Provided herein are methods for increasing telomere length comprising contacting a cell with an agent that activates the ataxia telangiectasia mutated (ATM) kinase pathway or a cyclin dependent kinase pathway, thereby elongating telomeres in the cell. Also provided is a method for treating disorders such as cancer and telomere syndromes.
FIG. 2A

5'-GAATT CCTGAGCCGCCGGGATCGCTAGGGATACGCGTCGAGTAAAT
3'-CTTACGACGATGCTGGGGGCCCATCTAGGATCTGTTTGTCCATTA

I-Sce1 recognition site
I-Sce1 cleavage

5'-GAATTCCTGACGCCCAGGGATGCTTTAGGTGATCAA
3'-CTTACGAGCCTCGGGGCCCATCTAGGATCCC

5'-TTAGGTTAGGTTAGGTTAGGTTAGGTTAG

Telomere sequence

CUUUUAGUCCCAUUCC

3' mTR sequence
Primer alignment Template

FIG. 2B

Class 1
(n = 205, 13.5%)

5'-GAATT CCTGAGCCGCCGGGATCGCTAGGGATACG

CUUUUAGUCCCAUUCC

5'-GAATTCCTGACGCCCAGGGATGCTTTAGGTGATCAA

Class 2
(n = 135, 8.9%)

5'-GAATTCCTGACGCCCAGGGATGCTTTAGGTGATCAA

CUUUUAGUCCCAUUCC

5'-GAATTCCTGACGCCCAGGGATGCTTTAGGTGATCAA

Class 3
(n = 728, 48.0%)

5'-GAATT CCTGAGCCGCCGGGATCGCTAGGGATACG

CUUUUAGUCCCAUUCC

5'-GAATTCCTGACGCCCAGGGATGCTTTAGGTGATCAA

Class 4
(n = 174, 11.5%)

5'-GAATT CCTGACGCCCAGGGATCGCTAGGGATACG

CUUUUAGUCCCAUUCC

5'-GAATTCCTGACGCCCAGGGATGCTTTAGGTGATCAA

Class 5
(n = 232, 15.3%)

5'-GAATT CCTGACGCCCAGGGATCGCTAGGGATACG

CUUUUAGUCCCAUUCC

5'-GAATTCCTGACGCCCAGGGATGCTTTAGGTGATCAA

Class 6
(n = 42, 2.8%)

5'-GAATT CCTGACG

CUUUUAGUCCCAUUCC

5'-GAATTCCTGACG
METHOD FOR REGULATION OF TELOMERE LENGTH

RELATED APPLICATION DATA


STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made in part with government support under Grant No. AG09383 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 JHU3860_IWO_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 telomere maintenance and cell viability, and more specifically to activation of the ATM kinase pathway and telomere elongation.

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 normally expressed in germline, stem cells and embryonic tissues, immune cells, proliferative cells of renewal tissues. In addition telomerase is often upregulated in, cancer cells. In contrast, telomerase activity is difficult to detect in normal somatic human tissues. 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. Studies in mice have found that tumors lacking telomerase are not able to continue to grow indefinitely.

[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 transcriptase eliminates 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] Long-term cell viability is critically dependent on telomere length maintenance. 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.

[0010] 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.

[0011] In yeast, mouse 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.
[0012] 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 activates the ATM pathway while removal of POT1 primarily activates the ATR pathway.

[0013] 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 telomeres 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 transgenic or immortalized 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 are 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.

[0014] 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 Tel1 gene. In yeast, loss of Tel1 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 telomeres 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.

[0015] 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.

[0016] While there is a well-established role for ATM and ATR in signaling telomere dysfunction in human and mouse cells, less is known about the role of these kinases in normal telomere elongation. In yeast, Tel1 and Mec1 seem to play partially redundant roles at telomeres. The loss of Tel1 and Mec1 generates short, stable telomeres, while of Mec1 alone has no effect. However, together the loss of both Tel1 and Mec1 leads to further shortening than in the absence alone. This implies that Mec1 may partially compensate for the loss of Tel in telomere maintenance.

[0017] To examine the role of ATM in telomere maintenance, an assay was developed, called ADDIT (Addition of de novo initiated telomeres), which measures telomere addition at a single chromosome end. Using the ADDIT assay, the inventors have demonstrated that ATM is required for telomere addition. This assay will allow identification of additional telomere length regulators that may uncover other novel approaches to manipulating telomere length for treatment of disease.

SUMMARY OF THE INVENTION

[0018] The present invention is based on the discovery that activation of ATM kinase pathway results in telomere elongation. Further, inhibition of poly (ADP-ribose)polymerase 1 (PARP1) results in activation of ATM kinase pathway and telomere elongation. The inventors previously developed an assay to examine telomerase addition, called an ADDIT assay, which is useful in analyzing the effects of PARP1 inhibitors on telomere length. Short telomeres induce a DNA damage response, senescence and apoptosis; thus, maintaining telomere length equilibrium is essential for cell viability.

[0019] Telomerase addition of telomere repeats is tightly regulated in cells. To probe pathways that regulate telomere addition, the inventors used the ADDIT assay to measure new telomere addition at a single telomere in vivo. The inventors found that ATM kinase as well as Cdk1 are required for addition of new repeats onto telomeres in mouse cells using the ADDIT assay. Evaluation of bulk telomeres, in both human and mouse cells, showed that blocking ATM kinase and Cdk1 inhibited telomere elongation.

[0020] The activation of ATM, through the inhibition of PARP1, resulted in increased telomere elongation, supporting the central role of the ATM pathway in regulating telomere addition. Understanding this role of ATM may yield new areas for possible therapeutic intervention in telomere-mediated disease.

[0021] The ADDIT assay can be used to identify 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 expresses 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.
In one aspect, the invention provides a method of increasing telomere length. The method includes contacting a cell with an agent that activates the ataxia telangiectasia mutated (ATM) kinase pathway or a cyclin dependent kinase pathway, thereby elongating telomeres in the cell. In a preferred aspect, the agent is a poly (ADP-ribose) poly-merase 1 (PARP1) inhibitor. For example, the PARPi inhibitor may be Olaparib (AZD2281), Talazoparib (BMN-673), Rucaparib (ABT-888), SEP 9722, Niraparib (MK 4827), DOG-290, E7016, E7440 or ISO-1001.

In another embodiment, the invention provides a method of treating telomere syndrome in a subject. The method includes administering to the subject a PARP1 inhibitor, an activator of Cdk1, or a combination thereof, wherein administration leads to progressive telomere lengthening, thereby treating the telomere syndrome in the subject. In one aspect, the syndrome is selected from the telomere syndromes including dyskeratosis congenita, bone marrow failure, aplastic anemia, and pulmonary fibrosis.

In yet another embodiment, the invention provides a method of treating cancer in a subject by interfering with lengthening of telomeres in cancer cells. The method includes administering to the cells an effective amount of an inhibitor of a regulator of telomere lengthening, wherein the administration of the inhibitor leads to progressive telomere shortening in the cancer cells, thereby treating cancer in the subject. In one aspect, the cancer is selected from stomach cancer, osteosarcoma, lung cancer, pancreatic cancer, adenocortical carcinoma, melanoma, breast cancer, ovarian cancer, cervical cancer, skin cancer, connective tissue cancer, uterine cancer, anogenital cancer, central nervous system cancers, retinal cancer, blood and lymphoid cancers, kidney cancer, bladder cancer, colon cancer and prostate cancer.

**BRIEF DESCRIPTION OF THE DRAWINGS**

**0025** FIGS. 1A-1E are pictorial and graphical representations showing that de novo telomere addition occurs only in telomerase-positive cells. FIG. 1E includes SEQ ID NOs: 1-9 (ordered from top to bottom).

**0026** FIGS. 2A-2B are pictorial representations showing classification of de novo telomere addition. FIG. 2A includes SEQ ID NOs: 10-15 and FIG. 2B includes SEQ ID NOs: 16-33.

**0027** FIGS. 3A-3B are graphical representations showing that ATM kinase is required for telomere addition.

**0028** FIGS. 4A-4B are graphical representations showing that activation of ATM kinase pathway elongates telomeres.

**0029** FIGS. 5A-5B are pictorial and graphical representations relating to generation of cell line SL13 to assay de novo telomere addition.

**0030** FIG. 6 is a flow diagram depicting the pipeline of PacBio® sequence read analysis.

**0031** FIGS. 7A-7B are pictorial and graphical representations showing that de novo telomere addition is significantly reduced in siTERT-treated cells.

**0032** FIG. 8 is a graphical representation showing that blocking ATM shortens bulk telomeres in mouse cells.

**0033** FIGS. 9A-9C are pictorial and graphical representations showing that de novo telomere addition occurs in G2/M phase.

**0034** FIG. 10 is a graphical representation showing that inhibition of Cdk5 prevents de novo telomere addition.

**0035** FIGS. 11A and 11B are graphical representations showing that Cdk1 is required for de novo telomere addition.

**DETAILED DESCRIPTION OF THE INVENTION**

**0036** The present invention is based on discovery that activation of ATM kinase pathway or a Cdk1 pathway results in telomere elongation. The inventors previously developed an assay to examine telomerase addition, called an ADDIT assay, which is useful in analyzing the effects Cdk1 and ATM activators including PARPi inhibitors on telomere length.

**0037** 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.

**0038** 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.

**0039** 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.

**0040** In prior studies, the inventors described a new assay, named ADDIT, that can identify genes required for telomerase-dependent telomere elongation by measuring the de novo telomere addition at a single chromosome. Using this assay the inventors showed that the ATM kinase pathway and Cdk1 are required for telomerase-mediated telomere addition. Activation of ATM and Cdk1 also caused telomere elongation. Using an independent approach, they found that inhibition of Cdk1 and ATM kinase activity prevented bulk telomere elongation by telomerase and activation caused telomere elongation in cell culture experiment assayed by Southern blot.

**0041** The mechanism of telomere length maintenance involves many inter-dependent regulatory pathways that act together to establish and maintain telomere length. 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. The ATM kinase pathway is conserved from yeast to mammalian system. It is well documented that ATM is required for normal telomere maintenance in different yeast species including S. pombe in which ATM mediates the telomerase recruitment.

**0042** Since inhibition of ATM kinase gradually shortens telomeres, the inventors investigated whether activa-
tion of ATM kinase pathway would result in telomere elongation. 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 the phosphorylation levels of KAP1, in response to the PARP1 inhibitor, Olaparib was measured. Cells treated with Olaparib showed increased level of KAP1 phosphorylation, compared to DMSO-treated control cells. Olaparib inhibited PARylation in this cell line as shown by an antibody directed against poly ADP-ribose.

[0043] The inventors used the ADDIT assay to examine telomerase addition and found a small increase in telomere elongation, with Olaparib treatment compared to controls. Treatment with both Olaparib and KU55933 blocked this increased elongation suggesting that the effect of Olaparib was working through the ATM pathway. Further Olaparib treatment increased telomere length as assayed by Southern blotting. SL13 cells were grown in the presence of Olaparib for 55 population doublings and it was found that telomere length gradually increased after 25 PDs in the presence of Olaparib and were further elongated in later population doublings. It was not possible to culture cells for long term in both Olaparib and KU55933 due to the documented synthetic growth defects when these two pathways are inhibited. This highlights the utility of the ADDIT assay for short-term measurement of telomere elongation. These experiments suggest that inhibition of ATM blocks telomere elongation while activation of ATM stimulates telomere elongation.

[0044] Inhibition of PARP1 has been shown to activate ATM. PARP1 inhibitor Olaparib was used to activate ATM and found an increase in telomere length. Olaparib was one of the first PARP1/2 specific inhibitors in the clinical trials for the treatment of ovarian cancer. Although further validation is required, Olaparib appears to activate ATM kinase pathway and result in telomere elongation. Given that Olaparib is currently used in clinical trials, it is crucial to fully characterize the secondary effects of Olaparib on telomere length.

[0045] The PARP enzyme family currently has 17 members, and the specificity of PARP1/2 inhibitors may differ and have different consequences in mice and humans. In humans, tankyrase 1 and 2, members of PARP family, positively affect telomere length through the ADP ribosylation of TRF1. However, the sites for ADP ribosylation are not conserved in mouse. In vitro assays have shown that several PARP1/2 inhibitors in clinical trials, including Olaparib, have strong specificities to PARP1-4 but less are less active on other PARPs including 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.

[0046] 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/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 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.

[0047] The examples herein illustrate 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.

[0048] 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 Mre11 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 3’ strand resection.

[0049] 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.

[0050] Accordingly, in one aspect, the invention provides a method of increasing telomere length. The method includes contacting a cell with an agent that activates the ataxia telangiectasia mutated (ATM) kinase pathway or a cyclin dependent kinase pathway, thereby elongating telomeres in the cell.


[0052] Mutation(s) in both core component of telomerase as well as the accessory factors can cause critically short telomeres, dysfunctional telomeres, leading to age-related degenerative disorders. The first such mutation found in dyskerin, which is one of the critical accessory factors for human telomerase, causes a rare disorder called X-linked dyskeratosis congenita. Later it was found the mutations in human TR underlie bone marrow failure in autosomal dominant dyskeratosis congenita. Further studies identified additional mutations in telomerase components and telomere-related genes that lead to dysfunctional telomeres in autosomal dominant disease. It is now well characterized that short telomeres have a causal role in a broad spectrum of degenerative disorders called as “telomere syndromes,” including idiopathic pulmonary fibrosis, bone marrow failure, and other premature aging disorders. These disorders are primarily caused by defects that lead to short telomeres.

[0053] Short telomeres induce a DNA damage response, senescence and apoptosis; thus, maintaining telomere length equilibrium is essential for cell viability. In humans, syndromes of telomere shortening cause age-related degenerative diseases including dyskeratosis congenita, pulmonary fibrosis, aplastic anemia and others. The present invention may provide a novel approach to manipulate telomere length by using a PARP inhibitor.
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. The ATR pathway may also compensate for the loss of ATM function in these patients. These findings imply that AT patients may be at risk for the same spectrum of diseases seen in the telomere syndrome. It will be important to determine whether short telomeres can directly contribute to a more severe AT clinical phenotype and whether telomere lengthening could be of potential therapeutic value. Ultimately finding a safe way to elongate telomeres could benefit both AT patients and those with a Telomere syndrome.

Accordingly, the invention provides a method of treating telomere syndrome in a subject. The method includes administering to the subject a PARP1 inhibitor, an activator of CdK1, or a combination thereof, wherein administration leads to progressive telomere lengthening, thereby treating the telomere syndrome in the subject. In one aspect, the syndrome is selected from the telomere syndromes including, dyskeratosis congenita, bone marrow failure, aplastic anemia, and pulmonary fibrosis.

On the other hand, cancer cells divide continuously and increase or maintain telomere lengths to avoid cell death. The majority of human cancers maintain telomere lengths via up-regulated telomerase activity or activation of the alternative lengthening of telomeres (ALT) pathway. Recent studies associate certain mutations in TERT promoter and telomere binding proteins, such as POT1, with predisposition to cancer.

As such, in another embodiment, the invention provides a method of treating cancer in a subject by interfering with lengthening of telomeres in cancer cells. The method includes administering to the cells an effective amount of an inhibitor of a regulator of telomere lengthening, wherein the administration of the inhibitor leads to progressive telomere shortening in the cancer cells, thereby treating cancer in the subject.

In one aspect, the methods of the invention may further include measuring telomere length. Measuring may be accomplished 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 allows investigations that can target specific telomere ends, which is not possible with TRF analysis described below.

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 32P-(TTAGGG)n oligonucleotide probe to restriction enzyme digested genomic DNA embedded on a nylon membrane and subsequently exposed to autoradiographic film or phosphoimaging screen. Another histochemical method, termed Q-FISH, involves fluorescent in situ hybridization (FISH).

The term “subject” as used herein refers to any individual or patient to which the subject methods are performed. Generally the subject is human, although as will be appreciated by those in the art, the subject may be an animal. Thus other animals, including mammals such as rodents (including mice, rats, hamsters and guinea pigs), cats, dogs, rabbits, farm animals including cows, horses, goats, sheep, pigs, as well as primates (including monkeys, chimpanzees, orangutans and gorillas) are included within the definition of subject.

The terms “administration” or “administering” are defined to include an act of providing a compound or pharmaceutical composition of the invention to a subject in need of treatment. The phrases “parenteral administration” and “administered parenterally” as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarticular, intrathecal, intracar- pal, intraorbital, intracardiac, intradermal, intraperitoneal, transmucosal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intratrabecular injection and infusion. The phrases “systemic administration,” “administered systemically,” “peripheral administration” and “administered peripherally” as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the subject’s system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

The route of administration of a composition containing an agent as identified herein, will depend, in part, on the chemical structure of the molecule. Polypeptides and polynucleotides, for example, are not particularly useful when administered orally because they can be degraded in the digestive tract. However, methods for chemically modifying polynucleotides and polypeptides, for example, to render them less susceptible to degradation by endogenous nucleases or proteases, respectively, or more absorbable through the alimentary tract are well known. For example, a peptide agent can be prepared using D-amino acids, or can contain one or more domains based on peptidomimetics, which are organic molecules that mimic the structure of peptide domain; or based on a peptoid such as a vinyllogous peptoid. Where the agent is a small organic molecule such as a steroidal alkaloid (e.g., cyclopamine), it can be administered in a form that releases the active agent at the desired position in the body (e.g., the stomach), or by injection into a blood vessel that the agent circulates to the target cells (e.g., hematopoietic malignancy cells).

Regardless of the route of administration selected, the compounds of the present invention, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically acceptable dosage forms such as described herein or by other conventional methods known to those of skill in the art.

The total amount of an agent to be administered in practicing a method of the invention can be administered to a subject as a single dose, either as a bolus or by infusion over a relatively short period of time, or can be administered using a fractionated treatment protocol, in which multiple doses are administered over a prolonged period of time. One skilled in the art would know that the amount of agent depends on many factors including the age and general health of the subject as well as the route of administration and the number of treatments to be administered. In view of these factors, the skilled artisan would adjust the particular dose as necessary. In general, the formulation of the pharmaceutical composition and the routes and frequency of administration are determined, initially, using Phase I and Phase II clinical trials.
In general, a suitable daily dose of a compound of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. Generally, intravenous, intracerebroventricular and subcutaneous doses of the compounds of this invention for a patient will range from about 0.00001 to about 100 mg per kilogram of body weight per day which can be administered in single or multiple doses.

A “therapeutically effective amount” or “efficacious amount” means the amount of a compound that, when administered to a mammal or other subject for treating a disease, condition, or disorder, is sufficient to effect such treatment for the disease, condition, or disorder. The “therapeutically effective amount” will vary depending on the compound, the disease and its severity and the age, weight, and the like, of the subject to be treated. Typically a therapeutically effective amount should produce a serum concentration of drug of from about 0.1 mg/ml to about 50-100 μg/ml. In various embodiments, the dosage administered is sufficient to result in a serum concentration level of the drug in the subject of greater than about 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25 or 30 μM. The pharmaceutical compositions typically should provide a dosage of from about 0.01 mg to about 2000 mg of compound per kilogram of body weight per day. The drug may be administered at once, or may be divided into a number of smaller doses to be administered at intervals of time. It is understood that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by extrapolation from in vivo or in vitro test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions.

If desired, the effective daily dose of the active compound may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. There may be a period of no administration followed by another regimen of administration.

It will be understood, however, that the specific dose level and frequency of dosage for any particular patient may be varied and will depend upon a variety of factors including the activity of the specific compound employed, the metabolic stability and length of action of that compound, the age, body weight, general health, sex, diet, mode and time of administration, rate of excretion, drug combination, the severity of the particular condition, and the host undergoing therapy.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

Additionally, in various aspects, a physician or veterinarian having ordinary skill in the art can readily determine an appropriate subject for administration of the compounds described herein. For example, one of skill in the art is capable of routine diagnosis of diabetes. Also, it is routine for one of skill in the art to determine the appropriate compounds to be administered to the subject as well as the timing of administration depending on the diagnosis. Additionally, it may be appropriate to administer the compound in combination with other drugs, such as chemotherapeutic agents.

When other therapeutic agents are employed in combination with the compounds of the present invention they may be used for example in amounts as noted in the Physician Desk Reference (PDR) or as otherwise determined by one having ordinary skill in the art.

In certain embodiments, pharmaceutical compositions may include, for example, at least about 0.01 mg/g of body weight of a drug, such as an inhibitor. In other embodiments, the drug may comprise between about 0.1% to about 75% of the weight of the unit, or between about 2% to about 20%, for example, and any range derivable therein. In other non-limiting examples, a dose may also comprise from about 1 μg/kg/body weight, about 100 μg/kg/body weight, about 500 μg/kg/body weight, about 1 mg/kg/body weight, about 5 mg/kg/body weight, about 10 mg/kg/body weight, about 50 mg/kg/body weight, about 100 mg/kg/body weight, about 200 mg/kg/body weight, about 300 mg/kg/body weight, about 350 mg/kg/body weight, about 400 mg/kg/body weight, about 450 mg/kg/body weight, about 500 mg/kg/body weight, about 600 mg/kg/body weight, about 700 mg/kg/body weight, about 800 mg/kg/body weight, about 900 mg/kg/body weight, about 1000 mg/kg/body weight, about 2000 mg/kg/body weight to about 5000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 350 mg/kg/body weight to about 1000 mg/kg/body weight, about 50 μg/kg/body weight to about 500 mg/kg/body weight, and the like, can be administered.

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.

As used herein, an agent identified as a regulator of telomere length acts to increase extension of telomeres or inhibit telomere extension. 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.

While the examples highlight use of the PARP1 inhibitors Olaparib and KU5593, agents for use in the
methods of the invention may encompass those from numerous chemical classes, such as small organic molecules, peptides, saccharides, fatty acids, steroids, purines, pyrimidines and the like. 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, shRNA, and shRNA.

[0076] 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 pre-miRNA) is processed into a short stem-loop structure called a pre-miRNA and finally into a functional mature 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 miRNAs, long noncoding RNAs, snRNAs 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.

[0077] 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).

[0078] 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.

[0079] 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.

[0080] In general, the nucleotides comprising a polynucleotide are naturally occurring deoxyribonucleotides, such as adenosine, 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.

[0081] 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.

[0082] 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 oligonucleotides.
In various aspects modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorodithioates, phosphorodithiosters, aminophosphorothioesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminophosphoraminates, thionophosphoraminates, thionoalkylphosphonates, thionoethylphosphorothioesters, sele- nophosphates and borono-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.

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; ribonucleotides; and alkene containing backbones; sulfinate backbones; methyleneimino and methylenedihydrozino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

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 azan 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; O-; S-; or N-alkyl; O-; S- or N-alkenyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkenyl may be substituted or unsubstituted C₃ to C₁₀ alkyl or C₃ to C₁₀ alkenyl and alkynyl. Particularly preferred are O(CH₂)ₙOCH₃, O(CH₂)ₙOCH₂CH₃, O(CH₂)ₙONH₂, O(CH₂)ₙCH₃, O(CH₂)ₙON(CH₂)ₙCH₃, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2'-position: C₃ to C₁₂ lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkyl, alkynyl, alkyl, aralkyl, O-alkyl or O-aralkyl; NH; S-CH₃, OCN, Cl, Br, CN, CF₃, OC₆H₅, SO₂CH₃, CH₂(ONO₂), NO₂, N₃, NH₂, heterocy cloalkyl, heterocycloalkyl, aminooxy-

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'-O-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'-O- or 2'-O-amino ribonucleotide, 2'-C- or 2'-O- or 2'-C- or 2'-O- alkyl ribonucleotide, 2'-methoxethyl 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'-O-methyl ribonucleotide, 2'-fluoro ribonucleotide, 2'-amino ribonucleotide, 2'-O- or 2'-O-amino ribonucleotide, 2'-C- or 2'-O- or 2'-C- or 2'-O- alkyl ribonucleotide, 2'-methoxethyl ribonucleotide, 5'-C'-methyl ribonucleotide, or a combination thereof.

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.

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—) group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2.

Other modifications include 2'-methoxy(2'-O—CH₃), 2'-aminopropanoxy(2'-OCH₃CH₂CH₂NH₂), 2'-allyl(2'-CH—CH—CH₂), 2'-O-allyl(2'-O—CH₂—CH—CH₂),
2'-fluoro (2'-F), 2'-amino, 2'-thio, 2'-O-methyl, 2'-methoxyethyl, 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.

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-thioguanine 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 (pseduouracil), 4-thiouracil, 8-halo, 8-amo, 8-thio, 8-thiodikyl, 8-hydroxyl 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, 2-6-adene, 2-6-amino adenine, 8-azaguanine and 8-azaadenine, 7-deazaadenine and 7-deazaguanine and 3-deazaadenine and 3-deazaguanine. Further modified nucleobases include tri cyclic pyrimidines such as phenoxazine cytidine (1H-pyrimido[5,4-b][1,4]benzoxazolin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazizin-2(3H)-one), G-clamps as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-1H-pyrimido [5,4-b][1,4]benzoxazolin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (1H-pyrimido[3',2',4:5']pyrrolol[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-deza adenine, 7-dezaganosine, 2-amnipyr dine 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-azapuridines and N=2, N=6 and O=6 substituted purines, including 2-amino propyridadenine, 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'-O-methoxyethyl sugar modifications.

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, polymers, polyanimes, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phanantheridine, anthraquinone, acridine, fluorescines, rhodamines, coumarins, and dyes.

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

Addition of De Novo Initiated Telomeres Assay (ADDIT Assay) to Identify Telomere Length Regulators

To critically access the role of ATM, as well as other potential telomere length regulators, an assay was developed that measures telomere repeat addition in cells, referred to herein as the addition of de novo initiated telomeres assay, or ADDIT assay. Using the ADDIT assay, it was determined that that ATM is required for addition of new repeats onto telomeres in mouse cells. Evaluation of bulk telomeres, in both human and mouse cells, showed that blocking ATM inhibited telomere elongation. Finally, the activation of ATM through the inhibition of PARP1 resulted in increased telomere elongation, supporting the central role of the ATM pathway in regulating telomere addition. Under this role of ATM may yield new areas for possible therapeutic intervention in telomere-mediated disease.

Results

Addition of De Novo Initiated Telomeres—ADDIT Assay

To critically access the role of ATM, as well as other potential telomere length regulators, an assay was developed that measures telomere repeat addition in cells over 48 hr. CAST/EiJ mouse fibroblast cell line was engineered to (SL3) contain a conditional mTR allele and a modified chromosome 4 (chr4) with an internal 480 bp telomere ‘seed’ sequence followed by a unique 1-Sce1 endonuclease cut site (FIG. 5 and see Experimental Procedures). The internal telomere ‘seed’ sequence can be conditionally exposed by 1-Sce1 endonuclease cleavage (FIG. 1A) and then be elongated by telomerase in a manner similar to de novo telomere addition shown to occur in yeast (Diele and Gotschel, 1999, Cell 99, 723-733).

Expression of doxycycline-inducible HA-tagged 1-Sce1 endonuclease in this SL3 cell line exposed the telomere seed and allowed telomere addition by telomerase (data not shown). Chromosome cutting occurred by 8 hr after doxycycline treatment (data not shown). As a control, genomic DNA was isolated and digested it in vitro with purified 1-Sce1 endonuclease to compare the in vivo and in vitro cut DNA on a Southern blot. At 36 and 48 hr, a ‘smear’ above the in vivo cut telomere seed band was detected faintly on the Southern and suggested some de novo telomere addition (data not shown).

To better detect the telomere elongation, the single telomere length analysis (STELA) assay was modified (Baird et al, 2004, Hum Mol Genet 13, 1515-1524), to measure telomere length at the in vivo cut chr4. The linker, ‘telerette’, was ligated to the telomere and PCR amplified the telomere using the ‘telertail’ primer and an internal primer in the hygromycin resistance (HYG) sequence on the engineered chromosome (FIG. 1B). To determine the unextended cut chromosome length in vitro, a different linker was
designed, ‘IScErette’, which anneals to the 4-nt 3' overhang created by the I-Sce1 endonuclease (FIG. 1B). PCR using the forward primers, F1 or F2, and the telomere generated products of predicted size on a Southern blot (data not shown).

[0099] To examine whether elongation was telomerase dependent, mTR was deleted by Flp recombinase to generate mTR cells (FIG. 5B, other data not shown) and carried out the ADDIT assay. STELA PCR products in the mTR cells treated with doxycycline were similar to and shorter than the control IScErette cells in vitro cut DNA, likely because of in vivo resection by nucleases. In contrast, the STELA products from mTR cells were longer than the control IScErette products (data not shown), suggesting new telomeric sequence was added. Together, these data suggest the longer products in mTR cells are the result of telomerase elongation of the seed sequence in vivo.

[0100] Sequence Analysis of the Telomere Addition Products

[0101] To further verify telomere addition had occurred, the PCR products were sequenced with Pacific Biosciences (PacBio®) sequencing. PacBio® produces long sequence reads that allowed us to determine telomere length. While sequencing errors, predominantly point insertions and deletions, occurred as expected, the telomere repeats were easily recognizable. To assure products were full length, the PacBio® reads were filtered to examine only those that had the unique HIYG sequence followed by the seed telomere repeat sequence and also had the ‘telant’ primer sequence (FIG. 6). Individual reads are displayed as a horizontal line, where the wild-type TTAGGG (SEQ ID NO: 100) repeats are colored orange and variant telomeres repeat sequences are colored in darker orange. The I-Sce1 site is shown boxed (FIG. 1C). Three regions of variant telomere repeats were noted, evident as darker stripes in the aligned reads, that were present in the original SL13 clone, and served as useful sequence reference points (FIG. 1C). If there were degradation past these variants and then new TTAGGG (SEQ ID NO: 100) repeat synthesis in vivo, these landmarks would be removed.

[0102] 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-Sce1 cut site followed by additional telomeric sequences (FIGS. 1C and 1D). Telomerase will add telomere repeats onto primers (or sequences) that contain some non-telomeric sequence as described below. A number of reads were shorter than the reference sequence and likely arose from 5' end resection occurring in vivo at telomeres that were not elongated. The mTR samples showed only resection, and the I-Sce1 site was not present. Telomerase addition was defined as occurring when telomere sequence was added onto the I-Sce1 site. There were a few longer reads in the mTR cells, however these did not have telomere addition beyond the I-Sce1 site, suggesting these longer products occurred through slippage during STELA PCR and/or the PacBio® sequencing.

[0103] The sequence length distribution in the ADDIT assay represents telomere elongation, incomplete telomere replication and in vivo end resection (as well as PacBio® sequencing errors). To examine the telomerase interaction at the telomere, the percentage of reads that showed elongation past I-Sce1 were quantitated, which represents telomerase recruitment to the telomere.

[0104] In the mTR cells, around 20% of the reads had telomere sequence after the I-Sce1 site representing de novo addition, while the mTR sample showed no addition of repeats beyond the I-Sce1 site (FIG. 1D). In an additional control, siRNA against TERT also blocked repeat addition beyond the I-Sce1 site (FIG. 7). As expected, sequence reads from the in vitro IScErette control sample showed no elongation (FIGS. 1C and 1D). The small changes in length and sequence in this sample likely represent the PacBio® sequencing errors or slippage during PCRs.

[0105] De Novo Telomere Addition onto I-Sce1 Site

[0106] The sequence reads were examined to determine how telomerase added repeats to the I-Sce1 site. During telomere elongation, the RNA component of telomerase, mTR, anneals to the telomere through the primer-alignment region and uses the template region to add telomere repeats. For the mouse telomerase RNA, there is a 2-nt alignment region, while the human RNA contains 5 nucleotides in the alignment region. Evaluation of the I-Sce1 cleavage site showed that it has sequence complementarity to the mTR primer-alignment region (FIG. 2A).

[0107] The sequence junction between the I-Sce1 site and the de novo telomere repeats defined six different elongation classes, which have unique base pairing of the 3' end of the I-Sce1 site with the mTR (FIG. 2B). In Class 1, 205 of the 1514 (13.5%) PacBio® reads showed telomeric repeats directly added after the I-Sce1 3' overhang without any loss of nucleotides (FIG. 2B). The most common class of telomere addition, Class 5 (48.0%) had loss of 4 nucleotides from the I-Sce1 site, creating the most complementarity (AGGG) between the 3' end and the mTR sequence. The next most common, Class 5 (15.3%) resulted from base-pairing a G-rich sequence internal to the cleavage site forming 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 present in neither the I-Sce1 site nor the telomere sequence. Incorporation of a sequence in the alignment region has also been seen in vitro, and provides further evidence that telomere repeats are added by telomerase activity.

[0108] ATM Kinase is Essential for Telomere Addition

[0109] To probe the role of ATM, the ADDIT assay was used in cells treated with the ATM specific inhibitor KU55933 (as described in Hickson et al., 2004, Cancer Research 64, 9152-9159) or with siRNA to ATM. To confirm the inhibition of ATM, the phosphorylation level was examined of the ATM substrate Kap1 and, as a control, an ATM kinase substrate Chk1 by western blot. Cultured cells were pretreated with KU55933, siATM or DMSO control and then exposed to camptothecin (CPT), a DNA damaging agent. Western blot analysis with antibodies to the phosphorylated Kap1-S824 and Chk1-S345 indicated that KU55933 and siATM blocked Kap1 phosphorylation but not Chk1 phosphorylation (data not shown). This indicated that both KU55933 and siATM specifically inhibited ATM signaling, without affecting the ATR pathway.

[0110] PacBio® sequencing of the ADDIT assay indicated de novo telomere repeat addition beyond the I-Sce1 site was significantly reduced in three separate experiments when ATM was knocked down with siRNA (FIGS. 3A and 3B). Cells treated with the KU55933 had significantly fewer telomere elongation products compared to controls in replicated experiments (two-tailed P value=0.03, t-test). These
results indicate that blocking ATM activity prevents telomerase-mediated de novo telomere repeat addition. The difference in the pharmacologic inhibition of ATM with KU55933 and the siRNA knockdown might be due to the presence or absence of the ATM protein itself or a dominant effect of blocking ATM. Interestingly, expression of a kinase dead ATM is lethal in mice while ATM deletion is viable yet radiosensitive.

[0111] To determine whether ATM inhibition altered the mechanism of telomerase elongation, the distribution of the 6 different classes of elongation products described in FIG. 2 were examined. Although there was some variability in the distribution of products (see Table 3 below), the most dominant class of telomere addition was Class 3 that creates the most complementarity between the 3' end and the mTR sequence, suggesting that telomerase action is intact, but its recruitment is impaired by the loss of ATM.

<table>
<thead>
<tr>
<th>TABLE 3</th>
<th>Classification of de novo telomere addition, related to FIG. 2.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Class 1</td>
</tr>
<tr>
<td>Wild-type</td>
<td>Number of Reads</td>
</tr>
<tr>
<td></td>
<td>Percentage (%)</td>
</tr>
<tr>
<td>KU55933</td>
<td>Number of Reads</td>
</tr>
<tr>
<td></td>
<td>Percentage (%)</td>
</tr>
<tr>
<td>Olaparib</td>
<td>Number of Reads</td>
</tr>
<tr>
<td></td>
<td>Percentage (%)</td>
</tr>
</tbody>
</table>

PacBio® reads from wild-type, Olaparib or KU55933 treated samples are classified according to where the sequences aligned with the mTR template as described in FIG. 2. The percentages of reads in each class for each treatment are shown.

[0112] To further define the role of ATM telomere elongation, telomerase was overexpressed. Overexpression of telomerase in immortal cultured cells results in excessive telomere elongation: SL13 cells were treated with either KU55933 or the ATR inhibitor VE821 (as described in Reaper et al., 2011, Nature Chemical Biology 7, 428-430) and transduced them with a lentivirus expressing both mTR and mTERT. Controls showed the specificity of VE821 (data not shown). Telomere lengths examined on Southern blots showed rapid elongation in control cells as early as day 6. However, treatment with the ATM inhibitor KU55933 blocked this telomere elongation (data not shown). Cells treated with the ATR inhibitor VE821 showed subtle but reproducible decreased elongation at day 6, although the effect was not as great as with ATM inhibition (data not shown). Taken together, the results from the ADDIT assay and the Southern data indicate that ATM kinase is required for telomere elongation by telomerase. ATR may also play a role in telomere length regulation, though not to the same extent as ATM.

[0113] Inhibition of ATM Kinase Shortens Telomeres in Human Cells

[0114] ATM has been proposed to play different roles in signaling telomere dysfunction in human and mouse cells. Since it is not yet clear whether the damage signaling is mechanistically linked to telomere elongation, and given the discrepant reports of the role of ATM in human telomere length, the role of ATM in human cells was revisited. KU55933 shortens bulk telomeres in HT1080 cells, and telomere shortening in human HCT116 was also found (data not shown). When the mouse cell line SL13 was grown in KU55933, telomere shortening also occurred, although at a somewhat slower rate (data not shown), however this shortening was reproducible (FIG. 8B).

[0115] Activation of ATM Kinase Pathway Elongates Telomeres

[0116] To further probe the role of ATM in telomere length, ATM was activated in cultured cells. Cells treated with an inhibitor of poly (ADP-ribose) polymerase 1 (PARP1) were shown to activate ATM. PARP is an essential enzyme involved in recognition and repair of DNA breaks. Cells were treated with the PARP1 inhibitor, Olaparib (as described in Fong et al., 2009, The New England Journal of Medicine 361, 123-134), and also found that PARP1 inhibition correlated with increased ATM phosphorylation of Kap1 and increased phosphorylation of ATM-S1981 (data not shown). Using the ADDIT assay, the percentages of elongated telomeres were determined and found to be significantly increased in cells treated with Olaparib compared to controls, from 20% to 26%, in five independent experiments (two-tailed P value = 0.03, t-test) (FIGS. 4A and 4B). Similar to wild-type control, the most common class of telomere addition was Class 3 that creates the most complementarity between the 3’ end and the mTR sequence (Table 3). To examine whether this stimulation of telomere elongation acts through ATM, epistasis was examined by treating cells with both Olaparib and KU55933. Co-treatment significantly reduced telomere addition compared to Olaparib only (P value = 0.04, t-test) (FIGS. 4A and 4B), suggesting the increased telomere elongation by Olaparib treatment acts, at least in part, through the ATM pathway.

[0117] As an independent test of Olaparib on telomere length, telomeres were examined by Southern blot in cultured SL13 cells grown in the presence of Olaparib for an extended time. Telomere length gradually increased in the presence of Olaparib over the course of the experiment (data not shown). Taken together, the experiments described here indicate that inhibition of ATM blocks telomere elongation while activation of ATM stimulates telomere elongation.

[0118] Telomerase is known to be phosphorylated, so whether the KU55933 or Olaparib treatment of cells might directly affect telomerase activity was examined. Telomerase activity was measured by a quantitative direct activity assay in human cells treated with KU55933 or Olaparib and no change in telomerase activity was found (data not shown). ATM kinase is a positive regulator of telomerase elongation in both mouse and human cells, and the effects of ATM inhibition are not due to inhibition of telomerase enzyme activity.
FIGURE LEGENDS

[0119] FIG. 1 shows that de novo telomere addition occurs only in telomerase-positive cells. (A) Schematic of the ADDIT assay. I-SceI cutting at the endonuclease site (green box) exposes the 480 bp telomere ‘seed’ sequence (orange arrows). New telomere repeats (lighter orange arrows) are added by telomerase. (B) Representation of modified STELA, showing primers (arrows) and linkers either ‘telorette’ added to telomere or ‘Iscerette’ added to cleaved I-SceI end. Telomeres were PCR amplified with a HYG specific forward primer, either F1 or F2, and a reverse primer, teltail. S, SphI. (C) Analysis of PacBio® circular consensus sequence (CCS) reads. Each horizontal line represents one CCS read. Wild-type telomere repeats are shown in orange, divergent telomeric sequence in darker orange and the I-SceI site in green. X-axis indicates the length (bp) from the start of the telomere seed sequence. A maximum of 400 reads from each sample are shown for simplicity. (D) Bar graph shows percentage of PacBio® CCS reads with de novo telomere repeats from each sample. Asterisk indicates P value is <0.05 (n=number of separate experiments). (E) The sequences of PacBio® CCS reads boxed in (C) are shown. See also FIGS. 5-7.

[0120] FIG. 2 depicts the classification of de novo telomere addition immediately after telomere seed includes the 18-nt I-SceI recognition site (black box). I-SceI cutting 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) A total of 1514 PacBio® CCS reads from wild-type samples were classified by where the telomere repeat sequences were added, and the percentage of reads in each class are shown in parentheses. The different degree of 3′ end resection of the I-SceI site and potential positioning with mTR primer region is shown along with a representative PacBio® read of each class. The incorporation of a C residue in Class 2 is highlighted in yellow. De novo added wild-type telomere repeats are in pink. See also Table 3.

[0121] FIG. 3 shows that ATM kinase is required for telomere addition. (A) Representative analysis of PacBio® CCS reads (maximum of 200 shown for simplicity) from samples pretreated with DMSO, 10 μM KU55933 or 100 nM siATM™ prior to doxycycline treatment. (B) Bar graph shows percentage of CCS reads with de novo telomere addition. The means and standard error of mean (SEM) from multiple experiments (n) are the following: 19.5±2.06 for DMSO, 12.45±1.16 for KU55933 and 0±0 for siATM™. Asterisk indicates unpaired t-test two-tailed P value is <0.05.

[0122] FIG. 4 shows that activation of ATM kinase pathway elongates telomeres. (A) Representative analysis of PacBio® CCS reads (maximum of 350 reads shown for simplicity). Samples were pre-treated with either DMSO, 10 μM KU55933, 3 μM Olaparib or both prior to doxycycline treatment. (B) The mean percentages and SEM of CCS reads with de novo telomere addition from multiple experiments (n) are the following: 19.2±2.06 for DMSO, 12.45±1.16 for KU55933, 26.1±1.19 for Olaparib and 15.33±5.88 for Olaparib and KU55933. Asterisk indicates unpaired t-test one- or two-tailed P value is <0.05.

[0123] FIG. 5 relates to generation of cell line SL13 to assay de novo telomere addition, related to FIG. 1. (A) The chr4 subtelomeric targeting construct has two homology arms, hygromycin cassette (HYG), 480 bp telomere sequence (arrows) followed by the I-SceI endonuclease site and the HSV-tk cassette. The predicted sizes of Alf11-digested genomic DNA of wild-type mouse chr4 allele (5.2 kb) and the correctly targeted allele (8.2 kb) are shown. The restriction sites shown are A, Alf11; B, BstBI; N, Neol; X, Xhol. (B) The relative expression levels of mTR normalized to HPRT were measured by quantitative RT-PCR in cells sorted for GFP™ (mTR™), GFP- (mTR™) as well as in wild-type (WT) cells and the parental mTR™ cell line. Error bars indicate the SEM from triplicates of each sample.

[0124] FIG. 6 shows the pipeline of PacBio® sequence read analysis, related to FIG. 1C. (A) PacBio® sequencing reads were aligned to the last 50 bases of the hygromycin gene sequence (HYG) using the pairwise Alignment 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-SceI sequence with parameters: local, mismatch penalty -3, match bonus 2, gapOpening -2, gapExtension -2. A score of 36 was sufficient to identify the I-SceI 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.

[0125] FIG. 7 shows that de novo telomere addition is significantly reduced in siTER-TERT-treated cells, related to FIG. 1. (A) Representative PacBio® analysis of samples either treated with mock (siMock) or siRNA against TERT (siTER-TERT). Maximum of 300 CCS reads are shown for simplicity. X-axis indicates the length (bp) from the start of the telomere seed sequence. (B) The mean percentage and SEM of CCS reads with de novo telomere repeats from two experiments are: 13.9±2.47 for siMock and 3.6±1.22 for siTER. Asterisk indicates one-tailed P value=0.03 by unpaired t-test.

[0126] FIG. 8 shows that blocking ATM shortens bulk telomeres in mouse cells. Telomere lengths of SL13 cells treated with 10 μM KU55933 were measured at different population doublings (PD) using genomic Southern blot analysis. The figure shows densitometry tracing of the lanes generated via the analysis by graphic pixel counts (×103) versus DNA migration distance (Pixel Position). Boxed numbers indicate the pixel positions corresponding to the blot.

Discussion

[0127] The ADDIT assay provides a powerful tool to examine elongation of a single chromosome end over just one or two cell cycles. Using this assay, it was shown that blocking ATM activity led to decreased telomere elongation, while activation of ATM by Olaparib increased telomere elongation. Further, Southern blots validated that inhibition
of ATM telomere shortening in both human and mouse cells. The ADDIT assay may allow rapid identification of new regulators of telomere length, as even essential genes can be examined since long-term growth is not required.

[0128] Conservation of the Role of ATM in DNA Damage and at Telomeres

[0129] The role proposed for ATM in stimulating telomere elongation parallels its role in DNA damage. When DNA breaks are encountered, ATM phosphorylates specific mediator proteins such as Chk2 and p53 that arrest the cell cycle and also activate DNA repair pathways. If there is extensive DNA damage that cannot be repaired, cells undergo either apoptosis or cellular senescence. The role of ATM in signaling telomere dysfunction is well established. Here ATM signaling is linked to telomere elongation; it is proposed that telomere elongation is a form of ongoing repair that prevents telomeres from becoming critically short.

[0130] The decrease in telomere length when ATM was inhibited was not due to a decrease in telomerase enzyme activity. The results herein are consistent with results in S. cerevisiae where the loss of Tel1^ATM causes telomere shortening, but telomerase enzyme activity was not affected. Elegant work in S. pombe has shown that phosphorylation of the telomere binding protein Ccl4 by the ATM and ATR kinases allows Est1 to recruit telomerase to the telomere. While specific ATM substrates that affect telomere length in S. cerevisiae and mammalian cells are not yet fully known, ATM phosphorylates TRF1, which mediates telomere elongation, and other substrates may also play a role.

[0131] ATR May Compensate for Loss of ATM

[0132] ATM and ATR may have partially redundant functions in telomere length maintenance in mammals as they do in yeast. In S. cerevisiae, Mec1^ATM plays a minor, yet critical role in telomere maintenance. Cells lacking Tel1^ATM are completely defective in telomere extension in a de novo addition assay, yet bulk telomere lengths of tel1^ATM cells are not as short as tel1^ATM in AT^R cells. Interestingly, in S. pombe, the role of the two genes seems to be reversed. The rad^ATM mutant cells have much shorter telomere lengths compared to rad^ATM mutants, although like in S. cerevisiae, the double mutants have even shorter telomeres.

[0133] Previous work in mice showed that ATM is not required for rescue of the shortest telomeres in an intergenerational cross. When ATM^{+/--} mice were crossed to ATM^{+/+} mTR^{--} G5 late generation mice with short telomeres, the F1 progeny showed rescue of signal free ends in both ATM^{+/+} mTR^{--} and ATM^{+/--} mTR^{--} genotypes. It was concluded in that study that ATM is not essential for elongation of the shortest telomeres. Current work indicates ATM does play an important role; however, ATR may still play some role in telomere elongation. Perhaps in the earlier study the rescue of short ends was due to ATR compensating for the loss of ATM during the many cell divisions that occurred from one generation to the next in this animal model. The role of the ATR kinase on telomere length in mice in vivo has not been examined, as ATR null mice are not viable.

[0134] Activation of ATM Leads to Telomere Elongation

[0135] Understanding the pathways that regulate telomere elongation will allow future pharmacological manipulation of telomere length. Here it was shown that one method that activates ATM, PARP inhibition, can lead to increased telomere length. The PARP inhibitor Olaparib is FDA approved for the treatment of ovarian cancer. PARP inhibition blocks the repair of double stranded DNA breaks, and in cells that are already deficient for BRCA1/2, PARP inhibition leads to synthetic lethality. The mechanism for increased ATM activity with Olaparib treatment might be by increasing double strand DNA breaks in cells and thus sending increased DNA damage signal, or might operate through some other mechanism.

[0136] The effects of PARP1 inhibitors may have different consequences in mice and humans. In humans, tankyrase 1 and 2, members of PARP family, positively affect telomere length through the ADP ribosylation of TRF1. However, the sites for ADP ribosylation are not conserved in mouse tankyrase. In vitro assays indicate that PARP1 inhibitors, including Olaparib, are highly specific to PARP1-4 and have little effect on tankyrases. However, it will be important to verify whether these drugs affect tankyrase activity to understand their potential effects on telomere length regulation in humans.

[0137] The PARP activation of ATM shows that in principle one can pharmacologically manipulate telomere length. As the many nodes in the ATM pathway are probed for their effect on telomere length more deeply, development of more sophisticated methods to modulate telomere length as possible treatments of disease is possible. Ultimately, finding a safe way to elongate telomeres may benefit individuals with short telomere syndromes.

[0138] Materials and Methods

[0139] The following experimental procedures and protocols were utilized.

[0140] Plasmid Construction

[0141] Plasmid construction strategies are described as follows. Primers used are listed in Tables 1 and 2 below.

[0142] Chr4 Targeting Construct (pLSL25)

[0143] 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 JHUB821 plasmid (described in Morrish and Greider, 2009, PLoS Genet 5, e1000357) using primers pBlueSK_F and JHUB821 SàiJ_R. HSV-tk was PCR amplified from plasmid pGAP-HSV– tk (pTGB008, Adogene #24703) using primers HSVtK_F and HSVtK_R. All fragment sequences were verified and cloned in pMSCV-HYG vector (pLSL25). pLSL25 construct was linearized with XhoI and NotI to yield a ~16 kb fragment, which was gel purified prior to transformation.

[0144] mTR/EGFP Retroviral Construct (pLSL8)

[0145] To generate a mTR/conditional cell line, mTR driven by its endogenous promoter was amplified from pMSCV-mTR-HYG plasmid using primers HpaIFRT_ mTR_F and mTR_EcoR1_R. EGFP with FRT site was amplified from plasmid pCDNA5/FRT/TO (Invitrogen) using primers EcoR1_EGFP_F and Chl1_FRT_EGFP_R. These two fragments were cloned in a retroviral vector pMSCV– HYG to generate pLSL8.

[0146] Dox-Inducible HA-I-Scel Construct (pLSL39)

[0147] To generate a Dox-inducible HA-I-Scel1 expression system, a lentiviral construct containing HA-I-Scel1 driven by a tetracycline-inducible promoter (pSL39) was generated by assembling four fragments using the Gibson assembly cloning kit (NEB).

[0148] The selection marker GFP from the original Lentitet-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-Sce1 fragment amplified from pCBASce1 (Addgene #26477) using primers Isce1_F1 and Isce1_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 AgeI and BsrGI. PCR products as well as the final constructs were all sequence verified.

<table>
<thead>
<tr>
<th>TABLE 1-continued</th>
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<td>Oligonucleotide list, related to FIG. 1.</td>
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### Quantitative RT-PCR

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<td>HPRT_R</td>
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### TRAP

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<td>reverse primer</td>
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<td>K1 primer</td>
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<td>TSK primer</td>
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### HYG probe

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### TABLE 2

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<th>Modified STELA for chr4</th>
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<td>Oligonucleotide list, related to FIG. 1.</td>
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<td>Telosterone 2</td>
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<td>Telosterone 6</td>
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<td>Isocerette</td>
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TABLE 2-continued

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<td>Telorete B 4</td>
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<tr>
<td>Telorete E 3</td>
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[0149] Generation of SL13 Cell Line

[0150] To generate the telomerase-conditional cell line, mTR^- skin fibroblast cell line from CAST/EiJ mice (Morris and Greider, 2009, PLoS Genet 5, e1000357) were transduced with a retrovirus (pSIL8) containing mTR and the green fluorescence protein (GFP) that can be removed by FLP/FRT recombination and flow sorted for GFP-positive fluorescence (data not shown). In the chromosomally stable GFP-positive cells, chromosome 4 (chr4) was modified to generate an internal 480 bp telomere ‘seed’ sequence followed by a unique I-SceI endonuclease cut site (FIG. 5A). The length of seed sequence was based on an early study showing that 400 bp of telomere repeats can act as a functional telomere. Briefly, cells were transduced with linearized chr4 targeting construct (pSIL25) using XtremeGene 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 HYGRGVR CR 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. Two independent HYGR clones were identified, clone termed 1 L and 1 M, each containing a single Chr4 allele that was correctly modified (data not shown). 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 (pSIL39) was stably integrated, and later RFP-positive cells were flow sorted in 96-well plate as single clones. To induce I-SceI expression, doxycycline at final concentration of 2 μg/mL was added to cells. Typically, cells were collected post 48 hr of doxycycline treatment. A clone (SL13) was identified that expressed HA-I-SceI only in the presence of doxycycline by western blot (data not shown). To compare telomere addition in cells with and without telomerase, SL13 cells were transfected with a construct expressing the flp recombinase (pPGKFLPbPA, Addgene #13793). Approximately 10 days later, 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 presence of mTR was confirmed in GFP-positive cells, but absent in GFP-negative cells (FIG. 5B). These populations are referred to as mTR+ or mTR-, respectively.

[0151] Cell Culture and Treatments

[0152] Cell lines 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). Cells were treated with ATM inhibitor KU55933 (R&D Systems, #3544), ATR inhibitor VE821 (Selleckchem, #S8007) or PARP1 inhibitor Olaparib (Selleckchem, #1060) at indicated concentrations or DMSO as a control. For the ADDIT assay, SL.13 cells were treated with final concentration 2 μg/ml of doxycycline for typically 48 hr or as indicated.

[0153] Modified Single Telomere Length Analysis (STELA) for Mouse Chr4

[0154] The original STELA protocol used for human cells (Baer et al., 2004, *Hum Mol Genet* 13, 1515-1524) was modified to measure telomere lengths on the cut end of chr4 in SL.13 cells. Briefly, genomic DNA was extracted using Puregene Core Kit ATM (Qingren). 4 μg of genomic DNA was digested with Sph1 (NEB) and later diluted to 10 ng/μl in water. For the in vitro IScerette sample, genomic DNA was digested with Sphl and I-SceI (NEB) prior to ligation. The ligation was carried out at 35°C for at least 12 hr in a volume of 10 μl containing 10 ng of digested genomic DNA, 0.9 μM of telero tinker or IScerette linker and 0.5 μL of T4 DNA ligase (NEB) in 1×T4 ligation buffer. Multiple PCRs (typically 24 or 32 reactions per sample) were carried out for each sample in 25 μl containing 1 ng of ligated DNA, 0.2 μM HYG-specific and teltail primers, 1× Fail Safe PCR™ buffer H (Epicentre FSP9511), 1 U of Fail Safe Enzyme Mix (Epicentre FSP9100). The PCR reactions were pooled for each sample and purified using magnetic beads (Agencourt AMPure™ XP, Beckman Coulter). An equal fraction from each sample was analyzed by Southern blot using a HYG probe (as described below).

[0155] PacBio® Sequence Reads Analysis

[0156] A pipeline in R (see Fig. 6) was created that analyzes PacBio® sequencing data generated from modified STELA. 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-Sce1 site}÷{number of total CCS reads}). Unpaired t-test generated the one- or two-tailed P values. All PacBio® sequence data are available in the NCBI SRA database under the accession number SRP059426.

[0157] siRNA-Mediated Knockdown of ATM and TERT

[0158] ON-TARGET siRNA SMART™ pools from GE Healthcare were used; mouse TERT (L-08320-01-0005), mouse ATM (L-0920). SL.13 cells were transfected using Pepmap™ protocol (SignaGen Lab). The final concentration of siRNAs was 5-100 nM for each transfection. The efficiency of knockdown for each siRNA was assessed by immunoblotting or quantitative RT-PCR.

[0159] Direct Telomerase Activity Assay

[0160] Telomerase activity was assayed in whole cell lysates with a modified protocol (Nandakumar et al., 2012, *Nature* 492, 285-289). 293TREx-0611 cells overexpressing TPPI, POT1, and hTERT were seeded (3×105 cells) per well in a 96-well plate containing 0.5% FBS. The next day, cells were transfected with U1-hTERT using Lipofectamine™ 2000 and incubated overnight. Then, they were treated with 30 μM KU55933, 5 μM Olaparib, or DMSO for two hours before harvesting in 100 μl 1×CHAPS. Cells were lysed in ice for 30 minutes, vortexing occasionally, and cleared by spin (8000 rpm, 20 min, 4°C). For negative controls, 25 μl cleared lysate was incubated at 65°C with 1 μl RNaseA (1 mg/ml) for 10 minutes. For direct telomerase assays, lysates were incubated with primer aS (Wang et al., 2007) in 1× telomerase buffer (50 mM Tris-Cl, 30 mM KCl, 1 mM MgCl2, 1 mM spermidine, 0.5 mM dITP, 0.5 mM dATP, 2.92 μM dGTP, and 0.33 μM α32P-dGTP at 30°C). Reactions were stopped at 10 or 15 minutes with 20 mM EDTA, 10 mM Tris spiked with 500 cpm end-labeled 18mer. Telomerase products were purified by phenol chloroform extraction, ethanol precipitated, washed with 70% ethanol, and resuspended in 5 μl water and 5 μl 2× formamide loading dye. Products were denatured at 100°C, separated on a sequencing gel (10% acrylamide, 7M urea 1×TBE), at 90 W for 1.5 hour. The gel was dried, exposed to a phosphor screen overnight, and imaged using the STORM phosphimager.

[0161] Quantitative RT-PCR

[0162] To measure mTR and mTERT levels, RNA from different samples were 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 1×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 HPRT. Primers used were the following: RT_mTR_F, RT_mTR_R, RT_mTERT_F, RT_mTERT_R, HPRT_F and HPRT_R. Triplicates were run for each sample and the normalized average was reported.

[0163] Telomerase Repeat Addition Polymerase (TRAP)

[0164] TRAP was performed as described previously (Kim et al., 1994, *Science* 266, 2011-2014). Briefly, cells were spun down and washed, and cell extracts were generated by resuspending the pellets in 1×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 p-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 1×TRAP reaction buffer (200 mM Tris-HCl pH 8.3, 15 mM MgCl2, 630 mM KCl, 0.5% Tween20, 10 mM EGTA, pH 8.0), 0.1 mM dNTPs, and 34 μl 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, 1.1 of a master mix containing 1×laq buffer (Denville), 0.1 mM dNTPs (TaKaRa), 2 U Taq polymerase (Denville), 20 μM end-labeled TS primer (4 μCi γ-32P-ATP, 1×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 K1, 10-12 μM internal control template TSK1). A 2-step PCR amplification protocol was used (94°C ×10 min; 30 cycles of 94°C ×30 s, 59°C ×30 sec). PCR products were mixed with 1×GelPilot™ DNA loading dye (QIAGEN) and separated on a 10% non-denaturing polyacrylamide gel (1×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 200 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).

[0165] Antibodies and Western Blotting

[0166] To detect phospho-proteins, 1x lysis buffer made of final concentration of 1xRIPA™ (Cell Signaling, #98016S), 1x protease inhibitor cocktail, 1x PhosSTOP™ (Roche, 4906845001) was added directly to cells on the dish after washing with 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), membranes were incubated at 4°C overnight or 1 hr at RT with the following primary antibodies: anti-phospho-Kap1 (Bethyl Lab, #A300-767A); anti-phospho-CHK1 (Cell Signaling, #2348S); anti-phospho-ATM (Millipore, #05-740); anti-HA (Santa Cruz, #sc-7392); anti-Actin (Santa Cruz, #sc-1616); anti-PAR (Trevenen, #4335-MC-100). The following day, blots are washed with 1xPBS- T for 3x15 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 4th 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.

[0167] Southern Blot Analysis

[0168] To examine the in vivo chr4 cleavage, genomic DNA of SL13 treated with dioxycyline for various time points was extracted using the Puregene™ Core Kit A (Qiagen) and digested with NcoI restriction enzyme (NEB). For the in vitro control, genomic DNA without dioxycyline was digested with the purified I-Sce1 endonuclease (NEB) and NcoI. NcoI-digested genomic DNA was resolved by 1% Tris-acetate-EDTA (TAE) agarose gel electrophoresis. Following denaturization (0.5 M NaOH/5 M NaCl) and neutralization (1 M NaOH/0.5 M Tris-HCl pH 7.4), the DNA was transferred in 20x SSC to a Nylon Membrane (Amer sham Hybond N+) by weighting method overnight and cross-linked with UV Stratallinker™ (Stragene). Pre-hybridization was done at 42°C for 2 hr in prehybridization buffer (50% Formamide/6xSSC/1% SDS/5xDenhardt/ sperm DNA) freshly made. A radioactive HYG probe acquired from using the following primers, HYGprobe_F and HYGprobe_R, was made by random-prime labeling using Prime-It II™ (Stratagene) with a slight modification. Briefly, 25 ng of HYG probe was labeled using 33 µM of dATP, dTTP, 50 µCi of a-32P dCTP (3000 Ci/mmol) and 50 µCi a-32P dGTP (3000 Ci/mmol). Unincorporated nucleotides were removed using a G50 column (GE Healthcare). Labeled probe was counted and 1000 counts/ml (probe) or 1000 counts/ml (2-log 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 3x15 minutes each in 6xSSC and 1% SDS at 65°C, and 3x15 minutes each in 1xSSC and 1% SDS at 65°C, then exposed to a phosphorimager screen before scanning on a STORM phosphorimager. To analyze the chr4 STELA PCR products, equal amounts from each PCR sample were resolved by 1% TAE agarose gel electrophoresis and detected by Southern hybridization with a random primed a-32P-labeled HYG probe. The blot was hybridized and washed at 55°C, as described. Southern blots were performed as previously described (Morrish and Greider, 2009). Image processing software, Image Quant™ 1D v8.1 (GE Healthcare Life Sciences), was used to generate densitometry of Southern blot lanes by graphing pixel counts versus DNA migration distance.

Example 2
Role of Cyclin-Dependent Kinases in Telomere Length Regulation

[0169] 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.

[0170] 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). Cdkks 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 Cdkks 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.

[0171] This example illustrates that telomerase-mediated telomere addition is regulated in a cell-cycle-dependent manner also in mammalian cells, and that mammalian Cdkks, including Cdk1, are required for telomere elongation in vivo.

[0172] Results

[0173] De Novo Telomere Addition Occurs in G2/M Phase

[0174] To better understand how mammalian telomere length is regulated, the short-term ADD1 assay was used to visualize telomere addition in vivo. Briefly, an inducible I-Sce1 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 (Fig. 9A) 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 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 (Fig. 9C). No significant difference of de novo elongation was observed between cycling cells or cells arrested in G2/M phase. To ensure I-SceI induction is consistent across different cell cycle phases, I-SceI expression was measured in cells arrested at different phases and confirmed no significant difference (Fig. 9B). These results suggest that the requirements for telomere elongation are present in G2/M phase in mammalian cells similar to yeast.

**[0175]** Telomere Addition is Dependent on Cyclin-Dependent Kinases

**[0176]** Cdk1 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 Cdns and performed the short-term ADDIT assay. Cells were treated either with or without flavopiridol, which inhibits several members of the Cdk family, including Cdk1, 2, 4, 6, 7. Southern blot analysis of STELA PCR products from flavopiridol-treated samples suggested minimal elongation beyond the chr4 I-SceI-induced cut base line. PacBio® sequences from the STELA products indeed showed treatment with flavopiridol significantly reduced de novo telomere addition (Fig. 10), from 24% to less than 2%, suggesting Cdk activities are required for telomere elongation.

**[0177]** Cdk1 is Required for De Novo Telomere Addition

**[0178]** To determine which of the several Cdns in mammalian cells, is responsible for regulating telomere length, the effect of knocking down Cdk1 was first tested since the Cdk1 homologue in yeast has been shown to be required for telomere elongation. Cells were treated with siRNA against Cdk1 and confirmed more than 50% knockdown by measuring the phosphorylation level of Cdk1-Y15. ADDIT assay was performed in cells treated with or without siCdk1. PacBio® sequence analysis of the STELA PCR products indicated de novo telomere addition was completely blocked in cells with Cdk1 knockdown (Figs. 11A and 11B). These observations suggest the requirement of Cdk1 in telomere elongation is conserved from yeast to mammalian cells.

**FIGURE LEGENDS**

**[0179]** Fig. 9 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-Sce1 normalized to HPRT 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-Sce1 site)/(number of total CCS reads)}.

**[0180]** Fig. 10 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.

**[0181]** Fig. 11 shows Cdk1 is required for de novo telomere addition. Phosphorylation levels of Cdk1 were analyzed by immunoblotting cells treated with 10 μM RO3306 or different final concentration of siCdk1, 5 nM, 10 nM or 100 nM. Numbers at bottom of western indicate the relative expression levels of phosphorylated Cdk1-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 siCdk1 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.

**[0182]** Discussion

**[0183]** 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 Cdns, especially Cdk1, are required for telomere addition. Since Cdk1 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 Cdk1 in telomere addition.

**[0184]** Telomere Elongation is Cell Cycle Regulated

**[0185]** 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 α-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 telomerase addition was observed in SL.13 cells held in G2/M phase but not in G1, 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.

[0186] Cdk5 are excellent candidates for regulating cell cycle-dependent telomere elongation. The data indicate Cdk1 has an essential role in telomere elongation. A previous study reported that TRF2 (telomeric-repeat-binding factor 2) is a Cdk1 and Cdk2 substrate by high throughput in vitro kinase screening. Although further validation is necessary, Cdk1 and/or Cdk2 may regulate telomere length by interacting and phosphorylating TRF2 in vivo. It is possible other Cdk5 also regulate telomere length homeostasis.

[0187] 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 ADHIT assay.

[0188] The Essential Role of Cdk6 in Telomere Addition

[0189] Previous studies have shown that many of the key regulators required for telomere length maintenance are 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 Mre11 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 3' strand resection.

[0190] In addition, Cdk1 appears to be involved in regulating telomerase recruitment. In S. cerevisiae, phosphorylation of the single-strand DNA binding protein Cdc13 (T308) by Cdk1 promotes the interaction between Cdc13 and one of the telomerase components, Est1, resulting in telomerase recruitment and telomere elongation. A recent study showed S. cerevisiae Cdk1 also phosphorylates Stn1, one of the other components of CST complex (Cdc13-STN1-TEI1), 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 Cdc13. Recent studies purpose human CST (CTC1-STN1-TEI1) complex inhibits telomere elongation by competing with POT1-TTP1 and promotes telomere replication at C-strand by stimulating DNA polymerase α-primase. The results from ADHIT assay demonstrated that Cdk1 activity is also required for de novo telomere addition in mammalian cells (FIGS. 1H and 1C), indicating the conserved role of Cdk1 in telomere length regulation in higher eukaryotes. A previous study showed phosphorylation of TP1 (S111) 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 Cdk1 regulates telomere elongation, and further test whether Cdk1-mediated phosphorylation(s) of CST complex and/or shelterin complex components, such as TP1, exist.

[0191] Materials and Methods

[0192] Cell Culture and Treatments

[0193] SL.13 cells were grown in DMEM (Gibco) supplemented with 1% Penicillin/Streptomycin/Blountime and 10% Tet system approved FBS (Clontech, #63107). Final concentration of 2 μg/ml of doxycycline was added in the media to induce I-Soec1 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 μM Thymidin (Sigma, #T-1895).

[0194] Cell Cycle Profile Analysis

[0195] Cell cycle phase was accessed by DNA content using propidium iodide (PI) staining and flow cytometry analysis. Briefly, cell pellets were washed with 1×PBS at 500g for 5 minutes and resuspended in 500 ml of 1×PBS, 4.5 ml of ice cold 70% ethanol was added drop by drop, and incubated overnight at 4°C. Samples were washed with 5 ml 1×PBS and incubated with 500 μl of PI-Trition resuspension buffer at room temperature for 30 minutes protected from light. PI-Trition resuspension buffer was made of 0.1% Triton™ X-100, 200 μg/ml RNase A, 20 μg/ml PI (Invitrogen, #P3566) in 1×PBS. PI-stained samples were transferred to strainer tubes (BD Falcon) and run on FACSCalibur™ flow cytomtery (BD Biosciences). Cell cycle profiles were analyzed utilizing the Dean-Jet-FOX™ model in FlowJo™ software (FlowJo™).

[0196] Quantitative RT-PCR

[0197] To measure HA-tagged I-Soec1 expression levels, quantitative RT-PCR was performed as described in 2.4.5. Primers used are shown the following: HA-Isec1-FOR, 5'-5CTCTGACTATGCGGGTAGA-3' (SEQ ID NO: 100); Isec1-REV2, 5'-5CTTTCATACGGAAAGCAGAT-3' (SEQ ID NO: 101); HPRT_E, 5'-5TGATCATGTACCGGAGGCCA-3' (SEQ ID NO: 102); HPRT_R, 5'-5TCCAGAAGGCTCCTTTCACCA-3' (SEQ ID NO: 103).

[0198] Southern Blot Analysis

[0199] To examine the in vivo chr4 cleavage and de novo telomere addition, genomic DNA extracted from SL.13 cells arrested at different cell phases and treated with doxycycline for various time points were digested with NeoI restriction enzyme (NEB) and further analyzed by Southern blot as described previously in Example 1 with a random primed a-32p-labeled HYG probe.

[0200] siRNA-Mediated Knockdown of Cdk1

[0201] ON-TARGET™ siRNA SMART pools from GE Healthcare were used: mouse Cdk1 (L-058633-00-0005). SL.13 cells were subject to siRNA transfection using Pemate™ protocol (SignaGen Laboratories, #SL.100566) at a final concentration of 5 nM, 10 nM or 100 nM. The efficiency of knockdown was assessed by immunoblotting.

[0202] Western Blot Analysis and Antibodies

[0203] Cell lysates were made and processed as described in 3.4.3. Membranes were incubated at 4°C overnight with primary antibodies: anti-phospho-Cdk1 (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).

[0204] Modified Single Telomere Length Analysis (STELA) for Chr4

[0205] 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 Example 1.

[0206] PacBio® Sequence Analysis

[0207] PacBio® sequence reads were analyzed as described in Example 1.

[0208] 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.

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1. A method of increasing telomere length comprising contacting a cell with an agent that activates the ataxia telangiectasia mutated (ATM) kinase pathway or a cyclin dependent kinase pathway, thereby elongating telomeres in the cell.

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

3. The method of claim 2, wherein the nucleic acid molecule is an siRNA, shRNA, miRNA, Locked Nucleic Acid (LNA), antisense oligonucleotide, a chemically modified oligonucleotide, or a combination thereof.

4. The method of claim 1, wherein the agent increases expression or activity of ATM kinase.

5. The method of claim 1, wherein the agent increases expression or activity of cyclin dependent kinase 1 (Cdk1).

6. The method of claim 4, wherein the agent is an inhibitor of poly (ADP-ribose) polymerase 1 (PARP1).

7. The method of claim 6, wherein the PARP1 inhibitor is Olaparib (AZD2281), Talazoparib (BMN-673), Rucaparib (AG014699 or PF-01367338), Veliparib (ABT-888), CEP 9722, Niraparib (MK-4827), BGB-290, E7016, E7449 or INO-1001.

8. The method of claim 1, wherein the cell is a mammalian cell.

9. The method of claim 8, wherein the cell is a human or murine cell.

10. The method of claim 1, wherein telomere length is increased at least 50% as compared to telomere length prior to contacting with the agent.

11. The method of claim 1, further comprising measuring telomere length.

12. The method of claim 11, wherein telomere length is measured prior to, and after contacting with the agent.

13. The method of claim 11, wherein measuring comprises a technique that including PCR.

14. The method of claim 13, wherein the technique is a modified single telomere length analysis (STELA).

15. The method of claim 13, further comprising nucleic acid sequencing.

16. A method of treating a telomere syndrome in a subject, wherein the syndrome is characterized by shortened telomere length, the method comprising administering to the subject a poly (ADP-ribose) polymerase 1 (PARP1) inhibitor, an activator of cyclin dependent kinase 1 (Cdk1), or a combination thereof, wherein administration of the agent...
leads to progressive telomere lengthening, thereby treating the telomere syndrome in the subject.

17. The method of claim 16, wherein the syndrome is selected from dyskeratosis congenita, bone marrow failure, aplastic anemia, and pulmonary fibrosis.

18-19. (canceled)

20. The method of claim 16, wherein the PARP1 inhibitor is Olaparib (AZD2281), Talazoparib (BMN-673), Rucaparib (AG014699 or PF-01367338), Veliparib (ABT-888), CEP 9722, Niraparib (MK 4827), BGB-290, E7016, E7449 or INO-1001.

21-27. (canceled)

28. A method of treating cancer in a subject by interfering with lengthening of telomeres in cancer cells, comprising administering to the cells an effective amount of an inhibitor of a regulator of telomere lengthening, wherein the administration of the inhibitor leads to progressive telomere shortening in the cancer cell, thereby treating cancer in the subject.

29. The method of claim 28, wherein said cancer is selected from the group consisting of stomach cancer, osteosarcoma, lung cancer, pancreatic cancer, adrenocortical carcinoma, melanoma, breast cancer, ovarian cancer, cervical cancer, skin cancer, connective tissue cancer, uterine cancer, anogenital cancer, central nervous system cancers, retinal cancer, blood and lymphoid cancers, kidney cancer, bladder cancer, colon cancer and prostate cancer.

30-37. (canceled)

* * * * *