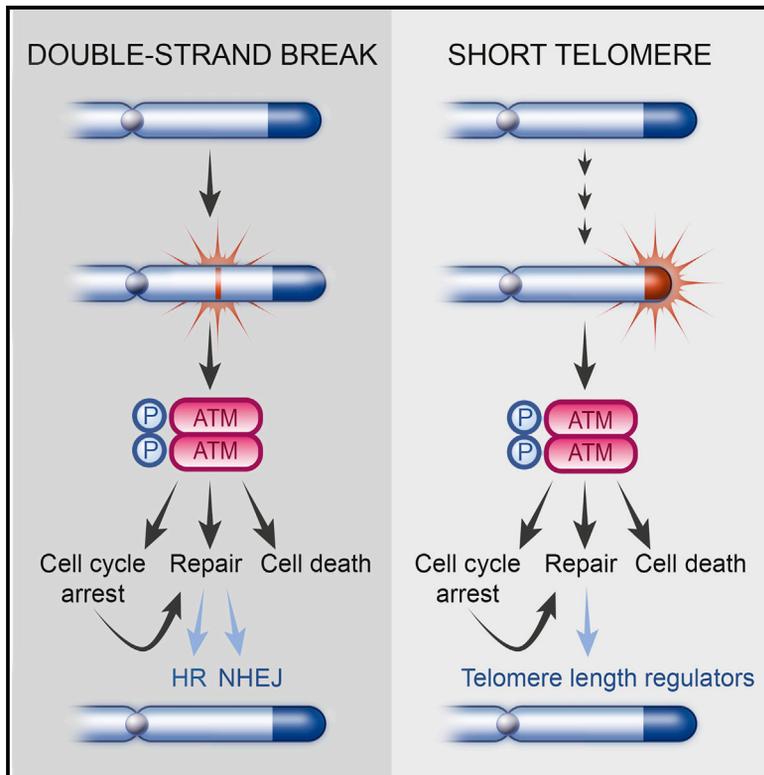


ATM Kinase Is Required for Telomere Elongation in Mouse and Human Cells

Graphical Abstract



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In Brief

Lee et al. develop an assay to identify telomere length regulators and show ATM inhibition shortens telomeres, whereas ATM activation elongates telomeres. The ADDIT assay will be a powerful tool to identify regulators of telomere length.

Highlights

- ADDIT assay measures telomerase-mediated addition at a single telomere
- De novo telomere addition in mouse cells requires ATM kinase
- ATM inhibition blocks bulk telomere elongation in both mouse and human cells
- Excess activation of ATM by inhibition of PARP1 increases telomere addition

ATM Kinase Is Required for Telomere Elongation in Mouse and Human Cells

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SUMMARY

Short telomeres induce a DNA damage response, senescence, and apoptosis, thus maintaining telomere length equilibrium is essential for cell viability. Telomerase addition of telomere repeats is tightly regulated in cells. To probe pathways that regulate telomere addition, we developed the ADDIT assay to measure new telomere addition at a single telomere *in vivo*. Sequence analysis showed telomerase-specific addition of repeats onto a new telomere occurred in just 48 hr. Using the ADDIT assay, we found 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. Understanding this role of ATM may yield new areas for possible therapeutic intervention in telomere-mediated disease.

INTRODUCTION

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 (Kastan and Lim, 2000; Paull, 2015). Ataxia telangiectasia (AT) patients, who lack ATM function, have immune system defects and neurological impairment and are cancer prone and radiosensitive (Shiloh and Ziv, 2013). A role for ATM in telomere length maintenance was suggested when the ATM gene was cloned (Savitsky et al., 1995) and shown to be the homolog of the yeast Tel1 gene (Greenwell et al., 1995). In yeast, loss of Tel1^{ATM} 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 (Sprung et al., 1997). However, other reports suggested cells might have shorter telomeres in the absence of ATM (Metcalfe et al., 1996; Xia et al., 1996; Vaziri et al., 1997; Hande et al., 2001; Tchirkov and Lansdorp, 2003). Modification of human TRF1 protein by both ATM and tankyrase regulates binding of TRF1 to the telomere, yet this regulation of TRF1 is not conserved in mice (Hsiao et al., 2006; Wu et al., 2007; Chiang et al., 2008).

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 (Greider and Blackburn, 1985). The delicate balance of shortening and lengthening is regulated by an intricate series of feedback mechanisms that establish a dynamic telomere length equilibrium (Smogorzewska and de Lange, 2004). In humans, syndromes of telomere shortening cause age-related degenerative diseases including dyskeratosis congenita, pulmonary fibrosis, aplastic anemia, and others (Armanios and Blackburn, 2012). Elucidating the molecular interactions that regulate telomere elongation is essential to understand telomere function and how it is disrupted in disease.

At the cellular level, loss of tissue renewal is caused by short telomeres that activate a DNA damage response, inducing apoptosis or senescence (Lee et al., 1998; Hao et al., 2005). Critically short telomeres activate the ATM and ATR kinase-dependent pathways in primary human cells, leading to senescence (d'Adda di Fagagna et al., 2001). In addition, induction of telomere dysfunction through the removal of shelterin components also activates ATM or ATR-dependent signaling and cell-cycle arrest (Palm and de Lange, 2008). Cancer cells avoid cell death through increased telomerase expression or other mechanisms that maintain telomere length (Greider, 1999; Artandi and DePinho, 2010).

Although, 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^{ATM} and Mec1^{ATR} play partially redundant roles at telomeres. The loss of Tel1^{ATM} generates short, stable telomeres, while loss of Mec1^{ATR} alone has no effect. However, the loss of both Tel1^{ATM} and Mec1^{ATR} leads to further shortening than in Tel1^{ATM} alone (Ritchie et al., 1999). This implies that Mec1^{ATR} may partially compensate for the loss of Tel1^{ATM} in telomere maintenance, as discussed in more detail below.

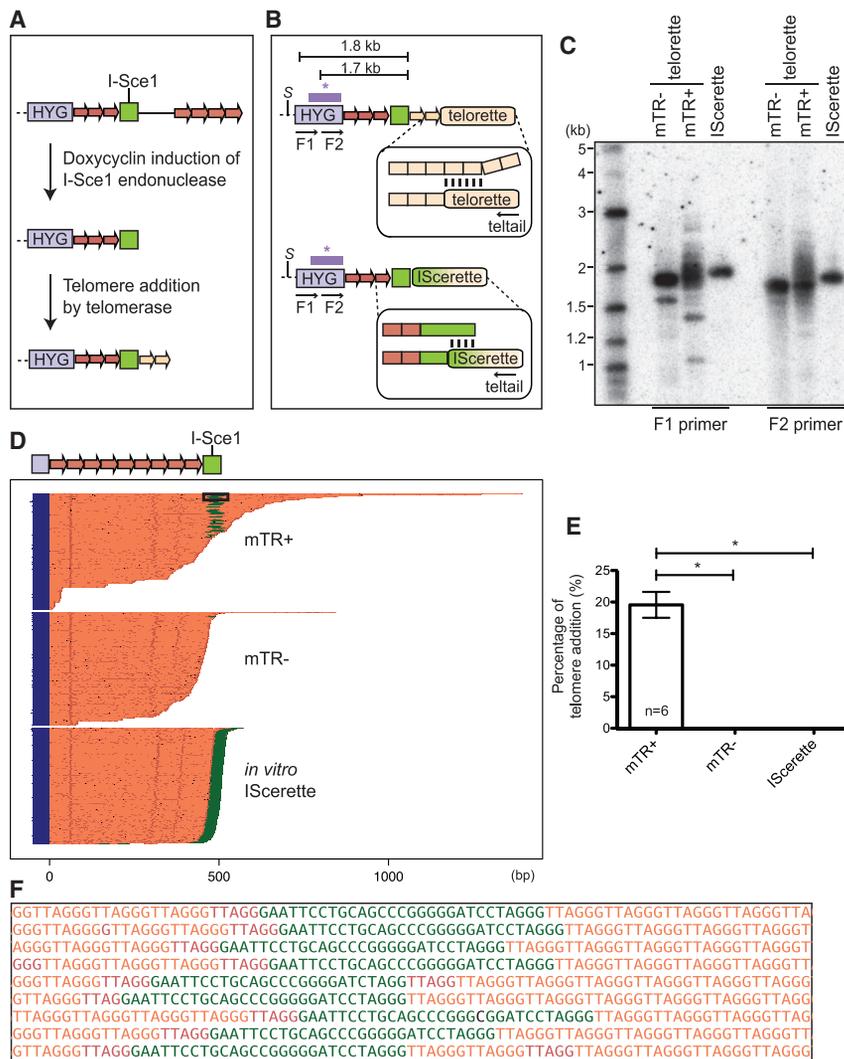


Figure 1. De Novo Telomere Addition Occurs Only in Telomerase-Positive Cells

(A) Schematic of the ADDIT assay. I-Sce1 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-Sce1 end. Telomeres were PCR amplified with a HYG-specific forward primer, either F1 or F2, and a reverse primer, teltail. S, Sph1.

(C) STELA PCR products, using either F1 or F2 primer, were analyzed by Southern hybridization using HYG probe (purple bar with asterisk shown in B).

(D) 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-Sce1 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.

(E) 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).

(F) The sequences of PacBio CCS reads boxed in (D) are shown.

See also Figures S1, S2, and S3.

To examine the role of ATM in telomere maintenance, we developed an assay that we term ADDIT (addition of de novo initiated telomeres), which measures telomere addition at a single chromosome end. Using this assay, we demonstrate 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.

RESULTS

Addition of De Novo Initiated Telomeres: ADDIT Assay

To critically assess the role of ATM, as well as other potential telomere length regulators, we developed an assay that measures telomere repeat addition in cells over 48 hr. We engineered a CAST/EiJ mouse fibroblast cell line (SL13) containing a conditional mTR allele and a modified chromosome 4 (chr4) with an internal 480-bp telomere “seed” sequence followed by a unique I-Sce1 endonuclease cut site (Figure S1; see Experimental Procedures). The internal telomere seed sequence can be conditionally

exposed by I-Sce1 endonuclease cleavage (Figure 1A) and then be elongated by telomerase in a manner similar to de novo telomere addition shown to occur in yeast (Diede and Gottschling, 1999).

Expression of doxycycline-inducible HA-tagged I-Sce1 endonuclease in this SL13 cell line exposed the telomere seed and allowed telomere addition by telomerase (Figures 1C and S1C). Chromosome cutting occurred by 8 hr after doxycycline treatment (Figure S1D). As a control, we isolated genomic DNA and digested it in vitro with purified I-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 (Figure S1D).

To better detect the telomere elongation, we modified the single telomere length analysis (STELA) assay (Baird et al., 2004), to measure telomere length at the in vivo cut chr4. We ligated the linker, “telorette,” to the telomere and PCR amplified the telomere using the “teltail” primer and an internal primer in the hygromycin resistance (HYG) sequence on the engineered chromosome (Figure 1B). To determine the un-extended cut chromosome length in vitro, we designed a different linker, “IScerette,” which anneals to the 4-nt 3' overhang created by the I-Sce1 endonuclease (Figure 1B). PCR using the forward primers, F1 or F2, and the teltail generated products of predicted size on a Southern blot (Figure 1C).

To examine whether elongation was telomerase dependent, we deleted mTR by Flp recombinase to generate mTR⁻ cells (Figures S1E and S1F) and carried out the ADDIT assay. The STELA PCR products in the mTR⁻ cells treated with doxycycline were similar to and shorter than the control IScurette 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 IScurette products (Figure 1C), 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.

Sequence Analysis of the Telomere Addition Products

To further verify telomere addition had occurred, we sequenced the PCR products with Pacific Biosciences (PacBio) sequencing. PacBio produces long sequence reads (Loomis et al., 2013) that allowed us to determine telomere length. While sequencing errors, predominantly point insertions and deletions, occurred as expected (Carneiro et al., 2012), the telomere repeats were easily recognizable. To assure products were full length, we filtered the PacBio reads to examine only those that had the unique HYG sequence followed by the seed telomere repeat sequence and also had the teltail primer sequence (Figure S2). Individual reads are displayed as a horizontal line, where the wild-type TTAGGG repeats are colored orange and variant telomere repeats are colored in darker orange. The I-Sce1 site is shown in green (Figure 1D). We noted three regions of variant telomere repeats, evident as darker orange stripes in the aligned reads, that were present in the original SL13 clone and served as useful sequence reference points (Figure 1D). If there were degradation past these variants and then new TTAGGG repeat synthesis in vivo, these landmarks would be removed.

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 telomere sequences (Figures 1D and 1F). Telomerase will add telomere repeats onto primers (or sequences) that contain some non-telomeric sequence (Greider, 1991; Harrington and Greider, 1991; Morin, 1991; Kramer and Haber, 1993) 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. We defined telomerase addition 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.

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, we quantitated the percentage of reads that showed elongation past I-Sce1, which represents telomerase recruitment to the telomere. 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 (Figure 1E).

In an additional control, small interfering RNA (siRNA) against TERT also blocked repeat addition beyond the I-Sce1 site (Figure S3). As expected, sequence reads from the in vitro IScurette control sample showed no elongation (Figures 1D and 1E). The small changes in length and sequence in this sample likely represent the PacBio sequencing errors or slippage during PCR.

De Novo Telomere Addition onto I-Sce1 Site

We examined the sequence reads 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 (Autexier and Greider, 1995). For the mouse telomerase RNA, there is a 2-nt alignment region, while the human RNA contains 5 nt in the alignment region (Chen and Greider, 2003a, 2003b). Evaluation of the I-Sce1 cleavage site showed that it has sequence complementarity to the mTR primer-alignment region (Figure 2A).

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 (Figure 2B). In class 1, 205 of the 1,514 (13.5%) PacBio reads showed telomeric repeats directly added after the I-Sce1 3' overhang without any loss of nucleotides (Figure 2B). The most common class of telomere addition, class 3 (48.0%) had loss of 4 nt 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 (Autexier and Greider, 1995) and provides further evidence that telomere repeats are added by telomerase activity.

ATM Kinase Is Essential for Telomere Addition

To probe the role of ATM, we used the ADDIT assay in cells treated with the ATM-specific inhibitor KU55933 (Hickson et al., 2004) or with siRNA to ATM. To confirm the inhibition of ATM, we examined the phosphorylation level of the ATM substrate Kap1 and, as a control, an ATR 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 (Figures 3A and 3B). This indicated that both KU55933 and siATM specifically inhibited ATM signaling, without affecting the ATR pathway.

PacBio sequencing of the ADDIT assay products 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 (Figures 3C and 3D). Cells treated with the KU55933 had significantly fewer telomere elongation products compared to controls in replicated experiments

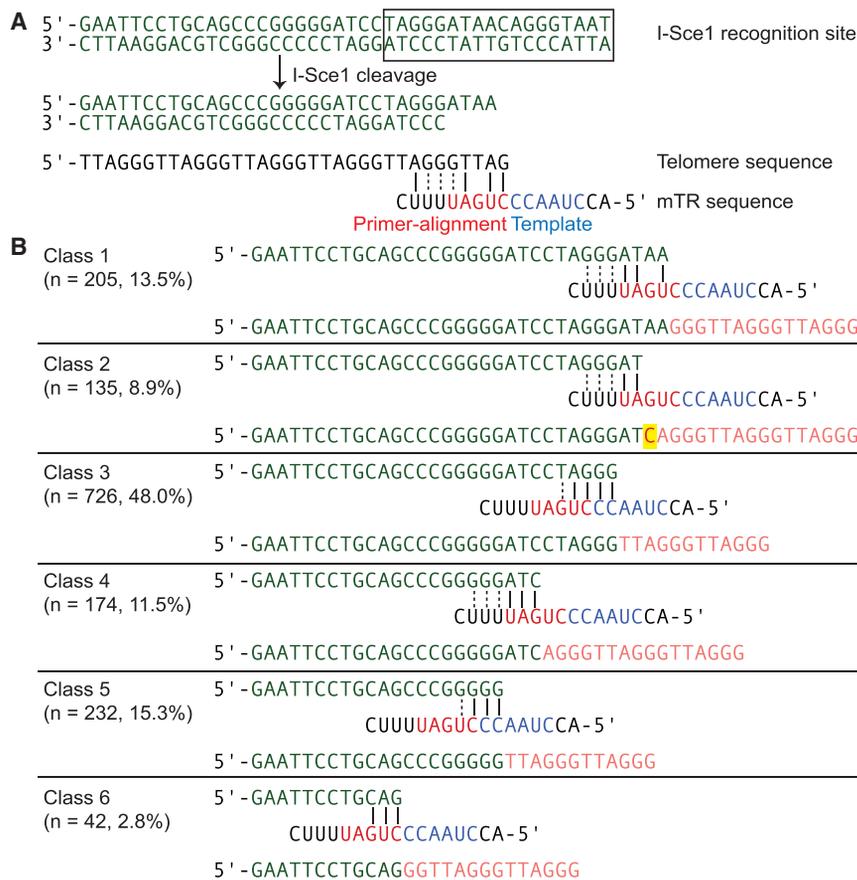


Figure 2. Classification of De Novo Telomere Addition

(A) The 42-nt unique sequence (green) located immediately after telomere seed includes the 18-nt I-Sce1 recognition site (black box). I-Sce1 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 1,514 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-Sce1 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 S1.

(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 (Ma and Greider, 2009) 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 (Barlow et al., 1996; Daniel et al., 2012; Yamamoto et al., 2012).

To determine whether ATM inhibition altered the mechanism of telomerase elongation, we examined the distribution of the six different classes of elongation products described in Figure 2. Although there was some variability in the distribution of products (Table S1), 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.

To further define the role of ATM in telomere elongation, we overexpressed telomerase. Overexpression of telomerase in immortal cultured cells results in excessive telomere elongation (Cristofari and Lingner, 2006). We treated SL13 cells with either KU55933 or the ATR inhibitor VE821 (Reaper et al., 2011) and transduced them with a lentivirus expressing both mTR and mTERT. Controls showed the specificity of VE821 (Figure S4A). 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 (Figure 4A). 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 (Figure 4A). 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.

Inhibition of ATM Kinase Shortens Telomeres in Human Cells

ATM has been proposed to play different roles in signaling telomere dysfunction in human and mouse cells (Smogorzewska and de Lange, 2002). 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 (Metcalf et al., 1996; Xia et al., 1996; Sprung et al., 1997; Vaziri et al., 1997; Hande et al., 2001; Tchirkov and Lansdorp, 2003), we wanted to revisit the role of ATM in human cells. KU55933 shortens bulk telomeres in HT1080 cells (Wu et al., 2007), and we also found telomere shortening in human HCT116 (Figure 4B). When the mouse cell line SL13 was grown in KU55933, telomere shortening also occurred, although at a somewhat slower rate (Figure 4C); however, this shortening was reproducible (Figures S4B and S4C).

Activation of ATM Kinase Pathway Elongates Telomeres

To further probe the role of ATM in telomere length, we sought to activate ATM in cultured cells. Cells treated with an inhibitor of poly (ADP-ribose) polymerase 1 (PARP1) were shown to activate ATM (Bryant and Helleday, 2006; Wahlberg et al., 2012). PARP is an essential enzyme involved in recognition and repair of DNA

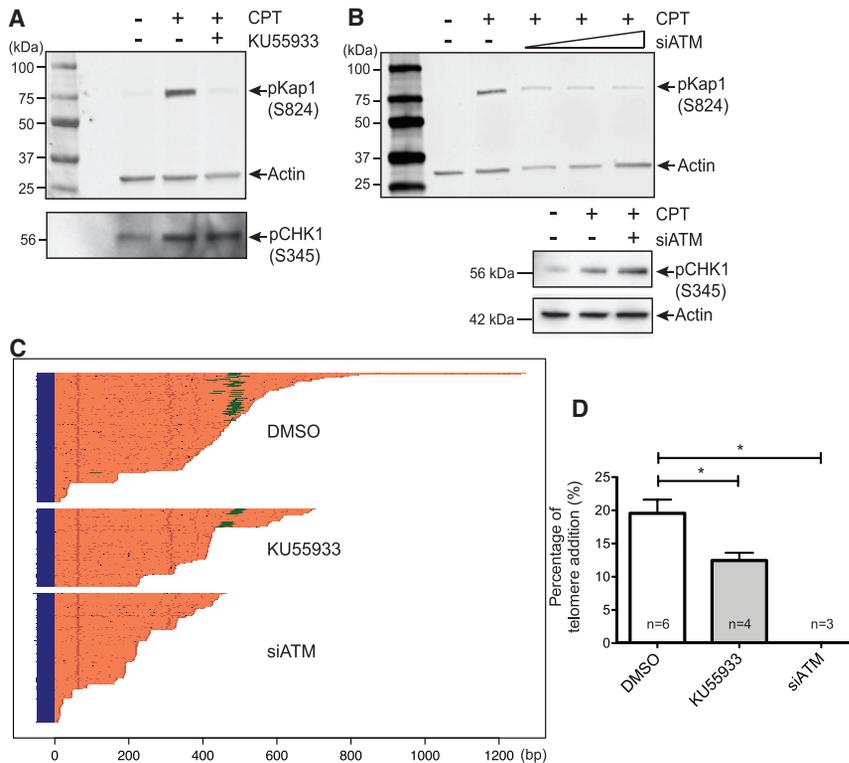


Figure 3. ATM Kinase Is Required for Telomere Addition

(A) Western blot analysis of SL13 cells pretreated with either 10 μ M KU55933 or DMSO and then exposed to 10 μ M CPT for 4 hr. After immunoblotting with anti-phospho-Kap1 (S824) and anti-Actin antibodies, the blot was stripped and re-probed with anti-phospho-CHK1 (S345).

(B) Western blot analysis of SL13 cells pretreated with either increasing concentration of siATM (5 nM, 10 nM, 100 nM) or DMSO and then exposed to 10 μ M CPT for 4 hr (top). Relative expression levels of phosphorylated Kap1-S824 normalized to Actin are the following from left to right: 0.03, 1.00, 0.64, 0.46, 0.22. In the bottom blot, a final concentration of 100 nM of siATM was used. After immunoblotting with anti-phospho-CHK1 (S345), the blot was stripped and re-probed with anti-Actin antibody.

(C) Representative analysis of PacBio CCS reads (maximum of 200 shown for simplicity) from samples pretreated with DMSO, 10 μ M KU55933, or 100 nM siATM prior to doxycycline treatment.

(D) Bar graph shows percentage of CCS reads with de novo telomere addition. The means and SEM from multiple experiments (n) are the following: 19.56 \pm 2.06 for DMSO, 12.45 \pm 1.16 for KU55933, and 0 \pm 0 for siATM. Asterisk indicates unpaired t test two-tailed p value is <0.05.

breaks. We treated cells with the PARP1 inhibitor, Olaparib (Fong et al., 2009), and also found that PARP1 inhibition correlated with increased ATM phosphorylation of Kap1 and increased phosphorylation of ATM-S1981 (Figure 5A). Using the ADDIT assay, we found the percentages of elongated telomeres were 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) (Figures 5C and 5D). 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 S1). To examine whether this stimulation of telomere elongation acts through ATM, we examined epistasis by treating cells with both Olaparib and KU55933. Co-treatment significantly reduced telomere addition compared to Olaparib only (p value = 0.04, t test) (Figures 5C and 5D), suggesting the increased telomere elongation by Olaparib treatment acts, at least in part, through the ATM pathway.

As an independent test of Olaparib on telomere length, we examined telomeres 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 (Figure 5B). Taken together, the experiments described here indicate that inhibition of ATM blocks telomere elongation, whereas activation of ATM stimulates telomere elongation.

Telomerase is known to be phosphorylated (Kang et al., 1999), so we wanted to examine whether the KU55933 or Olaparib treatment of cells might directly affect telomerase activity. We measured telomerase activity by a quantitative direct activity assay (Nandakumar et al., 2012) in human cells treated with

KU55933 or Olaparib and found no change in telomerase activity (Figure 6). We conclude that ATM kinase is a positive regulator of telomere elongation in both mouse and human cells, and that the effects of ATM inhibition are not due to inhibition of telomerase enzyme activity.

DISCUSSION

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, we showed 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 shortens telomere 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.

Conservation of the Role of ATM in DNA Damage and at Telomeres

The role we propose 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 (Shiloh and Ziv, 2013). 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 (Karlseeder et al., 1999; d'Adda di Fagnana et al., 2003; de Lange, 2009). Here, we link ATM signaling to telomere elongation; we propose that telomere

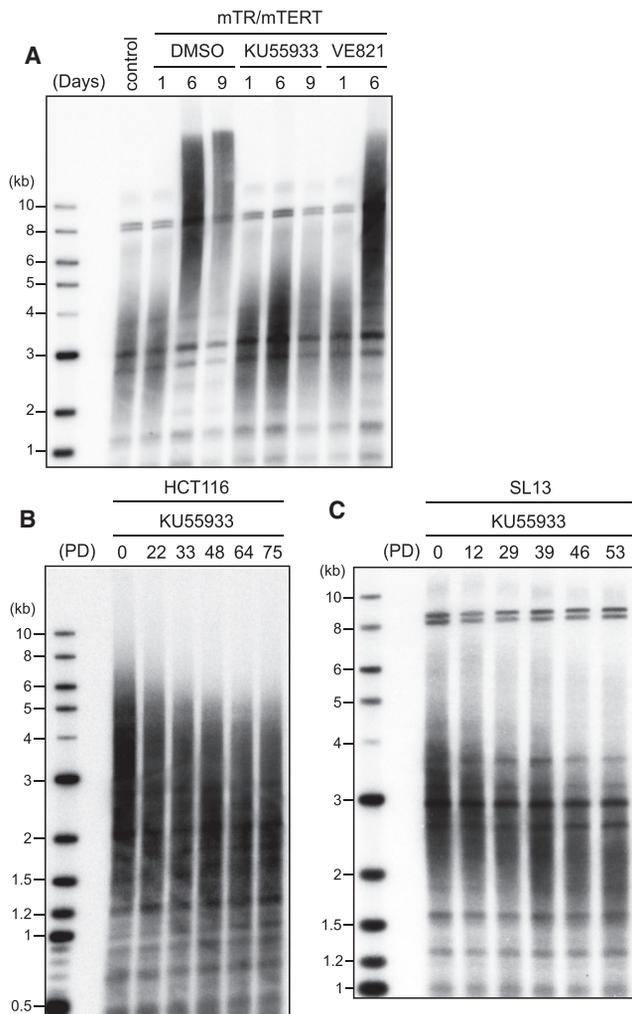


Figure 4. ATM Is Essential for Bulk Telomere Elongation in Both Human and Mouse Cells

(A) Telomere Southern blot of SL13 cells treated with DMSO, 10 μ M KU55933, or 5 μ M VE821 and then transduced with a lentivirus expressing both mTR and mTERT (mTR/mTERT) and grown for 1, 6, and 9 days.

(B) Telomere Southern blot of HCT116 cells treated with 10 μ M KU55933 and examined at different population doublings (PD).

(C) Representative telomere Southern blot of SL13 cells treated with 10 μ M KU55933.

See also Figure S4.

elongation is a form of ongoing repair that prevents telomeres from becoming critically short.

The decrease in telomere length when ATM was inhibited was not due to a decrease in telomerase enzyme activity. Our results are consistent with results in *S. cerevisiae* where the loss of Tel1^{ATM} causes telomere shortening, but telomerase enzyme activity was not affected (Chan et al., 2001). Elegant work in *S. pombe* has shown that phosphorylation of the telomere binding protein Ccq1 by the ATM and ATR kinases allows Est1 to recruit telomerase to the telomere (Moser et al., 2011; Yamazaki et al., 2012). While specific ATM substrates that affect telomere length in *S. cerevisiae* and mammalian cells are still not fully known, ATM phosphorylates TRF1, which mediates telomere elongation

(Wu et al., 2007), and other substrates may also play a role. In this issue of *Cell Reports*, Tong et al. (2015) also show that ATM regulates telomerase access to telomeres and find that TRF1 plays a role in that process.

ATR May Compensate for Loss of ATM

ATM and ATR may have partially redundant functions in telomere length maintenance in mammals as they do in yeast. In *S. cerevisiae*, Mec1^{ATR} plays a minor, yet critical role in telomere maintenance (Ritchie et al., 1999). Cells lacking Tel1^{ATM} are completely defective in telomere extension in a de novo addition assay (Frank et al., 2006; Ma and Greider, 2009), yet bulk telomere lengths of *tel1*^{ATM} Δ cells are not as short as *tel1*^{ATM} Δ *mec1*^{ATR} Δ cells (Ritchie et al., 1999). Interestingly, in *S. pombe*, the role of the two genes seems to be reversed (Nakamura et al., 2002). The *rad3*^{ATR} mutant cells have much shorter telomere lengths compared to *tel1*^{ATM} mutants, although like in *S. cerevisiae*, the double mutants have even shorter telomeres (Naito et al., 1998).

Our previous work in mice showed that ATM is not required for rescue of the shortest telomeres in an intergenerational cross (Feldser et al., 2006). 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. We concluded in that study that ATM is not essential for elongation of the shortest telomeres. Our 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 (Feldser et al., 2006) 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.

Activation of ATM Leads to Telomere Elongation

Understanding the pathways that regulate telomere elongation will allow future pharmacological manipulation of telomere length. Here, we showed that one method that activates ATM, PARP inhibition (Bryant and Helleday, 2006; Wahlberg et al., 2012), can lead to increased telomere length. The PARP inhibitor Olaparib is FDA approved for the treatment of ovarian cancer, although its side effects make it an unlikely candidate for telomere elongation in vivo (Fong et al., 2009; Banerjee et al., 2010; Kim et al., 2015). 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 (Underhill et al., 2011). 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.

The effects of PARP1 inhibitors may have different consequences in mice and humans. Our preliminary experiments with Olaparib treatment in human cells showed no alteration in telomere length (data not shown). In humans, tankyrase 1 and 2, members of PARP family, positively affect telomere length through the ADP ribosylation of TRF1 (Cook et al., 2002; Seimiya and Smith, 2002). However, the sites for ADP ribosylation are not conserved in mouse tankyrase (Hsiao et al., 2006; Chiang et al., 2008).

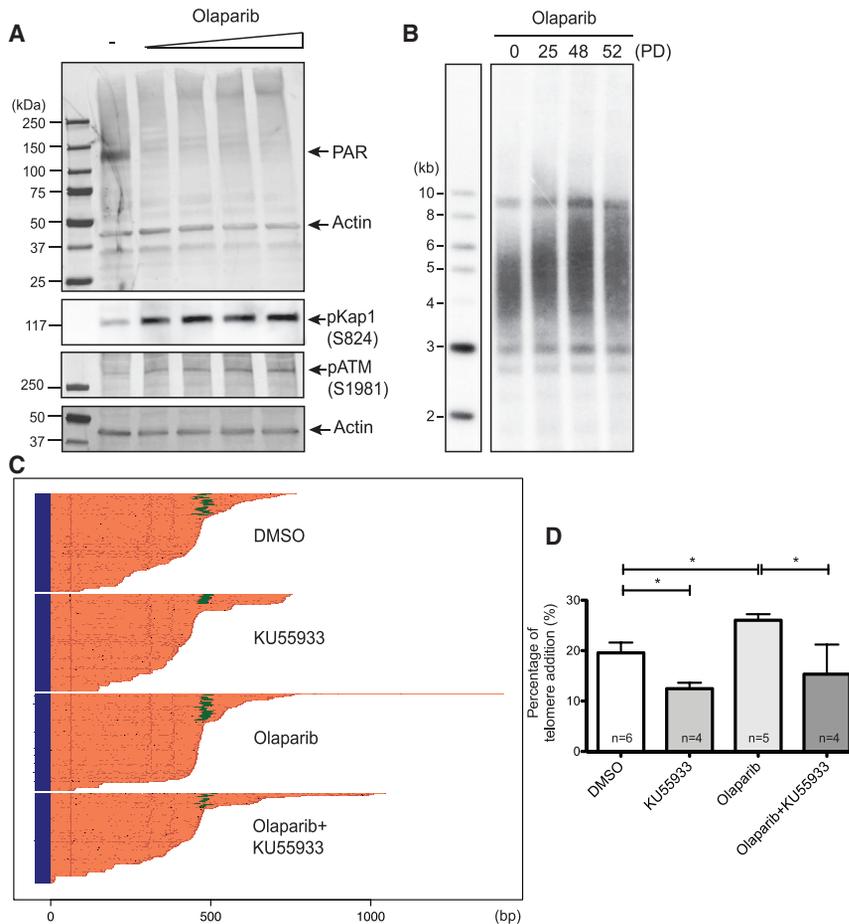


Figure 5. Activation of ATM Kinase Pathway Elongates Telomeres

(A) Western blot analysis of SL13 cells treated with either DMSO or Olaparib at final concentration 1, 3, 5, or 10 μM for 24 hr. After immunoblotting with anti-PAR and anti-Actin antibodies, the blot was stripped and re-probed with anti-phospho-Kap1 (S824). The same lysates were also analyzed with anti-phospho-ATM (S1981) and anti-Actin antibodies.

(B) Telomere Southern blot of SL13 cells treated with 3 μM Olaparib and examined at different population doublings (PD).

(C) Representative analysis of PacBio CCS reads (maximum of 350 reads shown for simplicity). Samples were pre-treated with DMSO (as control) or either 10 μM KU55933 or 3 μM Olaparib or both 10 μM KU55933 and 3 μM Olaparib prior to doxycycline treatment.

(D) 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 .

“seed” sequence followed by a unique I-Sce1 endonuclease cut site (Figure S1A). The length of seed sequence was based on an early study showing that 400 bp of telomere repeats can act as a functional telomere (Farr et al., 1991). In brief, cells were transfected with linearized chr4 targeting construct (pISL25) using XtremeGENE 9 (Roche). After 3 days of transfection, cells were selected for hygromycin resistance at final concentration of 500 $\mu\text{g}/\text{ml}$ for 1 week followed by an additional 1 week of negative selection with ganciclovir at 35 $\mu\text{g}/\text{ml}$ final concentration to select against Tk gene. The $\text{HYG}^{\text{R}}\text{GVC}^{\text{R}}$ 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. We identified two independent HYG^{R} clones, clone termed 1L and 1M, each containing a single chr4 allele that was correctly modified (Figure S1B). To cut the endogenous chromosome 4 at the engineered I-Sce1 site in vivo, we stably integrated a HA epitope tagged I-Sce1 endonuclease driven by a tetracycline-inducible promoter (pISL39), and later RFP-positive cells were flow sorted in 96-well plate as single clones. To induce I-Sce1 expression, doxycycline at final concentration of 2 $\mu\text{g}/\text{ml}$ was added to cells. Typically, cells were collected post 48 hr of doxycycline treatment. We identified a clone (SL13) that expressed HA-I-Sce1 only in the presence of doxycycline by western blot (Figure S1C). To compare telomere addition in cells with and without telomerase, SL13 cells were transfected with a construct expressing the flip recombinase (pPGKFLPobpA, Addgene #13793). Approximately 10 days later, flow cytometry was used to sort GFP-positive (with mTR) and GFP-negative (without mTR) cells. We measured mTR level by qRT-PCR and confirmed mTR was present in GFP-positive cells but absent in GFP-negative cells (Figure S1F). We refer to these populations as mTR^+ or mTR^- , respectively.

In vitro assays indicate that PARP1 inhibitors, including Olaparib, are highly specific to PARP1-4 and have little effect on tankyrases (Wahlberg et al., 2012). However, it will be important to verify whether these drugs affect tankyrase activity to understand their potential effects on telomere length regulation in humans.

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

EXPERIMENTAL PROCEDURES

Plasmid Construction

Plasmid construction strategies are described in the Supplemental Experimental Procedures. Primers used are listed in Table S2.

Generation of SL13 Cell Line

To generate the telomerase-conditional cell line, $\text{mTR}^{-/-}$ skin fibroblast cell line from CAST/EIJ mice (Morrish and Greider, 2009) were transduced with a retrovirus (pISL8) containing mTR and the green fluorescence protein (GFP) that can be removed by FLP/FRT recombination and flow sorted for GFP-positive fluorescence (Figure S1E). In the chromosomally stable GFP-positive cells, we modified chromosome 4 (chr4) to generate an internal 480-bp telomere

Cell Culture and Treatments

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)

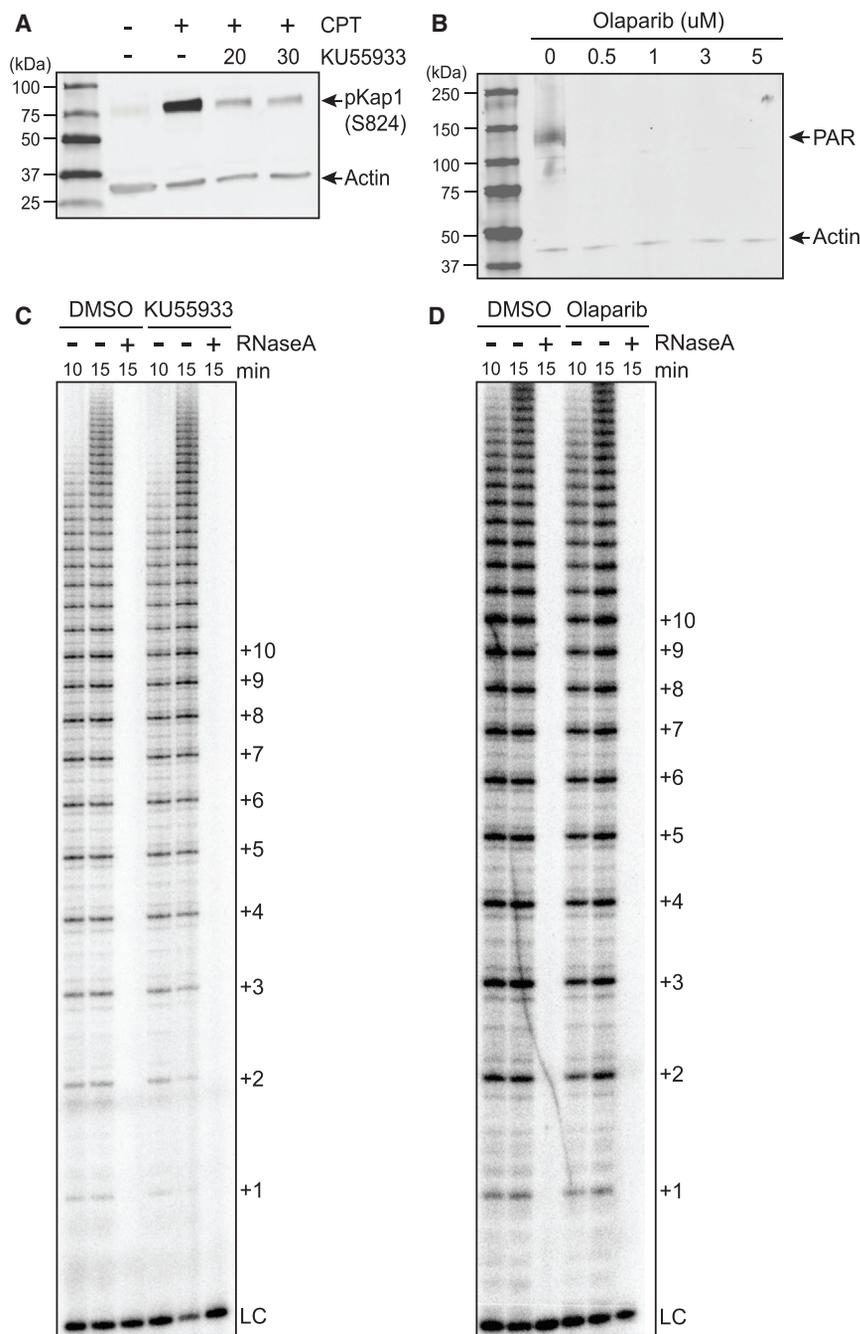


Figure 6. Telomerase Activity Is Unaffected by Either Olaparib or KU55933 Treatment

(A) Western blot analysis of 293TREx-0611 cells pretreated with either DMSO or 20 or 30 μ M KU55933 and then exposed to 10 μ M CPT for 4 hr. Immunoblotted with anti-phospho-Kap1 (S824) and anti-Actin antibodies.

(B) Western blot analysis of 293TREx-0611 cells treated with either DMSO or Olaparib at final concentration 0.5, 1, 3, or 5 μ M. Immunoblotted with anti-PAR and anti-Actin antibodies.

(C) Direct telomerase activity assay using whole cell lysates from 293TREx-0611 cells expressing hTERT, hPot1, and hTTP1 that were transfected with hTR overnight and treated with DMSO or 30 μ M KU55933. Reactions were stopped at 10 or 15 min. End-labeled 18-mer loading control (LC), RNaseA-treated control (RNaseA), and numbers of telomere repeats added to 18-mer primer are indicated.

(D) Direct telomerase activity assay using lysates from cells treated with DMSO or 5 μ M Olaparib.

digested genomic DNA, 0.9 μ M of telomere linkers or IScerette linker and 0.5 U of T4 DNA ligase (NEB) in 1 \times T4 ligation buffer. Multiple PCRs (typically 24 or 32 reactions per sample) were carried out for each sample in 25 μ l containing 1 ng of ligated DNA, 0.2 μ M HYG-specific and teltail primers, 1 \times Fail Safe PCR buffer H (Epicenter FSP995H), 1 U of Fail Safe Enzyme Mix (Epicenter FS99100). The PCRs 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 (see the [Supplemental Experimental Procedures](#)).

PacBio Sequence Reads Analysis

We created a pipeline in R (see [Figure S2](#)) 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: $100\% \times [(\text{number of CCS reads with telomere repeats added beyond the I-SceI site}) / (\text{number of total CCS reads})]$. Unpaired t test generated the one- or two-tailed p values.

siRNA-Mediated Knockdown of ATM and TERT

ON-TARGET siRNA SMART pools from GE Healthcare were used: mouse TERT (L-048320-01-0005), mouse ATM (11920). SL13 cells were transfected using Pepmute 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 qRT-PCR.

or PARP1 inhibitor Olaparib (Selleckchem, #1060) at indicated concentrations or DMSO as a control. For the ADDIT assay, SL13 cells were treated with final concentration 2 μ g/ml of doxycycline for typically 48 hr or as indicated.

Modified Single Telomere Length Analysis for Mouse chr4

The original single telomere length analysis (STELA) protocol used for human cells (Baird et al., 2004) was modified to measure telomere lengths on the cut end of chr4 in SL13 cells. Briefly, genomic DNA was extracted using Puregene Core Kit A (QIAGEN). Then, 4 μ g of genomic DNA was digested with SphI (NEB) and later diluted to 10 ng/ μ l in water. For the in vitro IScerette sample, genomic DNA was digested with SphI 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 μ l containing 10 ng of

Direct Telomerase Activity Assay

Telomerase activity was assayed in whole-cell lysates with a modified protocol (Nandakumar et al., 2012). 293TREx-0611 cells overexpressing TPP1, POT1, and hTERT were seeded (3×10^5 cells) per well in a poly-D-lysine-coated 6-well plate. The next day, cells were transfected with U1-hTR using Lipofectamine 3000 and incubated overnight. Then, they were treated with 30 μ M KU55933, 5 μ M Olaparib, or DMSO for 2 hr before harvesting in

100 μ l 1 \times CHAPS. Cells were lysed on ice for 30 min, vortexing occasionally, and cleared by spinning (8,000 rpm, 20 min, 4°C). For negative controls, 25 μ l cleared lysate was incubated at 65°C with 1 μ l RNaseA (1 mg/ml) for 10 min. For direct telomerase assays, lysates were incubated with primer a5 (Wang et al., 2007) in 1 \times telomerase buffer (50 mM Tris-Cl, 30 mM KCl, 1 mM MgCl₂, 1 mM spermidine), 0.5 mM dTTP, 0.5 mM dATP, 2.92 μ M dGTP, and 0.33 μ M α 32P-dGTP at 30°C. Reactions were stopped at 10 or 15 min with 20 mM EDTA, 10 mM Tris spiked with 500 cpm end-labeled 18-mer. Telomerase products were purified by phenol chloroform extraction, ethanol precipitated, washed with 70% ethanol, and resuspended in 5 μ l water and 5 μ l 2 \times formamide loading dye. Products were denatured at 100°C and separated on a sequencing gel (10% acrylamide, 7 M urea 1 \times TBE), at 90 W for 1.5 hr. The gel was dried, exposed to a phosphor screen overnight and imaged using the STORM phosphorimager.

ACCESSION NUMBERS

The accession number for the PacBio sequence data reported in this paper is NCBI SRA: SRP059426.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.10.035>.

AUTHOR CONTRIBUTIONS

Conceptualization, S.S.L. and C.W.G.; Methodology, S.S.L.; Software, C.B.; Validation, S.S.L.; Investigation, S.S.L. and A.M.P.; Formal analysis, C.B., S.J.W., and S.S.L.; Writing – Original Draft, S.S.L. and C.W.G.; Writing – Review and Editing, S.S.L., C.W.G., and A.M.P.; Visualization, S.S.L. and C.B.; Supervision, C.W.G. and S.J.W.; Funding Acquisition, C.W.G. and S.J.W.

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