorporation of nucleotides that are normally in the alignment region as a template has also been found in telomerase RNA mutant in vitro. This incorporation of the templated C residue further supports the conclusion that telomere repeats are being added by telomerase activity in vivo.


[00112] To measure how efficiently telomere addition occurs in vivo, cells were collected at different time points after doxycycline induction and performed the ADDIT assay. A high percentage of de novo telomere addition was observed 24 hours (hrs) post-doxycycline treatment but not in the early time points (Figure 5). Given that telomere seed is exposed in vivo as early as 8 hrs after doxycycline induction (data not shown) and population doubling time of SL13 cells is 24 hrs, the short period of time of 8 to 16 hrs required to detect de novo telomere addition suggests the elongation occurred in one cell cycle.

[00113] FIGURE LEGENDS

[00114] Figure 1 pertains to generation of cell line to assay de novo telomere addition. (A) The chr4 subtelomeric targeting construct has two homology arms, hygromycin cassette (HYG), 480 bp telomere sequence (arrows) followed by I-SceI restriction site as well as the HSV-tk cassette. The predicted sizes of AflII-digested genomic DNA of wild-type mouse chr4 allele and correctly targeted allele are shown. A, AflII; B, BstBI; X, Xhol. (B)
Schematic of the ADDIT assay. Doxycycline induction of I-Scel endonuclease exposes the telomere 'seed' sequence. New telomere repeats (lighter arrows) are added by telomerase. The predicted sizes of Ncol-digested genomic DNA of chr4 allele before and after I-Scel cutting are shown. N, Ncol.

[00115] Figure 2 shows that modified STELA PCR products indicate de novo telomere addition in mTR+ cells. Representation of modified STELA, showing primers (arrows) and linkers either 'telorette' added to telomere or 'IScerette' added to cleaved I-Scel end. Telomeres were PCR amplified with a forward primer, either F1 or F2, and a reverse primer, teltail. Orange boxes represent telomere repeats and green box represents I-Scel restriction sequence. S, Sphl.

[00116] Figure 3 shows that de novo telomere addition occurs only in telomerase-positive cells. (A) Analysis of PacBio circular consensus sequence (CCS) reads is shown. Reads were filtered for those that have both the unique HYG sequence followed by telomere sequence and also have the 'teltail' primer sequence, to assure only full length of STELA PCR products were analyzed. Wild-type telomere repeats are shown in orange, divergent telomeric sequence in darker orange and the I-Scel site is shown in green. X-axis indicates the length (bp) from the start of the telomere seed sequence. Maximum of 500 reads from each sample were shown for simplicity. (B) The percentage of PacBio CCS reads with de novo telomere repeats was calculated from each sample by using the following formula: 100 \% \times \text{(number of CCS reads with telomere repeats added beyond the I-Scel site) / (number of total CCS reads)}. n, number of independent samples analyzed. (C) The sequences of PacBio CCS reads boxed in A are shown.

[00117] Figure 4 shows that de novo telomere addition is absent in siTERT-treated cells. (A) Relative expression levels of mTERT normalized to UPRT measured by quantitative RT-PCR. Error bars indicate the SEM from triplicates of SL13 untreated and treated with siTERT at final concentration 100nM. (B) The percentage of PacBio® CCS reads with de novo telomere repeats was calculated from each sample by using the following formula: 100 \% \times \text{(number of CCS reads with telomere repeats added beyond the I-Scel site) / (number of total CCS reads)}. n, number of independent samples analyzed. (C) PacBio® analysis of samples either treated with or without siTERT (maximum of 400 CCS reads shown for simplicity). X-axis indicates the length (bp) from the start of the telomere seed sequence. Note increased size length of seed sequence and two additional darker stripes that serve as internal reference points were detected from both samples indicating possible duplication of the telomere seed sequence from the original SL13 cell line.
[00118] Figure 5 shows the classification of *de novo* telomere addition. (A) The 42-nt unique sequence (green) located immediately after telomere seed includes the 18-nt I-Scel recognition site (black box). I-Scel cut leaves a 3’ 4-nt overhang. The sequences of the telomerase mTR template (blue) and primer-alignment region (red) are shown. Potential Watson-Crick base-pairings indicated by vertical lines. Wobble pairing shown with dotted vertical lines. (B) Total \( n=697 \) of PacBio® reads from wild-type sample (mTR+ in Figure 3A) were classified by where the telomere repeat sequences were added, and the percentage of reads followed in each class are shown. The different degree of 3’ end resection of the I-Scel site and potential positioning with mTR primer region is shown along with a representative PacBio read of each class. The incorporation of a C in Class 2 is highlighted. *De novo* added wild-type telomere repeats are light.

[00119] Figure 6 shows that *de novo* telomere addition occurs as early as 24 hrs after seed sequence exposure. (A) PacBio® analysis of CCS reads from samples collected at different hours after doxycycline exposure is shown (maximum of 250 reads for simplicity). X-axis indicates the length (bp) from the start of the telomere seed sequence. Note increased size length of seed sequence and two additional darker stripes that serve as internal reference points indicating possible duplication of the telomere seed sequence from the original SL13 cell line. (B) The percentages of PacBio® CCS reads with *de novo* telomere repeats from A.

[00120] Figure 7 shows the pipeline of PacBio® sequence read analysis. (A) PacBio® sequencing reads were aligned to the last 50 bases of the hygromycin gene sequence (HYG) using the pairwiseAlignment function in the Biostrings™ package in Bioconductor™ (Pages H.) with parameters: local-global, mismatch penalty -3, match bonus 2, gapOpening -2, gapExtension -2. (B) Using the same parameters, the last X bp of the 3′ ends of reads (where X is 1.5 times the maximum-length teltail sequence) to the teltail sequence were aligned. In multiplexed runs, all possible barcoded teltail sequences were aligned and only the best alignment considered, excluding reads that aligned equally well to multiple barcoded teltail sequences. Reads with a HYG alignment score greater than or equal to 60 and a teltail alignment score greater than or equal to 20 were kept for subsequent analysis. (C) Reads were then aligned by the sequence between the end of the HYG alignment and the start of the teltail alignment to the I-Scel sequence with parameters: local, mismatch penalty -3, match bonus 2, gapOpening -2, gapExtension -2. A score of 36 was sufficient to identify the I-Scel sequence. The parsed read set was manually curated to ensure that seed and elongation sequences contained telomeric repeats. Additional functions used in the pipeline were provided by the R packages parallelMap and stringr.
DISCUSSION

In this example, successful development of the ADDIT assay that can identify novel mammalian genes required for telomerase-dependent telomere elongation by measuring de novo telomere addition at a single chromosome is discussed. It was verified that the telomere addition is telomerase-dependent and occurs in vivo over just one cell cycle. It takes roughly 6 to 8 weeks to observe bulk telomere length change in mammalian cells; however, by monitoring a single chromosome end, ADDIT assay can detect telomere changes less than a day. The very brief time required for the assay also allows the identification of essential genes that are difficult to probe for roles in telomere length regulation with the conventional method of long-term cell growth.

The ADDIT assay was designed in mouse CASTEiJ cells that have telomere length and distribution very similar to humans, in contrast to standard laboratory strains. This allows functional probing of telomeres in a setting similar to human telomere length regulation. The assay will provide insights into telomere length homeostasis and may allow identification of potential targets for future therapeutics.

MATERIALS AND METHODS

Plasmid construction.

Chr4 targeting construct (pSL25)

The chr4 subtelomeric targeting construct was made in multiple steps. The chr4 homology arms were amplified from BAC clone (#RP24-225H17) using the following primers: 1st arm_F, 1st arm_R, 2nd arm_F and 2nd arm_R. The telomere seed sequence was amplified from JHU821 plasmid (described in Morrish and Greider, 2009, PLoS Genet 5, el000357) using primers pBlueSK_F and JHU821_Sall_R. HSV-tk was PCR amplified from plasmid GFAP-HSV- tk (pTGB008, Addgene #24703) using primers HSVtk_F and HSVtk_R. All fragment sequences were verified and cloned in pMSCV-HYG vector (pSL25). pSL25 construct was linearized with XhoI and NotI to yield a -16 kb fragment, which was gel purified prior to transfection.

mTR/EGFP retroviral construct (pSL8)

To generate a mTR-conditional cell line, mTR driven by its endogenous promoter was amplified from pMSCV-mTR-HYG plasmid using primers HpalFRT_mTR_F and mTR_EcoRI_R. EGFP with FRT site was amplified from plasmid pcDNA5/FRT/TO (Invitrogen) using primers EcoRI_EGFP_F and Clal_FRT_EGFP_R. These two fragments were cloned in a retroviral vector pMSCV- HYG to generate pSL8.

Dox-inducible HA-I-Scel construct (pSL39)
To generate a Dox-inducible HA-I-Scel expression system, a lentiviral construct containing HA-I-Scel driven by a tetracycline-inducible promoter (pSL39) was generated by assembling four fragments using the Gibson assembly cloning kit (NEB).

The selection marker GFP from the original Lenti-tet-ON plasmid (Holland et al., 2012, Genes & Development 26, 2684-2689) was replaced with red fluorescence protein (RFP). The four fragments are the following: (1) HA-I-Scel fragment amplified from pCBAScel (Addgene #26477) using primers Iscel_F1 and Iscel_F2; (2) TetR fragment amplified from Lenti-tet-ON plasmid using primers rTetR_F2 and rTetR_R2; (3) RFP cassette amplified from dsRed-Express2 plasmid using primers dsRed-Express2_F3 and dsRed-Express2_R3_T; (4) vector fragment Lenti-tet-ON plasmid digested with Agel and BsrGI. PCR products as well as the final constructs were all sequence verified.

Cell culture and treatments.

Cell lines including 293T, 293FT, Pheonix, clone 1L and mTR. skin fibroblasts were grown in DMEM (Gibco) supplemented with 1% Penicillin/Streptomycin/Glutamine (PSG) and 10% heat inactivated FBS (Invitrogen). SL13 cells were grown in DMEM (Gibco) supplemented with 1% PSG and 10% Tet system approved FBS (Clontech, #631107).

Development of SL13 cell line.

First, to generate the telomerase-conditional cell line, mTR. skin fibroblasts from CAST/EiJ mice (Morris and Greider, 2009, PLoS Genet 5, el000357) were transduced with the mTR/GFP retrovirus (pSL8) (retrovirus transduction protocol described in more details below) and flow sorted for GFP-positive fluorescence. To modify the chr4 allele, GFP-positive cells were transfected with linearized chr4 targeting construct (pLSL25) using Xtreme 9™ (Roche). After 3 days of transfection, cells were selected for hygromycin resistance at final concentration of 500 μg/ml for 1 week followed by an additional 1 week of negative selection with ganciclovir at 35 μg/ml final concentration to select against Tk gene. The HYG^RGVC cells were plated at a very low density and grown for approximately 2 weeks until clonal populations were visible. Clonal populations were isolated with cloning cylinders (Sigma, #C1059) and screened for correct integration by Southern analysis. To integrate the doxycycline-inducible HA-I-Scel expression system, clone 1L was transduced with lentivirus pSL39. Later RFP-positive cells were flow sorted in 96-well plate as single clones. To induce I-Scel expression, doxycycline at final concentration of 2 μg/ml was added to cells. Typically cells were collected post 48 hours of doxycycline treatment. Clones were screened for doxycycline-dependent HA-I-Scel expression by Western blot. To collect
telomerase-negative cells, SL13 cells were transfected with a plasmid (pPGKFLPobpA, Addgene #13793) expressing flp, then approximately 10 days later GFP+ and GFP- cells were flow sorted.

[00137] Retrovirus packaging and transduction.

[00138] To generate the telomerase-conditional cell line, mTR<sup>−</sup> skin fibroblasts from CAST/EiJ mice (Morrish and Greider, 2009, PLoS Genet 5, e1000357) were transduced with the mTR/GFP retrovirus (plSL8). In brief, 2 x 10<sup>6</sup> Pheonix cells were plated onto 10-cfl polystyrend plates (BD Falcon) in 10 ml complete medium (DMEM (Gibco), 10% FBS (Gibco), 1X PGS (Gibco)) and allowed to double overnight. The following day, cells were transfected with 2 µg of plSL8 plasmid. Transfection was performed with FuGENE-6 (Promega) and Opti-MEM serum-free medium (Gibco). The next day following transfection, cells were fed with 3-4 ml of fresh media and incubated at 32°C. Viral supernatant was collected at 48 and 72 hrs post-transfection, and filtered through a 0.45 µm CN filter (Thermo Scientific) to eliminate any remaining non-viral debris. The viral supernatant was immediately used or stored at 4°C. For titering, 293FT cells were seeded in complete medium in 6-well plates at 10<sup>5</sup> cells/well and allowed to double overnight. The following day, polybrene-treated cells (8 µg/ml, Sigma) were infected with 1 and 2 µl as well as mock-infected controls with 1X PBS, and incubated overnight at 37°C, 5% CO<sub>2</sub>. The following morning, the medium was changed to eliminate the polybrene, which is toxic to cells. Cells were allowed to divide for an additional 36-48 hours and the percentage of GFP-positive cells determined by flow cytometry, a total of 48-60 hours post-infection. The viral titer (T.U./µl) was calculated using the following formula: {2x10<sup>5</sup> cells x (% GFP-positive cells - % GFP-positive mock-infected cells)} / 1 or 2 µl. To infect mTR/GFP retrovirus (plSL8) with MOI <1, 1x 10<sup>6</sup> mTR<sup>+</sup> skin fibroblasts were incubated with 3.5ml of 48 hrs viral supernatant as well as final concentration 8ug/ml polybrene at 32°C. After 6 hrs, supernatant was removed and refreshed with complete medium. 24 hrs after the first infection, the viral infection was repeated with the 72 hrs viral supernatant.

[00139] Lentivirus packaging and transduction.

[00140] To generate a doxycycline-inducible HA-I-Scel expression system, lentivirus plSL39 was first made. Briefly, 15-cm polystyrend plates (BD Falcon) were coated with 100 µg/ml sterile poly-D lysine and 6-8 x 10<sup>6</sup> 293FT cells were plated in complete medium (DMEM (Gibco), 10% FBS (Gibco), 1X PGS (Gibco)) and allowed to double overnight. The following day, the medium was changed to DMEM, 1% FBS and cells were co-transfected with 3 plasmids: plSL39 (containing the HA tagged I-Scel), pCMVA8.91
(containing the gag and pol lentiviral genes), and pVSV.G (containing the env lentiviral gene). Transfections were performed with Lipofectamine 2000™ (Invitrogen) and Opti-MEM™ serum-free medium (Gibco). After 48 hrs, the supernatant was collected, centrifuged for 5 minutes at 1000 rpm to get rid of large cell debris, and filtered through a 0.45 µm CN filter (Thermo Scientific) to eliminate any remaining non-viral debris. Aliquots of filtered supernatant were frozen at -80°C or used immediately. To transduce pSL39 lentivirus, clone IL cells were seeded in complete medium in 6-well plates at 10⁵ cells/well and allowed to double overnight. The following day, polybrene-treated cells (8 µg/ml, Sigma) were infected with pSL39 lentivirus and incubated overnight at 37°C, 5% CO₂. The following morning, the medium was changed to eliminate the polybrene and refreshed with complete medium. To estimate the efficiency of transduction, RFP intensity was measured by flow cytometry after 48 hrs.

[00141] Quantitative RT-PCR.

[00142] To measure mTR levels, total RNA (1 µg) from wild-type, mTR⁻/⁻, GFP+ and GFP- cells was reverse transcribed using random hexamers and Superscript III™ reverse transcriptase (Invitrogen), following the manufacturer’s instructions. Quantitative RT-PCR was performed using a CFX96 thermocycler (Bio-Rad). Each quantitative RT-PCR reaction contained 1X SYBR Green Supermix and 5 µM of each primer. Roughly, 5 ng cDNA were amplified per reaction. The expression in each sample was normalized to UPRT. The cycling conditions for mTR were as follow: 5 minutes at 95°C; 15 seconds at 95°C, 30 seconds at 68°C, 45 seconds at 72°C, 10 seconds at 82°C (35 cycles); 3 minutes at 72°C. For each cycle, fluorescence readings were performed at the 82°C step, to avoid generation of primer dimers. Primers used were the following: RT_mTR_F, RT_mTR_R, HPRT_F and HPRT_R. Triplicates were run for each sample and the normalized average was reported.

[00143] Telomeric repeat addition protocol (TRAP).

[00144] Telomerase activity was measured using the telomeric repeat amplification protocol (TRAP), a 2-step PCR-based assay. Briefly, cells were spun down and washed, and cell extracts were generated by resuspending the pellets in 1X CHAPS lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 1 mM EGTA pH 8.0, 0.1 mM benzamidine, 5 mM β-mercaptoethanol (BME), 0.5% CHAPS, 10% glycerol). After a 30 minute incubation on ice, cells were spun down for 20 minutes at 14,000 rpm at 4°C. The supernatant was collected and half of it was treated with 2 µg RNase A, incubated for 10 minutes at 65°C, then chilled on ice. In step 1, RNase A-treated and untreated lysates were incubated with 1X TRAP reaction buffer (200 mM Tris-HCl pH 8.3, 15 mM MgCl₂, 630 mM KCl, 0.5% Tween-20,
10 mM EGTA, pH 8.0), 0.1 mM dNTPs, and 34 µM TS primer for 1 hour at 30°C. For the second TRAP step, 2 µl of the extension products in step 1 were added to 48 µl of a master mix containing 1X Taq buffer (Denville), 0.1 mM dNTPs (TaKaRa), 2 U Taq polymerase (Denville), 20 µM end-labeled TS primer (4 µCi γ-32P-ATP, 1X PNK forward buffer (Invitrogen), 20 µM TS primer, 1 U T4 PNK (Invitrogen); 30 minutes at 37°C, 15 minutes at 65°C) and primer mix (8.5 µM reverse primer, 17 µM internal control reverse primer Kl, 10⁻¹² µM internal control template TSK1). A 2-step PCR amplification protocol was used (94°C x 10 min; 30 cycles of 94°C x 30s, 59°C x 30 sec). PCR products were mixed with 1X GelPilot DNA loading dye (QIAGEN) and separated on a 10% non-denaturing polyacrylamide gel (IX TBE, 10% acrylamide/bis solution (19:1) (BioRad), 2% glycerol, 0.144% ammonium persulfate (Sigma), 0.04% N,N,N’,N’-Tetramethylethylenediamine (Sigma)) by running the gel for 90 minutes at < 20 W and drying at 80°C for 1 hour. Due to the repetitive nature of the telomeric sequence, the resulting PCR products consist of fragments that are separated by six basepairs. The dried gel was exposed on a phosphorimager screen and scanned on a Storm 860™ imager (GE Healthcare).

[00145] Southern blot analysis.

[00146] To screen for clones with correct modification at chr4 subtelomeric allele, genomic DNA was extracted from clones using the Puregene Core Kit A™ (Qiagen). AflII-digested genomic DNA was resolved by 0.7% Tris-acetate-EDTA (TAE) agarose gel electrophoresis. Following denaturation (0.5 M NaOH/1.5 M NaCl) and neutralization (1.5 M NaCl/0.5 M Tris-HCL pH 7.4), the DNA was transferred in 20X SSC to a Nylon Membrane (Amersham Hybond N+) by weighting method overnight and cross-linked with UV Stratalinker™ (Stratagene). Pre-hybridization was done at 42°C for 2 hours in prehybridization buffer (50% Formamide/6X SSC/1% SDS/5X Dendart/sperm DNA) freshly made. A radioactive chr4 probe was made by random-prime labeling using Prime-It II™ (Stratagene) with a slight modification. Briefly, 25 ng of a 1.2 kb chr4 homology arm 2 containing probe, acquired from XcmI digestion of plSL13-7 plasmid or 1 KB Plus DNA ladder (Invitrogen) was labeled using 33 µM of dATP, dTTP, 50 µCi of α-32P dCTP (3000 Ci/mmol) and 50 µCi α-32P dGTP (3000 Ci/mmol). Unincorporated nucleotides were removed using a G50 column (GE Healthcare). Labeled probe was counted and 10⁶ counts/ml (chr4 probe) or 10⁴ counts/ml (ladder) was denatured at 100°C for 5 minutes and added to the pre-hybridization solution and hybridized overnight at 42°C. Membranes were washed 3x 15 minutes each in 6X SSC and 1% SDS at 65°C, and 3x 15 minutes each in 1X SSC and 1% SDS at 65°C and exposed to a phosphorimager screen and detected on a
Fuji phosphorimager. To examine the HYG-specific bands, the blot was stripped by incubating with 0.4M NaOH at 45°C for 30 minutes followed by incubation in 0.1XSSC/0.1%SDS/0.2M Tris-HCl pH 7.5 at room-temperature for 15 minutes. The blot was prehybridized and re-probed with random primed a-32P-labeled HYG probe. HYG probe fragment was made by using the following primers: HYGprobe_F and HYGprobe_R. To examine the in vivo chr4 cleavage, genomic DNA extracted from SL13 cells treated with doxycycline for various time points were digested with Ncol restriction enzyme (NEB) and further analyzed by Southern as described with a random primed a-32P-labeled HYG probe.

[00147] **Western blot analysis and antibodies.**

[00148] To detect HA-tagged I-Scel, IX lysis buffer made of final concentration of IX RIPA (Cell Signaling, #98016S), IX protease inhibitor cocktail (Roche) was added directly to cells on the dish after washing with IX PBS. Protein concentration was measured with a BCA protein assay kit (Thermo, #23227). Typically 10 µg of protein from each sample was applied to SDS-PAGE gels (Biorad, #456-1084) and transferred to nitrocellulose membranes in transfer buffer. After blocking with the Odyssey™ blocking buffer (LI-COR, #927-40000) for 1 hour at room temperature (RT), membranes were incubated at 4°C overnight with primary antibodies: anti-HA (Santa Cruz, #sc-7392) and anti-Actin (Santa Cruz, #sc-1616). After incubation with secondary antibodies conjugated to near-infrared dyes (IRDye® 680 anti-goat, 800 anti-mouse, LI-COR), blots were scanned on a two-channel near-infrared Odyssey™ scanner (LI-COR).

[00149] **Modified single telomere length analysis (STELA) for chr4.**

[00150] The original STELA protocol used for human cells (Baird et al., 2004, *Hum Mol Genet* 13, 1515-1524) was modified to measure telomere lengths on the de novo end of chr4 in SL13 cells. Briefly, genomic DNA was extracted using Puregene Core Kit A™ (Qiagen). 4 µg of genomic DNA was digested with Sphl (NEB) and later diluted to 10 ng/µl in water. For the in vitro IScerette sample, genomic DNA was digested with Sphl and I-Scel (NEB) prior to ligation. The ligation was carried out at 35°C for at least 12 hrs in a volume of 10 µl containing 10 ng of digested genomic DNA, 0.9 µM of telorette linkers (mixture of telorette 1 to 6) or IScerette linker and 0.5 U of T4 DNA ligase (NEB) in IX T4 ligation buffer. Multiple PCRs (typically 24 or 32 reactions per sample) were carried out for each test DNA in volumes of 25 µl containing 1 ng of ligated DNA, 0.2 µM HYG-specific and teltail primers, IX Fail Safe™ PCR buffer H (Epicentre FSP995H), 1 U of Fail Safe™ Enzyme Mix (Epicentre FS99100). The PCR conditions were the following: 94°C for 15 sec, 25 cycles of 95°C for 15 sec, 58°C for 20 sec and 68°C for 4 min, followed by 68°C for 10
min. The PCR reactions were pooled for each sample and purified using magnetic beads (Agencourt AMPure XP™, Beckman Coulter). The concentration was measured and an equal amount of fraction from each sample was analyzed by Southern blot using a HYG probe.

**siRNA-mediated knockdown of TERT.**

**ON-TARGET™** siRNA SMART pools from GE Healthcare were used: mouse TERT (L-048320-01-0005). SL13 cells were subject to siRNA transfection using Pepmute™ protocol (SignaGen Laboratories, #SL100566) at a final concentration of 10 nM. The efficiency of knockdown was assessed by quantitative RT-PCR.

**PacBio® sequence analysis.**

A pipeline in R (Figure 7) was created that analyzes Pacific Biosciences sequencing data generated from modified STELA. First, reads were aligned to the last 50 bases of the HYG sequence using the pairwise Alignment function in the Biostrings™ package in Bioconductor (Pages H.) (A), with parameters: local-global, mismatch penalty -3, match bonus 2, gapOpening -2, gapExtension -2. Using the same parameters, the last X bp of the 3' ends of reads were aligned (where X is 1.5 times the maximum-length Teltail sequence) to the Teltail sequence (B). In multiplexed runs, all possible barcoded Teltail sequences were aligned and only the best alignment considered, excluding reads that aligned equally well to multiple barcoded Teltail sequences. Reads with a HYG alignment score greater than or equal to 60 and a Teltail alignment score greater than or equal to 20 were kept for subsequent analysis. The sequence was then aligned between the end of the HYG alignment and the start of the Teltail alignment to the I-Scel sequence (C) with parameters: local, mismatch penalty -3, match bonus 2, gapOpening -2, gapExtension -2. A score of 36 was considered sufficient to identify an I-Sce-1 sequence. The parsed read set was manually curated to ensure that seed and elongation sequences contained telomeric repeats. Additional functions used in the pipeline were provided by the R packages parallelMap and stringr. The percentage of PacBio® CCS reads with de novo telomere repeats are calculated from each sample by using the following formula: 100% x {((number of CCS reads with telomere repeats added beyond the I-Scel site) / (number of total CCS reads))}. 
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<th>Table 1. Primer list</th>
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### To clone chr4 targeting construct (pSL25)

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### pbHuSK_F

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### To clone mTR/EGFP retroviral construct (pSL8)

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### To clone Dox-inducible HA-I-See1 construct (pSL39)

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### Modified STELA for chr4

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EXAMPLE 2

ROLE OF ATM KINASE IN TELOMERE ELONGATION

[00156] The ATM and ATR kinase-dependent DNA damage response pathways are activated in primary human cells when telomeres are critically short. Induction of telomere dysfunction through a different mechanism, the removal of shelterin components, also activates ATM or ATR-dependent signaling. Which pathway is activated is dependent on which shelterin component is removed. Deletion of TRF1 (telomeric-repeat-binding factor 1) activates the ATM pathway while removal of POT1 primarily activates the ATR pathway.

[00157] While there has been significant progress and ongoing studies to understand the role of ATM and ATR in telomere dysfunction, less is known about the role of these kinases in normal telomere elongation, when telomeres are not critically short. A role for ATM in telomere length maintenance was first evident when the ATM gene was cloned and shown to be the homolog of the Tell gene in yeast. Loss of Tell<sub>ATM</sub> function leads to short telomeres. Interestingly, while deletion of the related kinase Mecl<sub>ATR</sub> does not itself cause telomeres shortening, the double mutant of Tell<sub>ATM</sub> Mecl<sub>ATR</sub> shows further shortening not seen in Tell<sub>ATM</sub> mutant alone. This implies that Mecl<sub>ATR</sub> may partially compensate for the loss of Tell<sub>ATM</sub>.

[00158] The role of ATM in regulating telomere elongation in mammalian cells has been more controversial than in yeast. In human cells, a prominent, early paper suggested that ATM plays no role in human telomere maintenance. However other reports suggested cells might have shorter telomeres in the absence of ATM. The different methods for measuring telomeres and the small number of samples analyzed left this unresolved. Mouse studies on ATM and telomere elongation have also failed to find a definitive role for ATM. To detect telomere shortening in the absence of telomerase, it requires four to six generations of interbreeding telomerase null mice. Two groups showed that first generation ATM null mice do not have short telomeres. Progressive breeding of ATM<sup>+/−</sup> heterozygotes did not show telomere shortening. Since ATM<sup>+/−</sup> mice are sterile, it is not possible to interbreed them to examine telomere length over many generations. Thus the failure to see short telomeres in these mice might be simply due to the limitations of breeding. In addition, as discussed below, ATM and ATR play partially overlapping roles in several species, thus to see major changes in telomere length in mice may require reduction in both pathways.

[00159] Given the conserved role of ATM in telomere length regulation in <i>S. cerevisiae</i>, <i>S. pombe</i> and Arabidopsis, the role of ATM kinase in telomere elongation in mammalian
cells was examined. To avoid the issues of breeding ATM<sup>-</sup> mice and missing small effects of telomere length changes on long telomeres, the ADDIT assay (described in Example 1) was used so that telomerase repeat addition can be monitored over one cell cycle. Using this assay, it was demonstrated that ATM kinase pathway regulates telomerase-mediated telomere elongation. This highlights the conserved nature of the pathways that regulate telomere length across species and suggests novel approaches to manipulating telomere length.

RESULTS

ATM kinase is essential for de novo telomere addition.

Having established the robust ADDIT assay that can measure telomere elongation over one cell cycle, it was desired to examine whether the ATM kinase activity is required for telomere elongation. Two different methods were used to inhibit ATM: the ATM specific inhibitor KU55933 and siRNA knockdown. To confirm the inhibition of ATM kinase activity, the phosphorylation level of ATM substrate Kapl as well as ATR kinase substrate Chkl was examined by western blot. Cultured cells were pretreated with KU55933, siATM or DMSO control and later exposed to a DNA damaging reagent Camptothecin (CPT). Western blot analysis with antibodies to the phosphorylated Kapl-S824 and Chkl-S345 indicated that KU55933 and siATM blocked Kapl phosphorylation but not Chkl phosphorylation (data not shown). This indicated that both KU55933 and siATM specifically inhibited the ATM kinase-dependent signaling pathway, while the ATR pathway was not affected.

Telomere elongation was measured by the ADDIT assay in cells treated for 48 hrs with doxycycline to induce I-Scel in the presence or absence of KU55933 or siATM. PacBio sequencing of the STELA products indicated that addition of de novo telomere repeats beyond the I-Scel site was significantly reduced when ATM was inhibited or knocked down (Figures 8A and 8B). Cells treated with the KU55933 had fewer and significantly shorter elongation products while cells treated with siATM showed no telomere elongation (Figures 8A and 8C). These results indicated that ATM activity is required for telomerase-mediated de novo telomere repeat addition.

Inhibition of ATM kinase prevents telomere elongation by telomere overexpression.

To examine the role of ATM in telomere elongation by an independent method, telomerase was overexpressed in the cell line, in the presence of ATM inhibition. It has been shown that overexpressing telomerase elongates telomere lengths in human cells. Cells
were treated with KU55933 and then transduced with a lentivirus expressing both mTR and mTERT and grown for 2 or 5 days in culture and telomere lengths were assayed by Southern blot. Telomere lengths in cells overexpressing telomerase were rapidly elongated in just 5 days (data not shown). However, treatment with KU55933 significantly blocked the telomere elongation. Significant blocking of elongation in the siATM treated cells was not seen even though the expression levels of ATM protein and ATM-dependent phosphorylation of KAP1 were decreased. This is likely due to the transient effect of knockdown by siRNA, which may explain why telomere shortening has not previously been reported in siRNA experiments with ATM. In addition, as discussed below, when cells are grown for several days, ATR may also compensate for the loss of ATM. The results from the ADDIT assay showing abrogation of telomere elongation and the Southern data collectively indicate that ATM kinase is required for telomere elongation by telomerase.

Inhibition of ATM kinase shortens telomere length.

Given that ATM inhibitor KU55933 treatment prevents telomere elongation by telomerase overexpression, whether KU55933 treatment shortens telomere lengths in continuously growing cells was tested. SL13 cells were grown in the presence of KU55933 and measured the telomere lengths at various population doublings (PDs). Telomeres of KU55933-treated cells gradually shortened with increasing cell PDs (Figure 9B). Densitometry of the Southern lanes showed shorter telomeres in the later PDs (Figure 9B, pixel position 2). The distinct non-telomeric bands served as useful loading controls, confirming all lanes were equally loaded (Figure 9B, pixel position 1 and 3). To examine whether telomere shortening in ATM inhibition also occurs in human cells, human HCT116 cells were treated with KU55933 drug. Consistent with the observations from mouse cells, telomere lengths of HCT116 shortened significantly in the presence of KU55933 with increasing cell PDs. Altogether these results further indicate that ATM kinase is a positive regulator of telomere elongation in both mouse and human cells.

Activation of ATM kinase pathway elongates telomere lengths.

Since inhibition of ATM kinase gradually shortened telomere lengths, whether activation of ATM kinase pathway would result in telomere elongation was investigated. A previous study showed that the ATM kinase pathway is activated following inhibition of poly (ADP-ribose) polymerase 1 (PARP1), an essential enzyme involved in DNA repair pathway. To examine activation of ATM kinase pathway in response to PARP1 inhibition in our cells, the phosphorylation levels of KAPI, a known target of ATM kinase, in response to PARP1 specific inhibitor, Olaparib was measured. Cells treated with Olaparib
indeed had a 5-fold increased level of Kapl phosphorylation, compared to DMSO-treated control cells. Although the level of KAP1 phosphorylation was not as robust as cells treated with DNA damaging reagent CPT, some induction was seen. To test whether this PARP inhibitor-induced ATM activation would stimulate telomere elongation, SL13 cells were grown in the presence of Olaparib and collected at various PDs and telomere length was measured by Southern blot analysis. Strikingly, telomere lengths gradually increased after 25 PDs in the presence of Olaparib and were further elongated in later PDs. To verify whether telomere elongation in response to PARP inhibition is through activation of ATM kinase pathway, testing whether blocking ATM would block the elongation effect of the PARP inhibitors was desired. Unfortunately, similar to other studies, cells were too sensitive to combination of KU55933 and Olaparib treatment for long-term cell growth. As such, the ADDIT assay was used to examine de novo telomere elongation in cells treated with either DMSO, KU55933, Olaparib or both drugs. Cells treated with KU55933 alone had less de novo telomere addition compared to DMSO control consistent with previous results. In contrast, cells treated with Olaparib had significantly higher percentage of de novo telomere addition. In the cells treated with both Olaparib and KU55933, the percentage of reads with telomere elongation was reduced from 22.4% to 18.1%, suggesting the increased telomere elongation in response to PARP acts through the ATM kinase pathway. Since different primers were used for STELA in this experiment the percentage of elongation differs compared to previous results (Figure 3). Given this difference and the small magnitude of the change, this experiment has been repeated so that more definitive conclusions could be drawn. All of the results support the idea that PARP inhibition positively affects telomere length regulation by activating ATM kinase pathway.

00170 FIGURE LEGENDS

00171 Figure 8 shows inhibition of ATM blocks de novo telomere addition. (A) Analysis of PacBio® CCS reads (maximum of 300 shown for simplicity) is shown from samples pretreated with DSMO, 10 µM KU55933 or 5 nM siATM. X-axis indicates the length (bp) from the start of the telomere seed sequence. (B) The percentage of CCS reads with de novo telomere repeats are shown. n; number of independent biological replicates analyzed. (C) The sequences of PacBio® CCS reads from siATM treated sample boxed in (A) are shown.

00172 Figure 9 shows that telomere lengths shorten in the presence of ATM inhibitor KU55933. Telomere lengths of SL13 cells treated with 10 µM KU55933 were measured at different population doublings (PD) using genomic Southern blot analysis. Densitometry
tracing of the Southern blot lanes by graphing pixel counts (x10^2) versus DNA migration distance (Pixel Position). Boxed numbers indicate the pixel positions corresponding to the blot in A.

[00173] DISCUSSION

[00174] In this example, experiments are described using the ADDIT assay, that demonstrated the ATM kinase pathway is required for telomerase-mediated telomere addition. This conclusion was further substantiated using an alternative cell culture experiment approach; it was found that inhibition of ATM kinase activity prevents bulk telomere elongation by telomerase overexpression. In addition, blocking ATM kinase with KU55933 shortened telomeres in both mouse and human cells in long-term cell culture experiments. Furthermore, activation of ATM kinase pathway by using PARP inhibitor Olaparib significantly elongated telomeres, further supporting the role of ATM kinase promoting telomere elongation.

[00175] Conserved pathways of telomere length regulation.

[00176] The mechanism of telomere length maintenance involves many interdependent regulatory pathways that act together to establish length homeostasis. This process involves the interaction of telomere binding proteins such as shelterin with telomerase to regulate elongation. In addition to dedicated telomere binding proteins, DNA damage proteins including MRN complex and Ku are conserved players in length maintenance from yeast to mammals. Protein modification also plays a key role in regulating telomere length. It was demonstrated that one of the key regulators of telomere length, the ATM kinase pathway, is also conserved from yeast to mammalian system. ATM is required for normal telomere maintenance in different yeast species including S. pombe and in these species ATM mediates the telomerase recruitment. The ATM homologue in Arabidopsis also plays a role in regulating telomere length by promoting elongation of short telomeres. Drosophila telomeres do not require telomerase for length maintenance, yet remarkably, the role of ATM is conserved in this species. These data suggest that even when telomerase was lost from this evolutionary branch of Diptera, the processes that regulate telomere length were still retained.

[00177] ATR may compensate for loss of ATM.

[00178] The data, from both the ADDIT assay and Southern analysis, suggests the ATM kinase is required for telomere length maintenance. When ATM was inhibited by siRNA different results were seen in ADDIT assay and the longer term bulk culture experiment: telomere addition was completely blocked in ADDIT assay but not when cells were grown
continuously in culture. This difference may be due to the transient siATM knockdown that does not sufficiently block ATM in longer-term culture conditions. Another possibility is that ATR kinase may compensate for ATM loss in the longer-term culture experiments. Previous studies in S. cerevisiae suggest ATR plays a minor, yet critical role in telomere maintenance. Cells lacking Tell\textsuperscript{ATM} are completely defective in telomere extension within the first few hours after the creation of short telomere. The bulk telomere lengths of \textit{tell}^{ATM} cells are short yet stable. Progressive telomere shortening is only seen when both \textit{tell}^{ATM} and \textit{mecl}^{ATR} are deleted, suggesting Mecl\textsuperscript{ATR} kinase may play some role in telomere elongation when Tell\textsuperscript{ATM} is missing. Similar to S. cerevisiae, the shortest telomere phenotype of \textit{S. pombe} was seen in the Rad3\textsuperscript{ATR}/Tell\textsuperscript{ATM} double mutants (Naito et al., 1998). Interestingly, Rad3\textsuperscript{ATR} mutant cells have much shorter telomere lengths compared to Tell\textsuperscript{ATM}, indicating Rad3\textsuperscript{ATR} may play a more critical role in telomere length regulation than does \textit{S. pombe} Tell\textsuperscript{ATM}.

\textbf{00179} Previous studies in mice indicated that ATM is not required for elongation of the shortest telomeres in an intergenerational cross. When the ATM\textsuperscript{+/-} mice were crossed to ATM\textsuperscript{+/+} mTR\textsuperscript{+/-} G5 late generation mice with short telomeres, the F1 mice that resulted showed rescue of signal free ends in both ATM\textsuperscript{+/-} mTR\textsuperscript{+/-} and ATM\textsuperscript{+/-} mTR\textsuperscript{+/-} offspring, suggesting ATM is not essential for elongation of the shortest telomeres. This elongation, however, may be due to ATR compensating for the loss of ATM. The role of the ATR kinase in these pathways has not been examined as ATR null mice are not viable. In humans recent analysis indicated that Ataxia telangiectasia (AT) patients who have mutations in the ATM gene have shorter telomeres compared to their age-matched controls, but not as short as people who have telomerase mutations. This observation suggests ATR may also compensate for the loss of ATM function in telomere length maintenance in human. Dissecting the mechanism of telomere shortening in AT patients may have implications for individualized treatment. It is important to understand whether short telomeres can directly contribute to a more severe AT clinical phenotype and whether telomere lengthening could be a potential therapeutic target.

\textbf{00180} \textit{Possible mechanisms of ATM regulated telomere elongation.}

\textbf{00181} Previous findings in S. cerevisiae imply that the primary function of ATM in telomere maintenance is by modulating the access of telomerase to its substrate, telomere, rather than by altering the enzyme activity level of telomerase. The kinase activity of ATM is required for telomere maintenance as kinase dead mutant show short telomeres. While specific ATM substrates that affect telomere length have been characterized in S. pombe,
the key substrates in *S. cerevisiae* are still not fully understood. In *S. pombe*, Tel1 and/or Rad3ATR phosphorylate a shelterin component Ccql that then interacts with telomerase subunit Estl to mediate telomerase recruitment. In *S. cerevisiae*, while Tel1ATM can phosphorylate the single-strand telomere binding protein Cdc13, this phosphorylation apparently is not responsible for recruitment of Estl. While the precise functional homologues of Ccql and Estl in mammalian cells are not fully established, likely due to sequence divergence and/or convergent evolution, shelterin components are still excellent candidates for ATM substrates in mammals given the conservation of length maintenance mechanisms across phyla. Previous studies in human cells suggest ATM phosphorylation of TRF1 can alter TRF1 association with telomeres, which can affect length regulation as well as end protection. Further, identification of ATM kinase target(s) will help us understand how ATM regulates telomerase recruitment and telomere elongation in mammalian cells.

**[00182]** PARP regulation of ATM regulated telomere length.

**[00183]** PARP1 plays a critical role in DNA repair pathways, especially in base-excision repair, by binding to the single-strand break and forming poly (ADP-ribose) (PAR) polymer chains on itself and other proteins. PAR formation is thought to be important to protect DNA break and recruit DNA repair proteins to the site of DNA damage. In spite of these roles, PARP1 is not essential for cellular survival as PARP1 knockout mice are viable. Recently the PARP 1/2 inhibitors have been developed for the treatment of cancer with the concept of using synthetic lethality to kill cancer cells. The idea is to treat certain cancers that have mutations in the DNA repair components, particularly BRCA1 or BRCA2, with PARP inhibitors and the additive effect of deficiency in two DNA damage pathways will cause cell death.

**[00184]** A previous study indicated that PARP inhibition would activate the ATM pathway. It was necessary to determine if PARP inhibitors would affect telomere length by activating the ATM kinase pathway. Olaparib was one of the first PARP 1/2 specific inhibitors in the clinical trials. Treatment with Olaparib activated ATM and increased telomere length. It is very striking to observe such significant telomere elongation in cells treated with Olaparib, especially because Olaparib has become one of the first drugs approved by FDA to treat advanced ovarian cancers in December 2014 (2015). Although further validation is required, Olaparib may activate ATM kinase pathway resulting a positive effect on telomere length equilibrium. Given that Olaparib is used to treat cancer patients, it is crucial to fully characterize the secondary effects of Olaparib on telomere lengths.
The PARP enzyme family has 17 members, and the specificity of the PARP1/2 inhibitors may differ and have different consequences in mice and humans. For instance, in humans, tankyrase 1 and 2, members of PARP family, positively affect telomere length through the ADP ribosylation of TRF1. However, the interaction between tankyrases and TRF1 in human and mice differ significantly, resulting in different effects on telomere length. Previous study using in vitro assays indicated that several PARP1/2 inhibitors in clinical trials, including Olaparib, have strong specificities to PARP 1-4 but less for others such as tankyrases. It will be important to verify whether these PARP inhibitors affect tankyrase activity in vivo to further understand the role of these drugs in telomere length regulation in humans.

ATM kinase pathway can also be activated by other ways such as oxidative stress without dsDNA breaks. Although chronic oxidative stress has been shown to enhance telomere shortening and cellular senescence in cultured cells, it will still be interesting to test whether very low level of oxidative stress, just enough to activate ATM kinase pathway, increases telomere lengths in mammalian cells. Ultimately finding a safe way to elongate telomeres, by discovery of a drug that activates the ATM kinase pathway, could benefit patients with telomere syndromes.

MATERIAL AND METHODS

Cell culture and treatments.

Cell lines such as 293FT and HCT116 were grown in DMEM (Gibco) supplemented with 1% Penicillin/Streptomycin/Glutamine (PSG) and 10% heat inactivated FBS (Invitrogen). SL13 cells were grown in DMEM (Gibco) supplemented with 1% PSG and 10%, Tet system approved FBS (Clontech, #631107). Final concentration of 2 μg/ml of doxycycline was added in the media to induce I-SceI expression. Typically cells were collected post 48 hours of doxycycline treatment. To inhibit ATM kinase activity, the ATM specific inhibitor KU55933 (R&D Systems, #3544) was added at the final concentration of 10 μM. To activate ATM kinase pathway, PARP1/2 inhibitor Olaparib (Selleckchem.com, #S1060) was used at the final concentration of 1 μM, 3 μM or 5 μM.

siRNA-mediated knockdown of ATM and ATR.

ON-TARGET™ siRNA SMART pools from GE Healthcare were used: mouse ATM (11920), mouse ATR (245000). SL13 cells were subject to transfection using Pempute™ protocol (SignaGen Laboratories, #SL100566). The final concentrations of siRNAs were 5 nM, 10 nM or 100 nM for each transfection. The efficiency of knockdown was assessed by immunobloting.
Western blot analysis and antibodies.

To detect phospho-proteins, IX lysis buffer made of final concentration of IX RIPA (Cell Signaling, #98016S), IX protease inhibitor cocktail, IX PhosSTOP™ (Roche, #4906845001) was added directly to cells on the dish after washing with cold IX PBS. Protein concentration was measured with a BCA protein assay kit (Thermo, #23227). Typically 10 µg of protein from each sample was applied to SDS-PAGE gels, 4-15% MiniPROTEAN TGX™ gels (Biorad, #456-1084), and transferred to nitrocellulose membranes in transfer buffer. After blocking with the Odyssey™ blocking buffer (LI-COR, #927-40000) for 1 hour at RT, membranes were incubated at 4°C overnight with the following primary antibodies multiplexed: anti-phospho-Kapl (Bethyl Lab, #A300-767A); anti-ATM (Novusbio, #NB100-220); anti-phospho-CHK1 (Cell Signaling, #2348S); anti-Actin (Santa Cruz, #sc-1616). The following day, blots are washed with IX PBS-T 3x 15 minutes, and then incubated with secondary antibodies conjugated to near-infrared dyes (IRDye® 680 anti-goat, 800 anti-rabbit, 800 anti-mouse, LI-COR). Blots were scanned on a two-channel near-infrared Odyssey scanner (LI-COR). Band intensities were quantified using the Odyssey™ software (LI-COR) and normalized to Actin levels. Certain antibodies were not multiplexed, instead after 4°C overnight incubation with the primary antibody, blot was washed and incubated with the following HRP-conjugated secondary antibodies: anti-mouse IgG HRP-linker antibody (Cell Signaling, #7076) and anti-goat IgG HRP-conjugate antibody (Biorad, #172-1034). The blot was scanned using the ImageQuant LAS 4000™ imager (GE Healthcare). To re-probe the same blot with a loading control antibody, the blot was stripped with a stripping buffer (Thermo, #46430) and processed as described above.

Telomere Southern analysis.

To measure telomere lengths by Southern blot, genomic DNA was extracted from cell pellets collected at different population doublings using the Puregene Core Kit A™ (Qiagen). Equal amounts of genomic DNA were digested overnight with Msel restriction enzyme (NEB) and loaded on a 0.7% TAE agarose gel. Samples were run at 100V for roughly 6 hours. Following denaturation (0.5 M NaOH/1.5 M NaCl) and neutralization (1.5 M NaCl/0.5 M Tris-HCL pH 7.4), the DNA was transferred in 20X SSC to a Nylon Membrane (Amersham Hybond N+) by weighting method overnight and cross-linked with UV Stratalinker (Stratagene). Pre-hybridization was done at 65°C in Church's buffer for 2 hours. A radioactive telomere probe was made by random-prime labeling using Prime-It II (Stratagene) with a slight modification. Briefly, 25 ng of a 500 bp telomeric 5'-TTAGGG containing probe acquired from EcoRI digestion of JHU821 or 1 KB Plus DNA ladder
(Invitrogen) was labeled using 33 µM of dATP, dTTP, dGTP and 50 µCi of α-32P dCTP (3000 Ci/mmol). Unincorporated nucleotides were removed using a G50 column (GE Healthcare). Labeled probe was counted and 10^6 counts/ml (telomere probe) or 10^4 counts/ml (ladder) was denatured at 100°C for 5 minutes and added to the pre-hybridization solution and hybridized overnight at 65°C. Membranes were washed 3x 15 minutes each in 6X SSC and 1% SDS at 65°C, and 3x 15 minutes each in 1X SSC and 1% SDS at 65°C and exposed to a phosphorimager screen and detected on a Fuji phosphorimager. Image processing software, ImageQuant™ ID v8.1 (GE Healthcare Life Sciences), was used to generate densitometry of Southern blot lanes by graphing pixel counts versus DNA migration distance.

[00196] Lentivirus transduction.

[00197] SL13 cells were seeded in complete medium with either DMSO or KU55933, or pre-transfected with siATM (siRNA transfection described in 4.4.2) in 6-well plates. The following day, polybrene-treated cells (8 µg/ml, Sigma) were infected with SVA (mTR/mTERT double construct) lentivirus, MOI ≤ 1, and incubated overnight at 37°C, 5% CO₂. The following morning, the medium was changed to eliminate the polybrene and refreshed with complete medium either with DMSO or KU55933. After 2 days of transduction, aliquots of cells were collected for Southern analysis and the rest of cells were re-plated in the presence of DMSO, KU55933 or transfected with siATM.

[00198] Modified single telomere length analysis (STELA) for chr4.

[00199] To multiplex samples treated with different conditions for PacBio® sequencing, the STELA PCR for chr4 described in 2.4.9 was performed with slight modification. Briefly, the 20-nt unique sequence of telorette was randomized to make several different sets of unique telorette linkers. The different sets of telorette have corresponding teltail primers that recognize the unique sequence. In some experiments, degenerated forward HYG primers and reverse teltail primers were used.

[00200] PacBio® sequence analysis.

[00201] PacBio® sequence reads were analyzed as described in Example 1.
### Table 2. Oligonucleotide list

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</tr>
<tr>
<td>Telorette F 3</td>
<td>TGCTCCGTGATCTGCGACATTCCTAAC (SEQ ID NO: 125)</td>
</tr>
<tr>
<td>Telorette F 4</td>
<td>TGCTCCGTGATCTGCGACATTCCTAAC (SEQ ID NO: 126)</td>
</tr>
<tr>
<td>Telorette F 5</td>
<td>TGCTCCGTGATCTGCGACATTCCTAAC (SEQ ID NO: 127)</td>
</tr>
<tr>
<td>Telorette F 6</td>
<td>TGCTCCGTGATCTGCGACATTCCTAAC (SEQ ID NO: 128)</td>
</tr>
<tr>
<td>N-Teltail</td>
<td>TGTCCGACTGTGATCTGCGACATTCCTAAC (SEQ ID NO: 129)</td>
</tr>
<tr>
<td>N-Teltail B</td>
<td>TGTCCGACTGTGATCTGCGACATTCCTAAC (SEQ ID NO: 130)</td>
</tr>
<tr>
<td>N-Teltail C</td>
<td>TGTCCGACTGTGATCTGCGACATTCCTAAC (SEQ ID NO: 131)</td>
</tr>
<tr>
<td>N-Teltail D</td>
<td>TGTCCGACTGTGATCTGCGACATTCCTAAC (SEQ ID NO: 132)</td>
</tr>
</tbody>
</table>
**EXAMPLE 3**

**ROLE OF CYCLIN-DEPENDENT KINASES IN TELOMERE LENGTH REGULATION**

[00203] Telomeres are specialized structures at the ends of the linear chromosome that allow cells to distinguish the natural ends from double-strand DNA break. Failure to maintain telomere structure can result in chromosome fusions and genomic instability. Telomere structure, and the enzyme telomerase that is responsible for synthesizing telomeric repeats, are highly conserved. Telomere DNA consists of a short tandem repeats of a short G-rich sequence with a stretch of 3' overhang. This G-rich 3' overhang structure of telomeres is conserved throughout eukaryotes, including ciliates, fungi and mammals. The proteins that interact with the 3' single-strand overhang and those that bind the double-strand telomeric repeats have important roles in maintaining telomere lengths as they are involved in protecting telomere ends and modulating telomerase access to telomeres.

[00204] Most of the key players and requirements for telomere length regulation have been investigated in the model organism *S.cerevisiae*, including cyclin-dependent kinase (Cdk). Cdns are serine/theomine kinases responsible for various cellular processes such as cell cycle progression and transcription. In both *S.cerevisiae* and *S.pombe*, a single Cdk, Cdk1, is responsible for regulating cell cycle transitions. In addition to its critical role in cell cycle regulation, a previous study from our lab showed that Cdk1 is required for telomere elongation by regulating the generation of the 3' overhang. In higher eukaryotes, there are a growing number of Cdk family members identified. Functional characterization of these Cdns elucidated specific roles in different cellular processes such as cell cycle regulation, transcription and others, but the role in telomere length regulation is not well elucidated.

[00205] This example illustrates that telomerase-mediated telomere addition is regulated in a cell-cycle dependent manner in mammalian cells, and that mammalian Cdns, including Cdk1, are required for telomere elongation *in vivo*.

[00206] RESULTS

[00207] *De Novo Telomere Addition Occurs In G2/M Phase.*

[00208] To better understand how mammalian telomere length is regulated, the short-term ADDIT assay was used to visualize telomere addition *in vivo*. Briefly, an inducible I-Scel
site is used to create a double-strand DNA break adjacent to a stretch of telomere 'seed' sequence near the end of a single chromosome (chr4). Given that telomere elongation in cycling cells was observed, whether telomere addition could be visualized in cells arrested at distinct cell cycle phases was tested to determine if telomere addition is cell cycle regulated in a similar manner to yeast. SL13 cells were arrested in G2/M phase using colcemid, a drug that depolymerizes microtubules limiting microtubule formation during M phase, or in G1/early S phase by thymidine block. Cells accumulated at the expected cell phases using PI staining and flow cytometry analysis (Figure 10A) and then induced doxycycline to expose the telomere seed sequence. The 'smear' representative of de novo telomere addition is not robustly detected by Southern blot analysis. However even less 'smear' from cells arrested in G1 phase was observed compared to controls cells and cells held in G2/M phase suggesting that telomere elongation may be dependent on cell cycle. To better visualize the de novo telomere addition, STELA PCR products of SL13 cells pretreated with colcemid or DMSO were sequenced. Consistent with the results from yeast, cells held in G2/M phase had significant percentage of PacBio® reads with de novo telomere addition (Figure IOC). No significant difference of de novo elongation was observed between cycling cells or cells arrested in G2/M phase. To ensure I-Scel induction is consistent across different cell cycle phases, I-Scel expression was measured in cells arrested at different phases and confirmed no significant difference (Figure 10B). These results suggest that the requirements for telomere elongation are present in G2/M phase in mammalian cells similar to yeast.


[00210] Cdkl positively regulates telomere elongation in S.cerevisiae. To examine whether Cdk activity plays a similar role in telomere elongation in mammalian cells, several methods were tested to inhibit Cdks and performed the short-term ADDIT assay. Cells were treated either with or without flavopiridol, which inhibits several members of the Cdk family, including Cdkl, 2, 4, 6, 7. Southern blot analysis of STELA PCR products from flavopiridol-treated sample suggested minimal elongation beyond the chr4 I-Scel-induced cut base line. PacBio® sequences from the STELA products indeed showed treatment with flavopiridol significantly reduced de novo telomere addition (Figure 11), from 24% to less than 2%, suggesting Cdk activities are required for telomere elongation.

[00211] Cdkl Is Required For De Novo Telomere Addition.

[00212] To determine which of the several Cdks in mammalian cells, is responsible for regulating telomere length, the effect of knocking down Cdkl was first tested since the
Cdkl homologue in yeast has been shown to be required for telomere elongation. Cells were treated with siRNA against Cdkl and confirmed more than 50% knockdown by measuring the phosphorylation level of Cdkl-Y15. ADDIT assay was performed in cells treated with or without siCdkl. PacBio® sequence analysis of the STELA PCR products indicated de novo telomere addition was completely blocked in cells with Cdkl knockdown (Fig 11A and 11B). These observations suggest the requirement of Cdkl in telomere elongation is conserved from yeast to mammalian cells.

**FIGURE LEGENDS**

**Figure 10** shows that de novo telomere addition occurs in G2/M phase. (A) SL cells treated with either DMSO, 200 ng/ml colcemid or 2.5 mM thymidine for 24 hrs were analyzed for cell cycle profile by propidium iodide staining and flow cytometry. The numbers indicate the percentage of cells in each cell cycle phase. (B) Relative expression levels of HA-tagged I-Scel normalized to UPRT measured by quantitative RT-PCR. Error bars indicate the standard error of mean (SEM) from triplicates of SL13 cells treated with 200 ng/ml colcemid, 2.5 mM thymidine or DMSO for 48 hrs. (C) Analysis of PacBio CCS reads (maximum of 300 shown for simplicity) of STELA PCR products made from cells treated with either DMSO or 200 ng/ml colcemid for 48 hrs. X-axis indicates the length (bp) from the start of the telomere seed sequence. The percentage of PacBio CCS reads with de novo telomere repeats are calculated from each sample by using the following formula: 100% × \{[(number of CCS reads with telomere repeats added beyond the I-Scel site) / (number of total CCS reads)]\}.

**Figure 11** shows that inhibition of Cdns prevents de novo telomere addition. Cells were treated with final concentration 400 nM of flavopiridol or DMSO together with or without 200 ng/ml colcemid, and exposed to doxycycline for 48 hrs. STELA PCR products made with a F2 primer were analyzed by Southern hybridization using HYG probe. Analysis of PacBio CCS reads (maximum of 200 reads for simplicity) from samples treated with either 400 nM flavopiridol or DMSO for 48 hrs. X-axis indicates the length (bp) from the start of the telomere seed sequence. The percentages of reads with de novo telomere addition are shown.

**Figure 12** shows Cdkl is required for de novo telomere addition. Phosphorylation levels of Cdkl were analyzed by immunobloting cells treated with 10 µM RO3306 or different final concentration of siCdkl, 5 nM, 10 nM or 100 nM. Numbers at bottom of western indicate the relative expression levels of phosphorylated Cdkl-T15 normalized to Actin. (A) Percentages of CCS reads with de novo telomere addition from samples treated
with either DMSO or final concentration of 5 nM siCdkl for 48 hrs. n; number of independent biological replicates analyzed. (B) Analysis of PacBio CCS reads (maximum of 200 reads for simplicity) from (A) shown. X-axis indicates the length (bp) from the start of the telomere seed sequence.

**DISCUSSION**

In the experiments presented in this example, the ADDIT assay was used to visualize telomerase-mediated de novo telomere addition occurred in cycling cells but also in cells held in G2/M phase. On the other hand, although further validation is required, Southern blot analysis suggested cells arrested in G1-phase has inefficient telomere elongation. In addition, it was shown that mammalian Cdks, especially Cdkl, are required for telomere addition. Since Cdkl is an essential gene required for early embryonic development, it would not be possible to address the role of this kinase with the conventional method of measuring bulk telomere length changes for over 2 months; however, the very brief time required for ADDIT assay overcame this issue and allowed us to examine the critical role of Cdkl in telomere addition.

Telomere Elongation Is Cell Cycle Regulated.

Previous work using the de novo telomere addition assay in S.cerevisiae demonstrated that when cells are arrested in G1 phase using the mating pheromone a-factor, the telomere seed is not elongated whereas the end was efficiently elongated in vivo in cells arrested in M phase by treatment with the microtubule depolymerizing drug nocodazole. Telomerase activity was present in extracts made from all cell cycle phases; however, telomere addition was restricted to late S/G2 phase. Consistent with the results from yeast, efficient telomere addition was observed in SL13 cells held in G2/M phase but not in Gl, suggesting cell cycle-coordinated telomere elongation is a conserved phenomenon. These observations suggest that essential component(s) mediating telomere elongation maybe absent or inactivated in certain cell cycle phases. The precise timing and coordination of telomerase recruitment to telomeres are regulated by ATM kinase (as discussed herein) and other mechanisms that have not been fully elucidated.

Cdkl are excellent candidates for regulating cell cycle-dependent telomere elongation. The data indicate Cdkl has an essential role in telomere elongation. A previous study reported that TRF2 (telomeric-repeat-binding factor 2) is a Cdkl and Cdk2 substrate by high throughput in vitro kinase screening. Although further validation is necessary, Cdkl and/or Cdk2 may regulate telomere length by interacting and phosphorylating TRF2 in vivo. It is possible other Cdks also regulate telomere length homeostasis.
Previous studies suggest DNA polymerase and primase, which are involved in telomere lagging strand synthesis, are also required for functional telomere elongation. It will be interesting to further dissect the telomere replication machinery to identify cell cycle-dependent components required for mammalian telomere elongation using the ADDIT assay.

The Essential Role Of Cdkl In Telomere Addition.

Previous studies have shown that many of the key regulators required for telomere length maintenance are identical between the natural telomeres and de novo telomere ends. The yeast Cdkl activity is also required to generate the 3’ G-rich overhang, which is important for telomere length maintenance, at both de novo telomere ends as well as the natural telomeres. It is possible that Cdkl activates a nuclease(s) that is involved in single-strand resection, such as Mrel1 that is involved in resection at double-strand DNA break sites. In yeast, Cdkl phosphorylates the nuclease Dna2, which is involved in generating the 3’ overhang at telomeres. It will be interesting to find whether mammalian Cdkl is also responsible for generating the 3’ overhang by activating a nuclease(s) responsible for 5’ strand resection.

In addition, Cdkl appears to be involved in regulating telomerase recruitment. In S.cerevisiae, phosphorylation of the single-strand DNA binding protein Cdc3 (T308) by Cdkl promotes the interaction between Cdc3 and one of the telomerase components, Estl, resulting in telomerase recruitment and telomere elongation. A recent study showed S.cerevisiae Cdkl also phosphorylates Stnl, one of the other components of CST complex (Cdc3-STN1-TEN1), which stabilizes the complex at telomeres. The CST complex is conserved in higher eukaryotes with an exception that mammalian CST complex consists CTC1 instead of Cdc3. Recent studies purpose human CST (CTC1-STN1-TEN1) complex inhibits telomere elongation by competing with POT1-TPP1 and promotes telomere replication at C-strand by stimulating DNA polymerase a-primase. The results from ADDIT assay demonstrated that Cdkl activity is also required for de novo telomere addition in mammalian cells (Figure 12B and 12C), indicating the conserved role of Cdkl in telomere length regulation in higher eukaryotes. A previous study showed phosphorylation of TPP1 (Si 11) affects telomerase interaction in a cell cycle dependent manner and is lost in the presence of Cdk inhibitor. It will be interesting to elucidate how mammalian Cdkl regulates telomere elongation, and further test whether Cdkl -mediated phosphorylation(s) of CST complex and/or shelterin complex components, such as TPP1, exist.
Cell Culture and Treatments.

SL13 cells were grown in DMEM (Gibco) supplemented with 1% Penicillin/Streptomycin/Glutamine and 10% Tet system approved FBS (Clontech, #631 107). Final concentration of 2 μg/ml of doxycycline was added in the media to induce I-Scel expression. Typically cells were collected post 48 hours of doxycycline treatment. To arrest cells in different cell cycle phases, cells were treated with either final concentration of 200 ng/ml colcemid (Gibco KaryoMAX Colcemid Solution, #15212-012) or 2.5 mM Thymidine (Sigma, #T-1895).

Cell Cycle Profile Analysis.

Cell cycle phase was accessed by DNA content using propidium iodide (PI) staining and flow cytometry analysis. Briefly, cell pellets were washed with 1X PBS at 500 x g for 5 minutes and resuspended in 500 μl of 1X PBS. 4.5 ml of ice cold 70% ethanol was added drop by drop, and incubated overnight at 4 °C. Samples were washed with 5ml 1X PBS and incubated with 500 μl of Pi-Triton resuspension buffer at room temperature for ≥ 30 minutes protected from light. Pi-Triton resuspension buffer was made of 0.1% Triton™ X-100, 200 μg/ml RNase A, 20 μg/ml PI (Invitrogen, #P3566) in 1X PBS. Pi-stained samples were transferred to strainer tubes (BD Falcon) and run on FACSCalibur™ flow cytometry (BD Biosciences). Cell cycle profiles were analyzed utilizing the Dean-Jet-Fox™ model in FlowJo™ software (FlowJo™).

Quantitative RT-PCR.

To measure HA-tagged I-Scel expression levels, quantitative RT-PCR was performed as described in 2.4.5. Primers used are the following: HA-I-Scel-FOR, 5'-TCCTGACTATGCGGTTGA-3' (SEQ ID NO: 143); I-Scel-REV2, 5'-CCTTCATCAGAGAACGG-3' (SEQ ID NO: 144); HPRT_F, 5'-TGATCAGTCACGAGAGG-3' (SEQ ID NO: 145); HPRT_R, 5'-TTCCGAGGCTCCTTTCACC-3' (SEQ ID NO: 146).

Southern Blot Analysis.

To examine the in vivo chr4 cleavage and de novo telomere addition, genomic DNA extracted from SL13 cells arrested at different cell phases and treated with doxycycline for various time points were digested with Ncol restriction enzyme (NEB) and further analyzed by Southern blot as described previously in Example 1 with a random primed a-32P-labeled HYG probe.

siRNA-mediated Knockdown Of Cdkl.
ON-TARGET™ siRNA SMART pools from GE Healthcare were used: mouse Cdkl (L-058633-00-0005). SL13 cells were subject to siRNA transfection using Pepmute™ protocol (SignaGen Laboratories, #SL100566) at a final concentration of 5 nM, 10 nM or 100 nM. The efficiency of knockdown was assessed by immunoblotting.

Cell lysates were made and processed as described in 3.4.3. Membranes were incubated at 4°C overnight with primary antibodies: anti-phospho-Cdkl (Cell Signaling, #9111) and anti-Actin (Santa Cruz, #sc-1616). After incubation with secondary antibodies conjugated to near-infrared dyes (IRDye® 680 anti-goat, 800 anti-rabbit, LI-COR), blots were scanned on a two-channel near-infrared Odyssey™ scanner (LI-COR).

The original STELA protocol used for human cells was modified to measure the de novo telomere addition on chr4 in SL13 cells as described in previously.

PacBio® sequence reads were analyzed as described in previously.

Although the invention has been described with reference to the above example, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.
What is claimed is:

1. A method of identifying a regulator of telomere length comprising:
   a) culturing a mammalian cell comprising a modified chromosome containing an internal telomere seed sequence and an endonuclease cleavage site downstream of the telomere seed sequence, wherein the cell conditionally expresses an endonuclease that cleaves and exposes the telomere seed sequence;
   b) contacting the cell of (a) with an agent that modulates expression of a selected gene or pathway in the cell; and
   c) measuring *de novo* telomere addition to the seed sequence in the presence and absence of the agent, wherein addition of telomere sequence in the presence of the agent, but not in the absence of the agent, and the degree of addition, is indicative of identification of the agent as being a regulator of telomere length, thereby identifying a regulator of telomere length.
2. The method of claim 1, wherein the mammalian cell further comprises a recombinant nucleic acid sequence encoding telomerase.
3. The method of claim 2, wherein the nucleic acid sequence is operably linked to a nucleic acid sequence encoding a reporter.
4. The method of claim 3, wherein the reporter is selected from the group consisting of luciferase (LUC), β-lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo, G418), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), β-galactosidase (β-gal), and xanthine guanine phosphoribosyltransferase (XGPRT), an affinity or epitope tag, and a fluorescent protein.
5. The method of claim 4, wherein the reporter protein is a fluorescent protein.
6. The method of claim 5, wherein the fluorescent protein is green fluorescent protein (GFP) or enhanced green fluorescent protein (eGFP).
7. The method of claim 2, wherein the mammalian cell further comprises a recombinant nucleic acid sequence encoding a recombinase.
8. The method of claim 7, wherein the recombinant nucleic acid sequence encoding telomerase further comprises a recombinase target sequence allowing excision of the nucleic acid sequence encoding telomerase.
9. The method of claim 1, wherein the mammalian cell further comprises a recombinant nucleic acid sequence encoding an endonuclease specific for the endonuclease cleavage site.

10. The method of claim 9, wherein the endonuclease is I-Scel.

11. The method of claim 9, wherein the nucleic acid sequence is operably linked to an inducible promoter.

12. The method of claim 11, wherein the promoter is inducible by a tetracycline antibiotic.

13. The method of claim 12, wherein the tetracycline antibiotic is doxycycline.

14. The method of claim 9, wherein the nucleic acid sequence is operably linked to a nucleic acid sequence encoding a reporter.

15. The method of claim 14, wherein the reporter is selected from the group consisting of luciferase (LUC), β-lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo, G418), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), β-galactosidase (β-gal), and xanthine guanine phosphoribosyltransferase (XGPRT), an affinity or epitope tag, and a fluorescent protein.

16. The method of claim 15, wherein the reporter is a human influenza hemagglutinin (HA) epitope tag.

17. The method of claim 1, wherein the measuring comprises PCR.

18. The method of claim 17, wherein the PCR is included in a modified single telomere length analysis (STELA).

19. The method of claim 17, wherein the measuring further comprises nucleic acid sequencing.

20. The method of claim 19, wherein the sequence is single molecule real time (SMRT) sequencing.

21. The method of claim 1, wherein the wherein the agent is a small molecule, a peptide, a nucleic acid molecule, or a protein.

22. The method of claim 21, wherein the nucleic acid molecule is an siRNA, shRNA, miRNA, Locked Nucleic Acid (LNA), antisense oligonucleotide, a chemically modified oligonucleotide, or a combination thereof.

23. The method of claim 1, wherein the pathway is a kinase pathway.

24. The method of claim 23, wherein the pathway is the ataxia telangiectasia mutated (ATM) kinase pathway.
25. The method of claim 24, wherein the agent activates the ATM kinase pathway.
26. The method of claim 25, wherein activation of the ATM kinase pathway is via inhibition of poly (ADP-ribose) polymerase 1 (PARP1) expression or activity.
27. The method of claim 23, wherein the pathway is cell cycle pathway.
28. The method of claim 27, wherein the agent modulates expression or activity of a cyclin dependent kinase.
29. The method of claim 28, wherein the agent activates expression or activity of cyclin dependent kinase 1 (Cdk1).
30. The method of claim 1, wherein the seed sequence comprises at least 400 base pairs of telomere repeats.
31. The method of claim 30, wherein the seed sequence is at least 480 base pairs in length.
32. The method of claim 1, wherein measuring is performed after one cell cycle.
33. An isolated mammalian cell line characterized by a genome having a modified chromosome containing a telomere seed sequence and an endonuclease cleavage site downstream of the telomere seed sequence.
34. The cell line of claim 33, wherein the genome further comprises a recombinant nucleic acid sequence encoding an endonuclease specific for the endonuclease cleavage site.
35. The cell line of claim 34, wherein the endonuclease is I-Scel.
36. The cell line of claim 33, wherein the genome further comprises a recombinant nucleic acid sequence encoding telomerase.
37. The cell line of claim 36, wherein the nucleic acid sequence is operably linked to a nucleic acid sequence encoding a reporter.
38. The cell line of claim 37, wherein the reporter is selected from the group consisting of luciferase (LUC), β-lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo, G418), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), β-galactosidase (β-gal), and xanthine guanine phophoribosyltransferase (XGPRT), an affinity or epitope tag, and a fluorescent protein.
39. The cell line of claim 38, wherein the reporter protein is a fluorescent protein.
40. The cell line of claim 39, wherein the fluorescent protein is green fluorescent protein (GFP) or enhanced green fluorescent protein (eGFP).
41. The cell line of claim 36, wherein the genome further comprises a recombinant nucleic acid sequence encoding a recombinase.
42. The cell line of claim 41, wherein the recombinant nucleic acid sequence encoding telomerase further comprises a recombinase target sequence allowing excision of the nucleic acid sequence encoding telomerase.
43. The cell line of claim 42, wherein the nucleic acid sequence is operably linked to an inducible promoter.
44. The cell line of claim 43, wherein the promoter is inducible by a tetracycline antibiotic.
45. The cell line of claim 44, wherein the tetracycline antibiotic is doxycycline.
46. The cell line of claim 42, wherein the nucleic acid sequence is operably linked to a nucleic acid sequence encoding a reporter.
47. The cell line of claim 46, wherein the reporter is selected from the group consisting of luciferase (LUC), β-lactamase, chloramphenicol acetyltransferase (CAT), adenosine deaminase (ADA), aminoglycoside phosphotransferase (neo, G418), dihydrofolate reductase (DHFR), hygromycin-B-phosphotransferase (HPH), thymidine kinase (TK), β-galactosidase (β-gal), and xanthine guanine phosphoribosyltransferase (XGPRT), an affinity or epitope tag, and a fluorescent protein.
48. The cell line of claim 47, wherein the reporter is a human influenza hemagglutinin (HA) epitope tag.
49. The cell line of claim 33, wherein the seed sequence comprises at least 400 base pairs of telomere repeats.
50. The cell line of claim 49, wherein the seed sequence is at least 480 base pairs in length.
51. The cell line of claim 33, wherein the cells are murine cells.
52. The cell line of claim 33, wherein the modified chromosome is chromosome 4.
53. A kit comprising:
   a) a cell of the cell line according to any of claims 33-52; and
   b) reagents for culturing the cell.
54. The kit of claim 53, further comprising reagents for measuring telomerase addition.
FIG. 1A

FIG. 1B
FIG. 3A

FIG. 3B

FIG. 3C
**FIG. 5A**

5' - GAATTCCTGCAGCCCCGCGGATCCATGAGGATTAACAGGGTAAT
3' - CTTAAGGACGTCGGCCCTAGGATCCCTATTGTCCCATTAA  

I-Sce1 recognition site

5' - GAATTCCTGCAGCCCCGCGGATCTTAGGGGATAA
3' - CTTAAGGACGTCGGCCCTAGGATCC

5' - TTAGGGTTAGGGTTAGGTTAGGGTTAG

Telomere sequence

CUUUUAGUCCCAAUCCA - 5' mTR sequence

Primer-alignment Template

**FIG. 5B**

| Class 1 | 5' - GAATTCCTGCAGCCCCGCGGATCTAGGGGATAA
       | (n = 205, 13.5%) | CUUUUAGUCCCAAUCCA - 5' |
|--------|----------------|-----------------------|
| Class 2 | 5' - GAATTCCTGCAGCCCCGCGGATCTAGGGGATAA
       | (n = 135, 8.9%) | CUUUUAGUCCCAAUCCA - 5' |
| Class 3 | 5' - GAATTCCTGCAGCCCCGCGGATCTAGGGGATAA
       | (n = 726, 48.0%) | CUUUUAGUCCCAAUCCA - 5' |
| Class 4 | 5' - GAATTCCTGCAGCCCCGCGGATCTAGGGGATAA
       | (n = 174, 11.5%) | CUUUUAGUCCCAAUCCA - 5' |
| Class 5 | 5' - GAATTCCTGCAGCCCCGCGGATCTAGGGGATAA
       | (n = 232, 15.3%) | CUUUUAGUCCCAAUCCA - 5' |
| Class 6 | 5' - GAATTCCTGCAGCCCCGCGGATCTAGGGGATAA
       | (n = 42, 2.8%) | CUUUUAGUCCCAAUCCA - 5' |
raw read

(A) Identify HYG end

HYG

(B) Identify tel-tail

HYG tel-tail

(C) Identify I-Sce1

HYG seed sequence tel-tail

No de novo telomere elongation

HYG seed sequence I-Sce1 elongation tel-tail

De novo telomere elongation

FIG. 7