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Conserved and Unique Features of Terminal Telomeric Sequences in ALT-Positive Cancer Cells

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eLife Assessment

This study demonstrates the application of END-seq, originally developed to study genomewide DNA double-strand breaks, to telomere biology; the work packs a punch, concisely demonstrating the utility of this approach and the new insights that can be gained. The authors confirm that telomeres in telomerase-positive cells terminate with 5'-ATC in a Pot1-dependent manner, and demonstrate that this principle holds true in telomerase-negative ALT cells as well. S1-END-seq is similarly developed for telomeres, showing that ALT cells harbor several regions of ssDNA. The study is well-executed and **convincing**, the new insights are **fundamental** and **compelling**, and the optimized END-seq approaches will be widely utilized. The work will prompt additional studies that the reviewers look forward to, including combining telomeric END-seq with long-read sequencing to address the distribution and origin of variant telomere repeats and ssDNA along telomeres in ALT and telomerase-positive settings.

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Abstract

A significant portion of human cancers utilize a recombination-based pathway, Alternative Lengthening of Telomeres (ALT), to maintain telomere length. Targeting the ALT is of growing interest as a cancer therapy, yet a substantial knowledge gap remains regarding the basic features of telomeres in ALT-positive cells. To address this, we adopted END-seq, an unbiased sequencing-based approach, to define the identity and regulation of the terminal sequences of chromosomes in ALT cells. Our data reveal that the terminal portions of chromosomes in ALT cells contain canonical telomeric sequences with the same 5’ terminus bias (-ATC) observed in non-ALT cells. Furthermore, as reported for non-ALT cells, POT1 is required to preserve the precise regulation of the 5’ end in cells that maintain telomere length using the ALT pathway. Thus, the regulation of the terminal 5’ of chromosomes occurs independently of the mechanism of telomere elongation. Additionally, we employed an S1 endonuclease-based

sequencing method to determine the presence and origin of single-stranded regions within ALT telomeres. These data shed light on conserved and unique features of ALT telomeres.

Introduction

Telomeres are the natural ends of eukaryotic chromosomes and are critical for maintaining chromosome integrity [1]. Human telomeres consist of 5-15 kilobases of repetitive DNA sequences (5'-TTAGGG-3'), which serve as a binding platform for an essential protective protein complex named shelterin [1]. The terminal portion of chromosomes is composed of a single-stranded 3' overhang of approximately 200 nucleotides, generated by the interplay between DNA replication and end-processing, and regulated by the telomere-associated protein POT1 [2, 3]. Noteworthy, the generation of the 3' overhang differs between leading and lagging strand telomeres. At leading strand telomeres, the newly synthesized DNA terminates in a blunt end and requires post-replicative resection by nucleases such as Apollo and Exo1 and a fill-in action promoted by CST complex to create the 3' overhang [4]. In contrast, lagging strand synthesis inherently generates a 3' overhang due to Okazaki fragment processing, where removal of the terminal RNA primer leaves a single-stranded gap that is partially filled in by DNA polymerase α-primase with the aid of the CST complex [5] (for reviews, see [2, 3]). Progressive loss of telomeric repeats during DNA replication leads to telomere shortening and represents a barrier to unlimited proliferation [6-9]. To overcome this tumor suppressive barrier, ~85% of cancers activate telomerase, a reverse transcriptase, to synthesize new telomeric repeats [10, 11]. The remaining ~15% of cancers engage the Alternative Lengthening of Telomeres (ALT) pathway, a recombination-based mechanism to elongate telomeres using existing telomeric DNA as a template [12, 13]. Major advancements in our understanding of the ALT pathway at the molecular level have been made over recent years, shedding light on the molecular pathways involved in telomere elongation as well as the genetic vulnerabilities of ALT-positive cells [14-24] (for reviews, see [25-28]).

Due to the repetitive nature of telomeres, some fundamental questions about the composition of ALT telomeres remain unanswered. Specifically, it is unclear if 5' telomere ends in ALT cells differ from telomerase-positive cells, whether they follow the same rules for end protection, and whether ALT telomeres contain any unique secondary structures. It is foreseeable that telomeres in telomerase-positive cells and ALT cells differ, as ALT can generate complex and non-canonical telomeric sequences, and secondary structures through recombination-based mechanisms. Interestingly, positive staining for the single-strand binding protein RPA and native FISH staining (ssTelo) of telomeres suggested the presence of single-stranded DNA regions in ALT-positive cells [19, 29]. However, the source or extent of ssDNA in ALT telomeres remains unclear. Intriguingly, EM data and rolling-circle amplification-based assays suggest that ALT frequently contain circular extrachromosomal telomeric DNA termed C-circles [30, 31].

Here, we report a novel approach to studying ALT telomeres using the high-resolution and unbiased method known as END-seq [32]. Using this method, we find that the terminal sequence of ALT chromosomes consists of canonical telomeric repeats, shares the same bias in the 5' terminus as telomeres maintained by telomerase, and terminates with ATC-5'. Furthermore, we demonstrated that this bias is influenced by the shelterin component, POT1.

Secondly, to assess the presence of ssDNA at ALT telomeres, we used S1 nuclease, an enzyme that specifically degrades single-stranded DNA including the single-stranded regions in duplex DNA. Using S1 nuclease treatment coupled with END-seq (S1-END-seq) [33], we show that ALT telomeres have tracts of ssDNA and the presence of ssDNA in telomeres can be used to distinguish ALT-positive from ALT-negative cells.

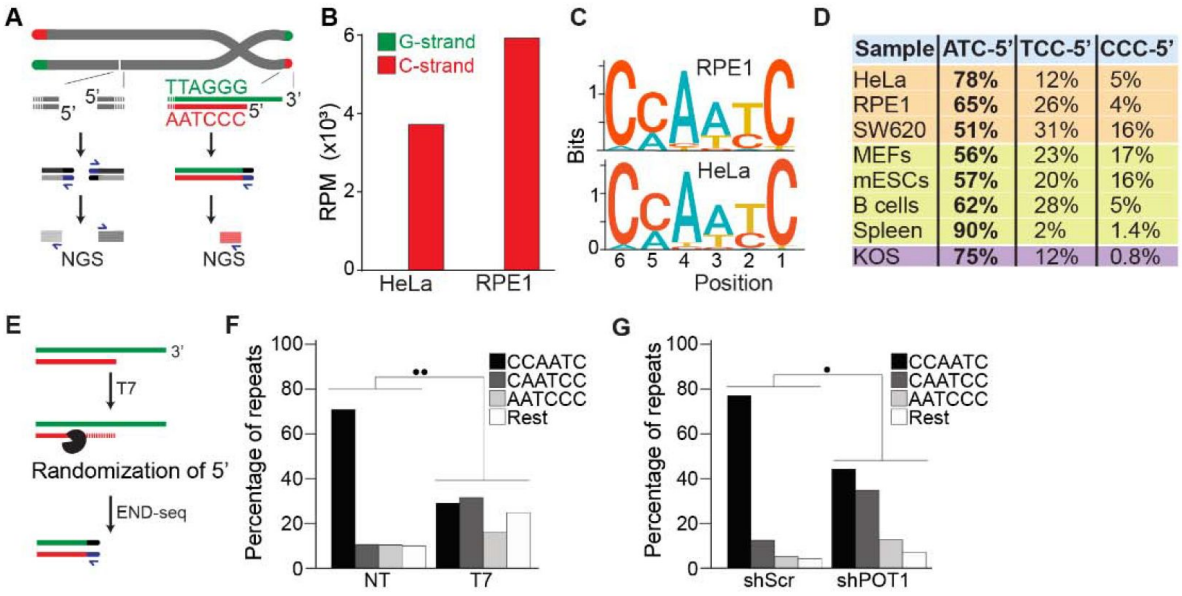
Results

END-seq efficiently captures 5’ chromosome termini

To study the terminal portion of chromosome, we employed an unbiased and high-resolution approach developed to map and investigate double-strand breaks (DSBs): END-seq [32]. In this method, biotin-labeled adaptors are ligated to DSBs, which enable their purification and subsequent sequencing by next-generation sequencing (NGS). Based on the directionality of DNA sequencing (5’ to 3’), DSBs are identified as unidirectional reads that originate from the lesion site on either side of the break (**Figure 1A**). Chromosome ends resemble one-ended DSBs, which by END-seq should be detected as unidirectional reads corresponding to the 5’ telomeric strand, the C-rich strand. To test this hypothesis, we performed END-seq on two telomerase-positive human cell lines, HeLa and telomerase expressing RPE1. Sequencing reads containing four consecutive TTAGGG repeats (G-rich) or CCCTAA repeats (C-rich) were defined as “telomeric reads” and were normalized to the total number of reads obtained in each individual library. Strikingly, virtually all (99.9%) telomeric END-seq reads correspond to the C-rich strand, in agreement with the fact that chromosome ends are one-ended DSBs (**Figure 1B**). To exclude possible artifacts derived from the repetitive nature of telomeric reads, we performed a similar analysis of reads corresponding to pan-centromeric repeats; this analysis shows that although the fraction of their presence in the human genome was similar (Figure S1A), the pancentromeric sequences are not enriched to the same level as telomeres nor show any strand bias when detected (Figure S1B).

Next, we performed a motif analysis on the C-rich telomeric END-seq reads to determine the prevalent terminal chromosomal sequence. This analysis shows that the vast majority (78% and 65% for HeLa and RPE1, respectively) of chromosomes end with ATC-5’ (**Figures 1C** and 1D). Strikingly, this is in agreement with previous data obtained using a PCR-based approach termed STELA on defined chromosome ends in HeLa cells [34]. A major advantage of END-seq over STELA is that it can be applied to telomeres of any length and chromosome location without the need of specific sub-telomeric primers. This excludes possible biases and allows the analysis of telomeres from any cell type and origin. As a proof of principle, we expanded the analysis to a collection of telomerase-positive human cell lines, as well as mouse and dog cell lines, and mouse tissues. In these experiments, we found that chromosome ends show a strong bias for ATC-5’ as a terminal sequencing (**Figure 1D**), in line with previous findings [35, 36], suggesting a conserved mechanism for its generation.

To further validate END-seq as a method to identify terminal telomeric repeats, we tested whether 5’-3’ T7 exonuclease treatment prior to END-seq would be sufficient to change the bias of the ATC-5’ sequence (**Figure 1E**). Since T7 exonuclease preferentially degrades 5’ ends of double-stranded DNA while leaving 3’ single-stranded overhangs intact, we expected that its treatment would randomize the 5’ telomeric ends by progressively resecting them, thereby disrupting the original sequence bias. If END-seq was biased or not sufficiently sensitive, T7-treatment would not significantly alter the detected sequence distribution. As shown in **Figures 1F** and S1C, T7-treated samples display an altered distribution of telomeric permutations. Quantitative measurement of the difference in distribution of possible telomeric permutations in the presence or absence of T7 treatment was performed using the Kullback-Leibler divergence (KL divergence) score. The result of this analysis indicated that the samples are extremely divergent (●● indicates a KL divergence value between 0.375 and 0.5). To further evaluate the distribution changes between these conditions, we compared the reads obtained from these samples with *in silico*-generated control reads representing various degrees of randomization from the ATC-5’ sequences (see Figures S1D and S1E). Using the KL divergence score, we determined that T7-randomized samples were most similar to a population where 100% of the reads were equally randomized. In



Sample	ATC-5'	TCC-5'	CCC-5'
HeLa	**78%**	12%	5%
RPE1	**65%**	26%	4%
SW620	**51%**	31%	16%
MEFs	**56%**	23%	17%
mESCs	**57%**	20%	16%
B cells	**62%**	28%	5%
Spleen	**90%**	2%	1.4%
KOS	**75%**	12%	0.8%

Fig 1.

END-seq successfully captures the 5' termini of human and mouse chromosomes

A. Schematic representation of the END-seq method applied to DSBs (left) and natural chromosome ends (right). Blunted dsDNA ends are ligated to biotinylated adaptors, purified, and subsequently sequenced by NGS. Reads originating from DSBs align to either side of the break (left), while telomeric reads align only to the 5' C-rich template (red). **B**. Number of reads containing at least 4 consecutive telomeric repeats corresponding to the G-rich (green) and C-rich (red) strands in HeLa and RPE1 cell lines. Telomeric reads are normalized to the total number of reads identified by END-seq and are represented as reads per million (RPM). **C**. Sequence logo representing the conservation (bits) of the last 6 nucleotides at the 5' end of chromosomes in HeLa and RPE1 cells. **D**. Distribution of the three most frequent telomeric repeats across human cell lines (orange), mouse cells (yellow) and a canine cell line (purple). **E**. Schematic representation of T7-mediated randomization of the 5' termini. **F**. Percentage of telomeric reads that have the indicated sequence as a 5' end. The following sequences (CCCAAT-5', TCCCAA-5', and ATCCCA-5') are grouped and labelled as "Rest". Kullback-Leibler divergence (KL divergence) analysis was used to compare the distributions of the individual conditions. KL divergence between 0.25 and 0.375 is represented by 2 dots. Prior to the END-seq protocol cells were either left untreated (NT) or were treated with T7. **G**. Percentage of telomeric reads are displayed as described in F. Cells expressing either a non-targeting shRNA (shScr) or a shRNA targeting POT1 (shPOT1) were harvested 3 days post induction and analyzed by END-seq. KL divergence between 0.125 and 0.25 is represented by 1 dot (•).

comparison, in the untreated samples, approximately 70% of the reads correspond to the perfect "canonical" CCAATC-5' sequence. These data indicate END-seq as a method to sensitively detect terminal telomeric repeats while excluding the potential artifacts.

Further, we investigated whether we could detect *in vivo* alterations of the terminal repeat, such as those reported upon depletion of the shelterin component, POT1 [37]. To deplete POT1, we generated a population of HeLa cells harboring a doxycycline-inducible POT1 short hairpin RNA (shPOT1-1) construct. Doxycycline treatment resulted in reduced POT1 expression, confirming efficient knockdown (Figure S1G). In agreement with previously published data, END-seq analysis showed that POT1-depleted cells have a significant reduction in terminal repeats ending with the canonical ATC-5' sequence (**Figure 1F**). Comparison with control reads generated *in silico* reveals that approximately 45% of reads from POT1-depleted cells are derived from random permutations of the telomeric repeat (Figure S1F).

Lastly, we tested how depletion of either or both POT1 paralogs affected the 5' termini of mouse telomeres. To this end, we engineered mouse embryonic stem cells (mESCs) expressing a FKBP-tagged POT1a protein (POT1a$^{dTAG/dTAG}$) that is degraded when cells are treated with dTAG13 compound (Figures S2A-C). Using this cell line, we then generated cell lines lacking POT1b through CRISPR knockout (POT1a$^{dTAG/dTAG}$ POT1b$^{-/-}$) (Figures S2D-S2F). This strategy allowed us to examine the 5' termini of POT1-proficient ESCs, as well as systematically analyze the effect of either single or double POT1 depletion in a genetically defined background. END-seq analysis revealed that while depletion of POT1a altered 5' termini, depletion of POT1b had no effect (**Figures 2A** and 2B). Using the KL divergence score, we determined that in the POT1-profient and POT1b-depleted samples, approximately 70% and 85% of reads, respectively, corresponded to the perfect canonical ends. In contrast, in POT1a-depleted samples, this proportion was around 25%, regardless of POT1b status. This striking result highlights a fundamental difference between POT1a and POT1b, demonstrating that POT1a plays a dominant role in maintaining proper 5' end processing, consistent with recently published work [35]. The stark contrast between their effects underscores the distinct functions of these paralogs and reveals an exciting new layer of telomere regulation. Collectively, these data demonstrate the effectiveness of END-seq to study terminal repeats of chromosomes.

The terminal 5' sequence in ALT-positive cells is canonical

Having established that END-seq efficiently captures the terminal repeat of chromosome ends, we sought to investigate whether ALT-positive cells have the same tight regulation of terminal repeats as telomerase-positive cells. We performed END-seq on three ALT-positive cell line: G292, SAOS2 and U2OS. Our analysis shows that in ALT cells, the vast majority of telomeric reads (82-98% across 3 cell lines and 11 biological repeats) correspond to the C-rich strand (**Figures 3A-3C**), consistent with non-ALT cells and the expected pattern for a one-ended-DSB.

Motif analysis revealed that the majority of telomeric reads in all three tested cell lines start with the canonical ATC-5' sequence (**Figures 3D** and S3A-S3C). These data suggest that regulation of the terminal 5' of ends of chromosomes occurs independently of the mechanism of telomere elongation. To test this hypothesis, we depleted POT1 in U2OS cells using two different doxycycline-inducible shRNAs (Figure S3D). POT1 depletion resulted in formation of 53BP1 foci (**Figures 3E** and **3F**), consistent with POT1's established role in suppressing the DNA damage response at chromosome ends [37, 38]. Next, we performed END-seq to evaluate the effect of POT1-depletion on the terminal sequence. The data show that, similarly to telomerase-positive HeLa cells, depletion of POT1 dramatically altered the 5' natural termini in ALT-positive cell lines (**Figure 3G**).

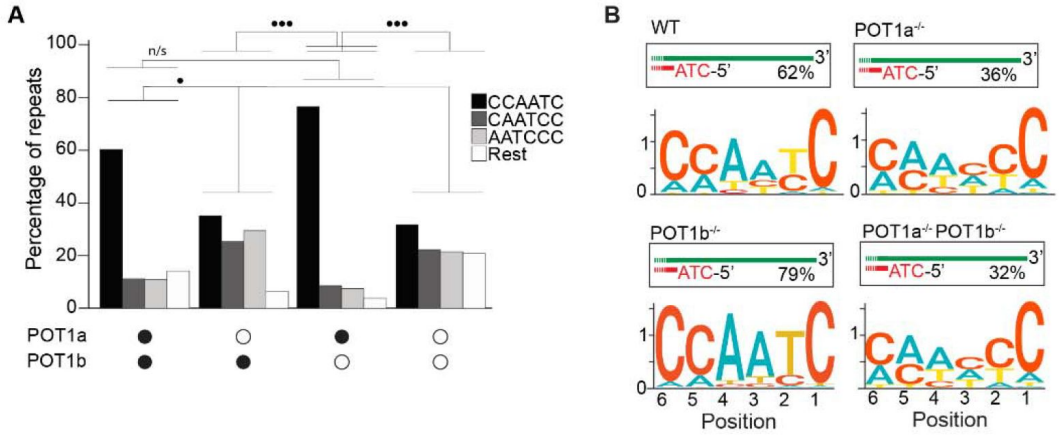


Fig 2.

POT1a is the key regulator of 5′ telomere end processing in mice, distinct from POT1b

A. Comparison of the effect of single and double depletion of POT1a and POT1b on the percentage of telomeric reads with the indicated 5′ end sequences. The following sequences (CCCAAT-5′, TCCCAA-5′, and ATCCCA-5′) are grouped and labelled as "Rest". Kullback-Leibler divergence (KL divergence) analysis was used to compare the distributions across conditions. KL divergence between 0.25 and 0.375 is represented by 2 dots. **B**. Sequence logo illustrating the conservation (in bits) of the last 6 nucleotides at the 5′ end of chromosomes in POT1-proficient, POT1a-depleted, POT1b-depleted, and POT1a/POT1b double-depleted mESCs.

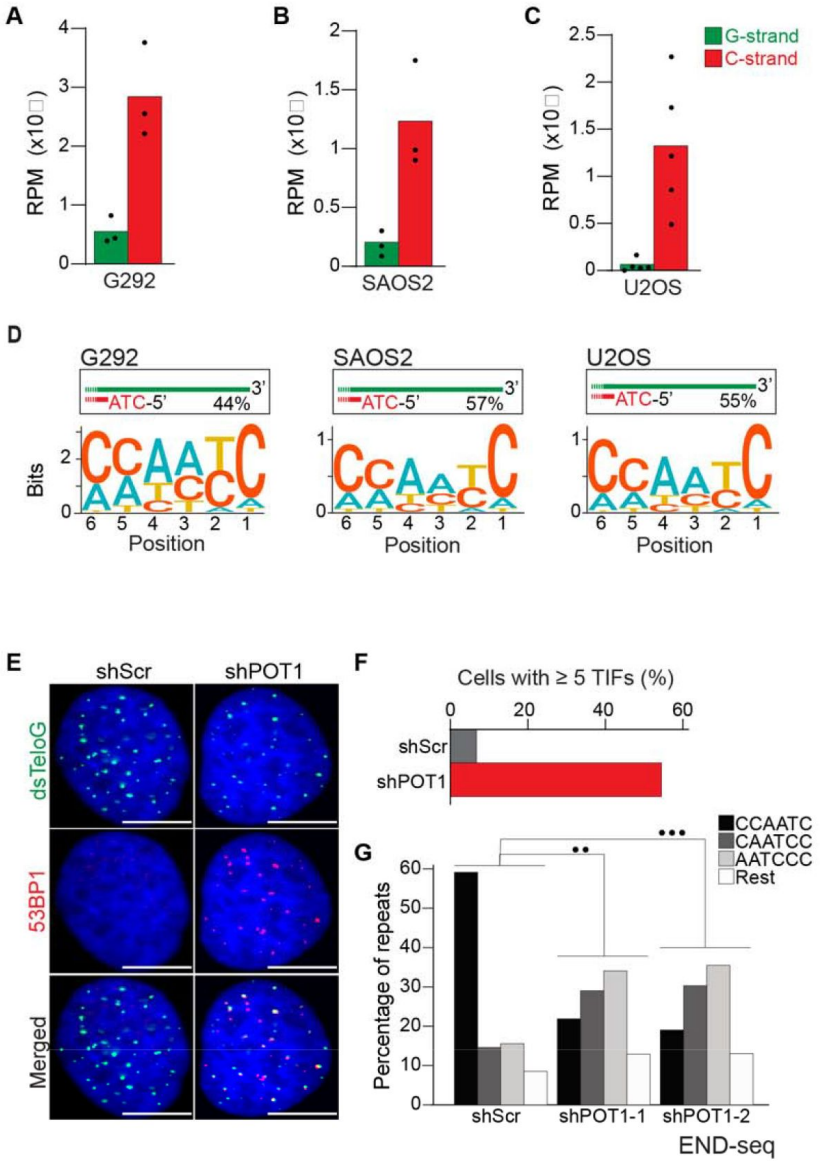


Fig 3.

ALT cells have precise 5′ termini

A-C. Number of telomeric reads containing at least 4 consecutive telomeric repeats corresponding to the G-rich (green) and C-rich (red) strands in the ALT-positive G292, SAOS2 and U2OS cell lines. Telomeric reads are normalized to the total number of reads identified by END-seq and are represented as reads per million (RPM). **D**. Sequence logo representing the conservation at chromosome ends. A sequence logo representing the conservation (bits) of the last 6 nucleotides at the 5′ end of chromosomes in ALT cells. Fraction of reads with CCAATC as 5′ end is displayed. **E**. Cells expressing either a non-targeting shRNA (shScr) or a shRNA targeting POT1 (shPOT1-1) were stained for 53BP1 (red) and telomeric DNA (TTAGGG, green). The scale bar represents 10 μm. **F**. Quantification of the data shown in panel E. Graphs indicated the percentage of cells that have at least 5 telomere dysfunctional foci (TIF) with 53BP1 co-localizing at telomeres. **G**. Percentage of telomeric reads that have the indicated sequence as a 5′ sequence that have as a 5′ end. The following sequences (CCCAAT-5′, TCCCAA-5′, and ATCCCA-5′) are grouped and labelled as "Rest". Cells expressing either a non-targeting shRNA (shScr) or a shRNA targeting POT1 (shPOT1-1 and shPOT1-2) were harvested 3 days post induction and analyzed by END-seq. Kullback-Leibler divergence (KL divergence) analysis was used to compare the distributions of the individual conditions. KL divergence between 0.25 and 0.375 is represented by 2 dots (●●), KL divergence greater than 0.375 is represented by 3 dots (●●●).

Next, we asked whether ALT cells are more likely to contain variant telomeric repeats (VTRs) in the terminal portions of chromosome ends. VTRs have been shown to occur frequently in telomeric reads isolated from ALT-positive cells [39]. To establish whether VTRs in ALT cells are contained within terminal repeats, we assessed the frequency of non-canonical telomeric repeats within the first 30 nucleotides from the 5' of the Illumina reads from U2OS (1.93%) and HeLa (1.93%) cells (Figure S3E). This result shows that in ALT-positive cells, VTRs do not occur more frequently than non-ALT cells within the very terminal portion of chromosome ends. Due to the short read lengths used in this approach, we cannot determine whether VTRs in ALT cells are specifically excluded from the terminal region of chromosome ends.

Frequent occurrence of ssDNA in ALT telomeres

Having demonstrated that the 5' terminal repeats in ALT cells are indistinguishable from non-ALT cells, we next asked whether the presence of single-stranded DNA (ssDNA) could be a unique feature of telomeric DNA in ALT-positive cells. Previous work provided indirect evidence for the presence of single-stranded telomeric DNA in ALT-positive cells, such as staining for RPA at telomeres, and FISH under native conditions (Figures S4A-S4D) [19, 29]. To test whether telomeres in ALT cells contain ssDNA, we employed S1-END-seq as a direct measure for the presence of ssDNA [33]. For this method, the S1 nuclease is used to convert any ssDNA into DSBs which are detected by END-seq. We anticipated that if telomeres do not contain any single-stranded telomeric DNA in addition to the 3' overhang, telomeric S1-END-seq reads will be indistinguishable from the signal obtained using standard END-seq, corresponding to one-ended DNA ends (**Figure 4A**). However, S1-mediated cleavage of ssDNA within the internal telomeric tract will generate two-ended DSBs within telomeres. A DSB within telomeres would results in telomeric reads corresponding both to the C-rich and the G-rich strands (**Figure 4A**). To test this prediction, we performed S1-END-seq on a panel of ALT-positive and ALT-negative (**Figures 4B** and S4E). S1-END-seq in ALT-negative cells revealed only C-strand telomeric reads excluding the presence of a significant amount of ssDNA within the telomeric tract in these cells (**Figures 4B** and S4E). By contrast, performing S1-END-seq on a panel of ALT-positive cell lines revealed that G-rich and C-rich telomeric reads were detected at similar frequencies (**Figures 4B** and S4E). These data show that telomeres in ALT-positive cells contain frequent regions of single-stranded DNA. Furthermore, our data shows that the ratio of C-rich to G-rich reads in S1-END-seq data can distinguish ALT-negative (0.98-0.99) from ALT-positive cell lines (0.4-0.58). Based on this ratio, we estimate that the ALT-positive cells analyzed here contain a minimum of three single-stranded telomeric DNA regions per chromosome end (Figures S4F and S4G).

We also investigated whether reads detected by S1-END-seq are more likely to contain VTRs compared to the terminal portions of telomeres. We compared the frequency of VTRs within the first 30 nucleotides of the S1-END-seq reads from U2OS libraries (3.8%) to their control END-seq U2OS libraries (1.93%). This shows that the frequency of VTRs is higher in the internal portions of chromosome ends compared to the terminal portions (Figure S4H).

To determine whether the presence of ssDNA at telomeres in ALT cells is a feature intrinsic to these cells or a feature of the ALT-mediated telomere elongation, we measured the levels of ssDNA at telomeres in cells lacking BLM. Depletion of BLM is sufficient to suppress ALT-mediated telomere elongation, as shown by the loss of ALT-associated features and progressive telomere shortening [14, 19, 40-42]. As expected, we found similar levels of C-rich and G-rich reads in S1-END-seq data from U2OS cells (C/G strand ratio: 0.55, **Figure 4D**), indicating a minimum of 5 ssDNA regions per chromosome end. By contrast, BLM-deficient U2OS cells show a bias towards C-rich reads (C/G strand ratio: 0.7, **Figure 4D**), indicating that these cells have reduced levels of ssDNA at chromosome ends compared to the parental ALT-positive U2OS cell line (at least 1 ssDNA region per chromosome end). Given that BLM helicase contributes to telomere stability by resolving secondary structures and ensures proper telomeres replication regardless of telomere maintenance mechanism [43-45], it is possible that ssDNA detected in the absence of BLM is due to replication fidelity. Contrary to this, most of the reads detected in both BLM-proficient and

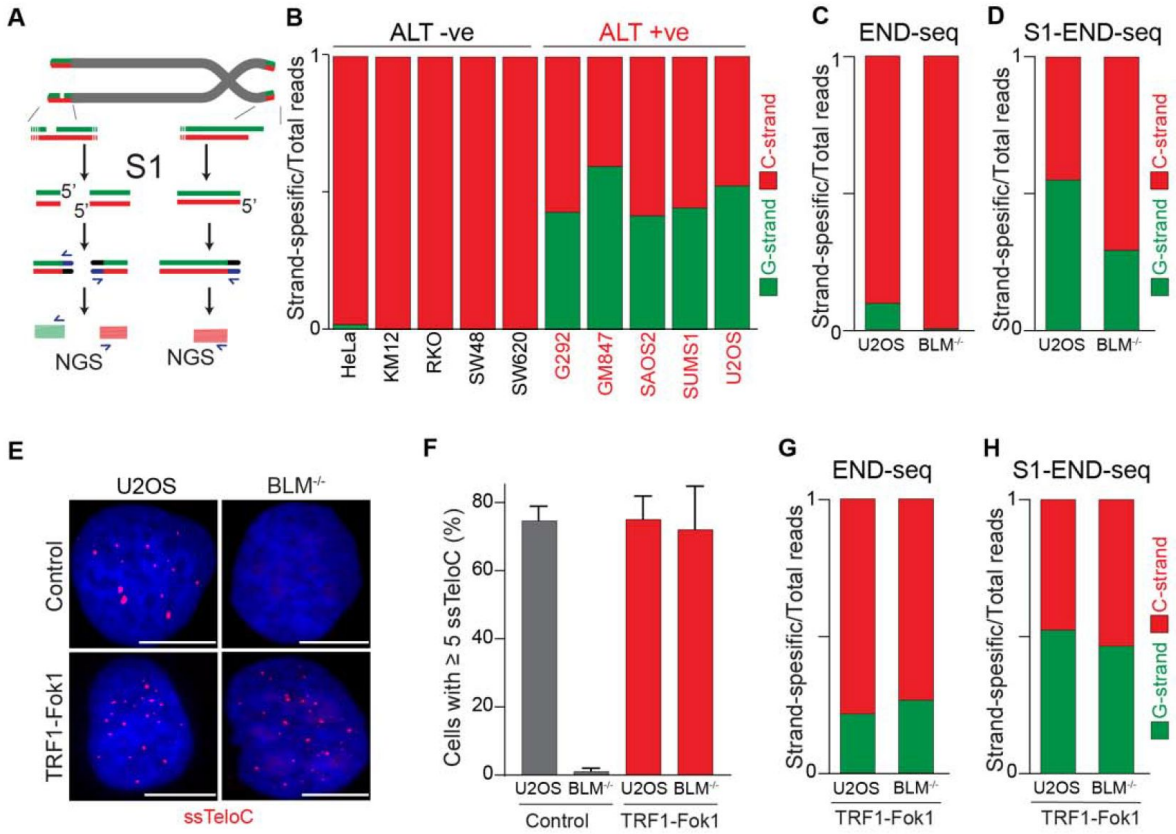


Fig 4.

ALT telomeres are readily distinguished due to presence of telomeric ssDNA by sequencing

A. Schematic Representation of the S1-END-seq method applied to telomeres containing either an internal ssDNA region (left) or only the natural G-rich overhang (right). S1 nuclease treatment cleaves ssDNA regions generating two ended DSB (left) or one ended DSB (right). DNA ends are ligated to biotinylated adaptors, purified, and subsequently sequenced by NGS. Reads originating from the double ended DSB align to either side of the break (left), resulting in C-rich (red) and G-rich (green) reads. Reads originating from the single-ended chromosome ends align only to the C-rich template (red). **B**. Proportion of telomeric reads (containing at least 4 consecutive telomeric reads) corresponding to G-rich reads (green) or C-rich reads (red). **C-D**. Proportion of telomeric reads (containing at least 4 consecutive telomeric reads) corresponding to G-rich reads (green) or C-rich reads (red) in BLM proficient (U2OS) or deficient ($BML^{-/-}$) cells by END-seq (**C**) or S1-seq (**D**). **E**. Native telomeric FISH (ssTelo) in BLM proficient (U2OS) or deficient ($BML^{-/-}$) cells either left untreated (control) or expressing TRF1-Fok1. The scale bar represents 10 μm. **F**. Quantification of the data shown in panel E, cells with 5 ssTelo signal (or grater) were scored as positive. When indicated, cells were induced to express the TRF1-Fok1 nuclease for 24 hours prior to harvesting. **G-H**. Proportion of telomeric reads (containing at least 4 consecutive telomeric reads) corresponding to G-rich reads (green) or C-rich reads (red) in BLM proficient (U2OS) or deficient ($BML^{-/-}$) cells expressing TRF1-Fok1 by END-seq (**G**) or S1-seq (**H**).

deficient cells by END-seq were coming from the C-strand (**Figure 4C**). As a control, we tested whether artificially introducing DSBs at telomeres using the TRF1-FokI construct [46] would abrogate the difference in BLM-proficient and deficient cells. Expression of TRF1-Fok1 results in DSBs at telomeres, which are detected as the appearance of G-rich reads by END-seq in both BLM proficient and BLM-deficient cells (**Figure 4G**). Expression of the TRF1-FokI nuclease in BLM-deficient cells was sufficient to induce single-stranded telomeric DNA at telomeres as seen by ssTelo signal (**Figures 4E** and 4F) as well as a C/G strand ratio close to what was seen in U2OS cells (**Figure 4H**).

Collectively, these data show that cells undergoing ALT have multiple single-stranded DNA regions that vastly exceed the number of chromosome ends. Accumulation of single-stranded telomeric DNA is tightly linked to the ALT process itself.

Discussion

END-seq as an unbiased tool to study the terminal telomeric repeat

Here, we show that END-seq can efficiently capture 5' chromosome termini, providing a high-resolution and unbiased approach to studying the terminal portion of chromosome ends. We found that by END-seq, chromosome ends are detected as one-ended DSBs corresponding to the 5' C-rich telomeric strand. Our data show that, in agreement with previous work [34], most chromosomes have the sequence ATC as the 5' end. END-seq has several advantages over alternative approaches: it is unbiased since it does not rely on capture oligos, it can be applied to telomeres of any length, and it does not require any prior knowledge of the subtelomeric sequence. While END-seq is designed to capture chromosome ends in an unbiased manner, we cannot exclude the possibility that certain telomeric structures may hinder detection, potentially leading to underrepresentation of a subset of telomeres. Nevertheless, we have been able to define the terminal identity of chromosome ends in cells with long telomeres, such as murine cells and ALT-positive cells, as well as cells with poorly characterized subtelomeric regions, such as canine cells. These data show that the ATC-5' sequence appears to be universal, highlighting a conserved mechanism of end-processing in mammalian cells. We also examined the effect of the depletion of POT1 paralogs on the 5' chromosome termini in mouse cells. While POT1a depletion altered the sequence at 5' ends, POT1b depletion had no effect. This finding adds to the growing evidence that POT1a and POT1b have distinct functions at mouse telomeres [35, 38, 47-50].

Canonical POT1-dependent regulation of the terminal sequences in ALT-positive cells

Our data show that ALT-positive cells, which use recombination-based mechanisms for telomere elongation, have the same regulation of terminal repeats as telomerase-positive cells. END-seq analysis of three different ALT-positive cell lines (G292, SAOS2 and U2OS) revealed that the majority of telomeric reads correspond to C-rich reads starting with the canonical CCAATC-5' sequence. This indicates that the regulation of the terminal 5' end of chromosome ends occurs independently of the telomere elongation mechanism and that ALT-based recombination does not alter this process. Furthermore, our data show that POT1 is critical, even in U2OS cells, to define the 5' end termini of telomeres.

Single-stranded DNA in ALT telomeres

Using the unbiased S1-END-seq approach, we show that the presence of single-stranded telomeric DNA characterizes ALT-positive cells. This property of ALT is unique in that cells can be readily classified as ALT-positive or ALT-negative based on the occurrence of S1-cuts within the telomeric

reads (**Figure 4B**).The observed single-stranded telomeric DNA may arise from various sources, including R-loops, D-loops, internal DNA loops (i-loops), and extrachromosomal circles, which are known to be prevalent in ALT [20, 30, 51, 52].

Notably, our findings align with previous indirect evidence suggesting that ALT-positive cells exhibit increased levels of single-stranded telomeric DNA, RPA-staining and FISH under native conditions [19, 29, 53, 54]. Based on our data, we can now conclude that these assays are all indeed detecting the telomeric ssDNA. Due to the nature of S1 cleavage and the repetitive nature of telomeric reads, it is impossible to establish how many regions within telomeres have ssDNA or the average length of the single-stranded DNA tract within telomeres. However, based on the relative ratio between G-rich and C-rich strands, we estimate that there are at least 5 distinct regions of single-stranded DNA for each chromosome end in ALT-positive cells (Figure S4G). The origin of the observed single-stranded telomeric DNA remains to be determined. It is possible that, in ALT cells, partially single-stranded extrachromosomal DNA in the form of C-circles is captured by the S1-END-seq approach. Alternatively, we may be detecting intermediates of the ALT process at chromosome ends or single-stranded DNA generated by strand displacement due to the presence of R-loops and/or G-quadruplexes. Future studies will be needed to elucidate the mechanisms underlying the generation of ssDNA at telomeres in ALT cells. Nevertheless, our findings provide a rationale for using ssTelo as a biomarker for ALT-positive cell detection, offering new avenues for studying and targeting ALT-driven cancers [19, 53-55].

Material and Methods

Cell culture

Human, mouse, and dog cell lines were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 0.5 mg/ml penicillin, streptomycin and L-glutamine (Gibco) and cultured at 37°C and 5%CO_2. HeLa cells used in this study were HeLa1.2.11. G292 cells were grown in McCoy medium supplement with 15% FBS and 0.5 mg/ml penicillin, streptomycin and L-glutamine (Gibco). Cell lines were tested for mycoplasma contamination. BLM knock-out cells were previously described in [19], canine cell line KOS was a kind gift of Dr. Amy LeBlanc (NCI).

Generation of mESCs lines

Knockout and knock-in cell lines were generated via CRISPR/Cas9 gene targeting by transient transfection using Lipofectamine 2000 (ThermoFisher Scientific). Cells were transfected with a plasmid encoding SpCas9 (px330) and a plasmid encoding specific gRNAs (backbone Addgene 41824). To introduce the dTAG13 controllable FKBP-tag POT1a in mESCs via homologous recombination-mediated repair, a synthetic repair template containing homology arms to the *Pot1a* locus, a blasticidin resistance marker, a 3xFLAG tag, and an FKBP tag was used, along with 2 sgRNA targeting the 5' region of *Pot1a* (guide 1: 5'-TTACATTAGTGCTAATCCAA-3', and guide 2: 5'-TTTTCATCAAACAATGTCTT-3'). To generate POT1b$^{-/-}$ cells two gRNAs were used: guide 1 (5'-GTTCCTTTGCTTAAGTACGG-3') and guide 2 (5'-GACATTTGATGGCACCTTAG-3'). Transfected cells were single-cell cloned, and resulting clones were screened by PCR to confirm the desired genomic deletion, which was further validated by Sanger sequencing.

Lentiviral-mediated gene manipulation

Lentiviral particles were generated by transfection into HEK293T followed by isolation and transduction as previously described [19]. Lentiviral expression was achieved with the following constructs and procedure:

POT1 depletion

sgRNA targeting POT1 (POT1-1: TGTAGCTTGATCAGACACTTA, POT1-2: TATGTATGCTAAATTGGATGG) were cloned into an inducible lentiviral vector (pRSITEP-U6Tet-sh-EF1-TetRep-2A-Puro) (Cellecta). Following infection, cells were treated with 1 μg/ml of doxycycline to induce shRNA expression and harvested 3 days later.

TRF1-Fok1 expression

pLenti-CMV-TRE3G-ER-TRF1-Fok1 expressing plasmids [20] were a kind gift from Agnel Sfeir. Infected cells were treated with 1 μg/ml of doxycycline and 0.6 μM of 4-hydroxytamoxifen (4-OHT) to induce TRF1-Fok1 and harvested 24 hours later.

RNA isolation and qRT-PCR

Total RNA was extracted from cells using the RNeasy Plus Mini Kit (Qiagen), and cDNA was made using SuperScript II Reverse Transcriptase (ThermoFisher). qPCR was performed using Power SYBR Green PCR Master (Fisher Scientific). Samples were run on a BioRad CFX Opus 96 and analyzed on BioRad CFX Maestro. The following primers were used in this study: hPOT1: 5'-CAGAACCTGACGACAGCTTTCC and 5'-GCACATAGTGGTGTCCTCTCCA. hGAPDH: 5'-GTCTCCTCTGACTTCAACAGCG and ACCACCCTGTTGCTGTAGCCAA. mPOT1b: 5'-TTCGGCCCCAGTAGCACCTT and 5'-TCTCTTGCTTAAAGTACGCAG. mGAPDH: 5'-TGTGTCCGTCGTGGATCTGA and 5'-TTGCTGTTGAAGTCGCAGGAG.

Immunofluorescence (IF) and FISH-based imaging

IF, IF-FISH and ssTelo staining were carried out as described previously [19, 56].

PNA probes used in this study were TelC-Cy3 (PNABio, Catalog number F1002) and TelG-Cy3 PNA probe (PNABio, Catalog number F1006).

Primary antibodies used in this study were TRF2 (NB110-57130, Novus Biologicals, rabbit), RPA32/RPA2 (2208, Cell Signaling, rat), Myc (2276, Cell Signaling, mouse) and 53BP1 (NB100-304, Novus Biologicals, rabbit).

Imaging and image analysis

Images were acquired using a Zeiss Axio Imager M2 with an Axiocam 702 camera and ZEN 2.6 (blue edition) software; for each experiment and condition, a minimum of 250 cells were imaged. Final figures were assembled using Adobe Photoshop 25.7.0 and Adobe Illustrator 28.5.

END-seq & S1-END-seq

For END-seq and S1-END-seq, 6–20 x10^6 cells were collected, embedded in 1% agarose plugs, and processed as previously reported [32, 33]. For mouse spleen library, mice were euthanized with CO_2 following ethical guidelines, and the spleens were surgically removed. Spleen tissues were mashed through a 70 μm cell strainer to obtain a single-cell suspension. The suspension was then centrifuged for 5 mins at 300x g, the pellet was washed with ice-cold PBS three times before proceeding with library preparation. Briefly, plugs were incubated with proteinase K solution for 1 hr at 50°C and then for 7 hrs at 37 °C, followed by consecutive washes in a wash buffer (10 mM Tris, pH 8.0, and 50 mM EDTA) and TE (10 mM Tris, pH 8.0, and 1 mM EDTA).

Washed plugs were then treated with RNAseA (Puregene, QIAGEN), washed again in Wash Buffer, and stored at 4°C for up to 2 weeks. End blunting, A-tailing, and ligation to biotinylated hairpin adaptor 1 (ENDseq-adaptor-1, 5'-Phos-GATCGGAAGAGCGTCGTGTAGGGAAAGAGTGUU[Biotin-

dT]U[Biotin-dT]UUACACTCTTTCCCTACACGACGCTCTTCCGATC*T-3' [*phosphorothioate bond]) were performed in the plug to minimize shearing and *in vitro* damage. Following this, high molecular weight DNA was isolated from agarose plugs and fragmented by sonication. Subsequently, adaptor-ligated fragments were captured with streptavidin-coated beads and sonication-created ends were repaired, A-tailed, and ligated with a distal adaptor (ENDseq-adaptor-2, 5'-Phos-GATCGGAAGAGCACACGTCUUUUUUUUAGACGTGTGCTCTTCCGATC*T-3' [*phosphorothioate bond]). Subsequent PCR amplification resulted in ready-to-sequence libraries where the sequenced first base corresponds precisely to the first base of the blunted DSB on either side of the break. For S1-END-seq, proteinase K and RNAseA treated plugs were treated with 100 U of S1 nuclease (Thermo Fisher) at 37 °C for 30 minutes and the reaction was terminated by the addition of EDTA to a final concentration of 10 mM. S1-treated plugs were then processed through the standard END-seq protocol. For T7 treatment, proteinase K and RNAseA treated plugs were incubated with 50 U of T7 exonuclease (NEB) at 37 °C for 30 minutes before processing through the standard END-seq protocol.

The libraries were sequenced on the Nextseq 550 or Nextseq 2000 platform (Illumina), using 75-bp single-end read kits. For each treatment condition, the control (non-treated) and sample libraries were sequenced in parallel in the same flow cell.

Telomeric Motif Detector and Quantifier

We developed a Python script, "getTelomereENDseq," to analyze gzipped FASTQ files for the presence of telomeric motifs in DNA sequences on a command-line environment using a Linux-based operating system and the statistical environment RStudio. The script identifies sequences containing G-rich and C-rich telomeric repeats, quantifies their occurrences, and calculates their relative frequencies. It takes a gzipped FASTQ file as input, and the minimum number of telomeric repeats is required. It generates two output files: one with sequences containing G-rich motifs and another with sequences containing C-rich motifs. For this study, we used a minimum of 4 consecutive TTAGGG repeats for G-rich reads and a minimum of 4 consecutive CCCTAA repeats for C-rich reads. Following this, in RStudio, using the stringi, ggseqlogo, ggplot2, ggsci, and tidyverse packages, the telomeric reads were trimmed by the last six nucleotides, reversed, and organized into data frames. These data frames were then used to plot the frequencies of telomeric repeats or motifs.

Statistical analysis and data visualization

We used RStudio and GraphPad Prim v10 for data visualization and statistical analysis. Statistical analyses are specified in the figure legends. For microscopy data, $P < 0.05$ was considered statistically significant, and P values were assessed by unpaired parametric t-test. The sample size was not predetermined. Statistical analyses are specified in the figure legends. We used Kullback-Leibler (KL) divergence to compare the distributions of frequencies of telomeric repeats between different samples. The KL divergence is a measure of the difference between two probability distributions. It quantifies how one probability distribution diverges from a second, expected distribution. To compute KL divergence, we used the philentropy package in RStudio. For this study, KL divergence values smaller than 0.125 are considered non-significant, and the rest assigned as follows: • indicates KL divergence value between 0.125 and 0.25, •• indicates a value between 0.25 and 0.375, and ••• indicates a value greater than 0.375.

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We are grateful to Agnel Sfeir, Amy LeBlanc, and Gianna Tricola for providing reagents. We thank the CCR Genomic Core for assistance with the NGS experiments performed in this study. We thank members of the Lazzerini Denchi laboratory, Sam John, Niek van Wietmarschen and Travis

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Additional information

Author contributions

B.A. and E.L.D. designed the project; B.A. performed experiments and bioinformatic analyses; R.P. and A.N. helped optimizing END-seq and S1-END-seq and provided critical suggestions; W.W. helped with bioinformatic analyses of END-seq and S1-END-seq data; R.S generated mESCs lines; T.M. isolated mouse tissues used in the study; E.L.D supervised the project; B.A and E.L.D wrote the manuscript.

Additional files

Supplementary text and figures

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Reviewer #1 (Public review):

Summary

This manuscript from Azeroglu et al. presents the application of END-Seq to examine the sequence composition of chromosome termini, i.e., telomeres. END-seq is a powerful genome sequencing strategy developed in Andre Nussesweig's lab to examine the sequences at DNA break sites. Here, END-Seq is applied to explore the nucleotide sequences at telomeres and to ascertain (i) whether the terminal end sequence is conserved in cells that activate ALT telomere elongation mechanism and (ii) whether the processes responsible for telomere end sequence regulation are conserved. With these aims clearly articulated, the authors convincingly show the power of this technique to examine telomere end-processing.

Strengths

(1) The authors effectively demonstrate the application of END-seq for these purposes. They verify prior data that 5'terminal sequences of telomeres in Hela and RPE cells end in a canonical ATC sequence motif. They verify that the same sequence is present at the 5' ends of telomeres by performing END-seq across a panel of ALT cancer cells. As in non-ALT cells, the established role of POT1, a ssDNA telomere binding protein, in coordinating the mechanism that maintains the canonical ATC motif is likewise verified. However, by performing END-Seq in mouse cells lacking POT1 isoforms, POT1a and POT1b, the authors uncover that POT1b is dispensable for this process. This reveals a novel, important insight relating to the evolution of POT1 as a telomere regulatory factor.

(2) The authors then demonstrate the utility of S1-END-seq, a variation of END-Seq, to explore the purported abundance of single-stranded DNA at telomeres within telomeres of ALT cancer cells. Here, they demonstrate that ssDNA abundance is an intrinsic aspect of ALT telomeres and is dependent on the activity of BLM, a crucial mediator of ALT.

Overall, the authors have effectively shown that END-seq can be applied to examine processes maintaining telomeres in normal and cancerous cells across multiple species. Using END-Seq, the authors confirm prior cell biological and sequencing data and the role of POT1 and BLM in regulating telomere termini sequences and ssDNA abundance. The study is nice and well-written, with the experimental rationale and outcomes clearly explained.

Weaknesses

This reviewer finds little to argue with in this study. It is timely and highly valuable for the telomere field. One minor question would be whether the authors could expand more on the application of END-Seq to examine the processive steps of the ALT mechanism? Can they speculate if the ssDNA detected in ALT cells might be an intermediate generated during BIR (i.e., is the ssDNA displaced strand during BIR) or a lesion? Furthermore, have the authors assessed whether ssDNA lesions are due to the loss of ATRX or DAXX, either of which can be mutated in the ALT setting?

Comments on revisions:

The authors addressed the comments. Thank you.

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Reviewer #2 (Public review):

This is a short yet very clear manuscript demonstrating that two methods (END-seq and S1-END-seq), previously developed in the Nussenzweig laboratory to study DSBs in the genome, can also be applied to the 5' ends of mammalian telomeres and the accumulation of telomeric single-stranded DNA.

The authors first validate the applicability of END-seq using different approaches and confirm that mammalian telomeres preferentially end with an ATC 5' end through a mechanism that requires intact POT1 (POT1a in mice). They then extend their analysis to cells that maintain telomeres through the ALT mechanism and demonstrate that, in these cells as well, telomeres frequently end in an ATC 5' sequence via a POT1-dependent mechanism. Using S1-END-seq, the authors further show that ALT telomeres contain single-stranded DNA and estimate that each telomere in ALT cells harbors at least five regions of ssDNA.

I find this work very interesting and incisive. It clearly demonstrates that END-seq can be applied with unprecedented depth and precision to the study of telomeric features such as the 5' end and ssDNA. The data are very clear and thoroughly interpreted, and the manuscript is well written. The results are carefully analyzed and effectively presented. Overall, I find this manuscript worthy of publication, as the optimized END-seq methods described here will likely be widely utilized in the telomere field.

Also, the authors have satisfactorily addressed my previous comments.

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Reviewer #3 (Public review):

Summary:

A subset of cancer cells attain replicative immortality by activating the ALT mechanism of telomere maintenance, which is currently the subject of intense research due to its potential for novel targeted therapies. Key questions remain in the field, such as whether ALT telomeres adhere to the same end-protection rules as telomeres in telomerase-expressing cells, or if ALT telomeres possess unique properties that could be targeted with new, less toxic cancer therapies. Both questions, along with the approaches developed by the authors to address them, are highly relevant.

Strengths:

Since chromosome ends resemble one-ended DSBs, the authors hypothesized that the previously described END-SEQ protocol could be used to accurately sequence the 5' end of telomeres on the C-rich strand. As expected, most reads corresponded to the C-rich strand and, confirming previous observation by the de Lange's group, most chromosomes end with the ATC-5' sequence, a feature that was found to be dependent on POT1 and to be conserved in both human ALT cells and mouse cells. Through a complementary method, S1-END-SEQ, the authors further explored ssDNA regions at telomeres, providing new insights into the characteristics of ALT telomeres. The study is original, the experiments were well-controlled and excellently executed.

Weaknesses:

A few additional experiments would have strengthened the results such as combining error-free long-read sequencing with END-SEQ to compare the abundance of VTRs within telomeres versus at their distal ends.
Along this line, are VTRs increased at ssDNA regions of ALT telomeres? What is the frequency of VTRs in the END-SEQ analysis of TRF1-FokI-expressing ALT cells? Is it also increased? Has TRF1-FokI been applied to telomerase-expressing cells to compare VTR frequencies at internal sites between ALT and telomerase-expressing cells?
To what extent do ECTRs contribute to telomeric ssDNA?
Future experiments may help shed light on this

https://doi.org/10.7554/eLife.106657.2.sa1

Author Response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Recommendations for the authors):***
>
> *One minor question would be whether the authors could expand more on the application of END-Seq to examine the processive steps of the ALT mechanism? Can they speculate if the ssDNA detected in ALT cells might be an intermediate generated during BIR (i.e., is the ssDNA displaced strand during BIR) or a lesion? Furthermore, have the authors assessed whether ssDNA lesions are due to the loss of ATRX or DAXX, either of which can be mutated in the ALT setting?*

We appreciate the reviewer's insightful questions regarding the application of our assays to investigate the nature of the ssDNA detected in ALT telomeres. Our primary aim in this study was to establish the utility of END-seq and S1-END-seq in telomere biology and to demonstrate their applicability across both ALT-positive and -negative contexts. We agree

that exploring the mechanistic origins of ssDNA would be highly informative, and we anticipate that END-seq–based approaches will be well suited for such future studies. However, it remains unclear whether the resolution of S1-END-seq is sufficient to capture transient intermediates such as those generated during BIR. We have now included a brief speculative statement in the revised discussion addressing the potential nature of ssDNA at telomeres in ALT cells.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *How can we be sure that all telomeres are equally represented? The authors seem to assume that END-seq captures all chromosome ends equally, but can we be certain of this? While I do not see an obvious way to resolve this experimentally, I recommend discussing this potential bias more extensively in the manuscript.*

We thank the reviewer for raising this important point. END-seq and S1-END-seq are unbiased methods designed to capture either double-stranded or single-stranded DNA that can be converted into blunt-ended double-stranded DNA and ligated to a capture oligo. As such, if a subset of telomeres cannot be processed using this approach, it is possible that these telomeres may be underrepresented or lost. However, to our knowledge, there are no proposed telomeric structures that would prevent capture using this method. For example, even if a subset of telomeres possesses a 5' overhang, it would still be captured by END-seq. Indeed, we observed the consistent presence of the 5'-ATC motif across multiple cell lines and species (human, mouse, and dog). More importantly, we detected predictable and significant changes in sequence composition when telomere ends were experimentally altered, either in vivo (via POT1 depletion) or in vitro (via T7 exonuclease treatment). Together, these findings support the robustness of the method in capturing a representative and dynamic view of telomeres across different systems.

That said, we have now included a brief statement in the revised discussion acknowledging that we cannot fully exclude the possibility that a subset of telomeres may be missed due to unusual or uncharacterized structures

> *I believe Figures 1 and 2 should be merged.*

We appreciate the reviewer's suggestion to merge Figures 1 and 2. However, we feel that keeping them as separate figures better preserves the logical flow of the manuscript and allows the validation of END-seq and its application to be presented with appropriate clarity and focus. We hope the reviewer agrees that this layout enhances the clarity and interpretability of the data.

> *Scale bars should be added to all microscopy figures.*

We thank the reviewer for pointing this out. We have now added scale bars to all the microscopy panels in the figures and included the scale details in the figure legends.

> ***Reviewer #3 (Recommendations for the authors):***
>
> *Overall, the discussion section is lacking depth and should be expanded and a few additional experiments should be performed to clarify the results.*

We thank the reviewer for the suggestions. Based on this reviewer's comments and comments for the other reviewers, we incorporated several points into the discussion. As a result, we hope that we provide additional depth to our conclusions.

> *(1) The finding that the abundance of variant telomeric repeats (VTRs) within the final 30 nucleotides of the telomeric 5' ends is similar in both telomerase-expressing and ALT cells is intriguing, but the authors do not address this result. Could the authors provide more insight into this observation and suggest potential explanations? As the frequency of VTRs does not seem to be upregulated in POT1-depleted cells, what then drives the appearance of VTRs on the C-strand at the very end of telomeres? Is CST-Pola complex responsible?*

The reviewer raises a very interesting and relevant point. We are hesitant at this point to speculate on why we do not see a difference in variant repeats in ALT versus non-ALT cells, since additional data would be needed. One possibility is that variant repeats in ALT cells accumulate stochastically within telomeres but are selected against when they are present at the terminal portion of chromosome ends. However, to prove this hypothesis, we would need error-free long-read technology combined with END-seq. We feel that developing this approach would be beyond the scope of this manuscript.

> *(2) The authors also note that, in ALT cells, the frequency of VTRs in the first 30 nucleotides of the S1-END-SEQ reads is higher compared to END-SEQ, but this finding is not discussed either. Do the authors think that the presence of ssDNA regions is associated with the VTRs? Along this line, what is the frequency of VTRs in the END-SEQ analysis of TRF1-FokI-expressing ALT cells? Is it also increased? Has TRF1-FokI been applied to telomerase-expressing cells to compare VTR frequencies at internal sites between ALT and telomerase-expressing cells?*

Similarly to what is discussed above, short reads have the advantage of being very accurate but do not provide sufficient length to establish the relative frequency of VTRs across the whole telomere sequence. The TRF1-FokI experiment is a good suggestion, but it would still be biased toward non-variant repeats due to the TRF1-binding properties. We plan to address these questions in a future study involving long-read sequencing and END-seq capture of telomeres.

> *Finally, in these experiments (S1-END-SEQ or END-SEQ in TRF1-Fok1), is the frequency of VTRs the same on both the C- and the G-rich strands? It is possible that the sequences are not fully complementary in regions where G4 structures form.*

We thank the reviewer for this observation. While we do observe a higher frequency of variant telomeric repeats (VTRs) in the first 30 nucleotides of S1-END-seq reads compared to END-seq in ALT cells, we are currently unable to determine whether this difference is significant, as an appropriate control or matched normalization strategy for this comparison is lacking. Therefore, we refrain from overinterpreting the biological relevance of this observation.

The reviewer is absolutely correct. Our calculation did not exclude the possibility of extrachromosomal DNA as a source of telomeric ssDNA. We have now addressed this point in our discussion.

The reviewer is correct in pointing out that we still do not know what causes ssDNA at telomeres in ALT cells. Replication stress seems the most logical explanation based on the work of many labs in the field. However, our data did not reveal any significant difference in the levels of ssDNA at telomeres in non-ALT cells based on telomere length. We used the HeLa1.2.11 cell line (now clarified in the Materials section), which is the parental line of HeLa1.3 and has similarly long telomeres (~20 kb vs. ~23 kb). Despite their long telomeres and potential for replication-associated challenges such as G-quadruplex formation, HeLa1.2.11 cells did not exhibit the elevated levels of telomeric ssDNA that we observed in

ALT cells (Figure 4B). Additional experiments are needed to map the occurrence of ssDNA at telomeres in relation to progression toward ALT.

> *(3) Based on the ratio of C-rich to G-rich reads in the S1-END-SEQ experiment, the authors estimate that ALT cells contain at least 3-5 ssDNA regions per chromosome end. While the calculation is understandable, this number could be discussed further to consider the possibility that the observed ratios (of roughly 0.5) might result from the presence of extrachromosomal DNA species, such as C-circles. The observed increase in the ratio of C-rich to G-rich reads in BLM-depleted cells supports this hypothesis, as BLM depletion suppresses C-circle formation in U2OS cells. To test this, the authors should examine the impact of POLD3 depletion on the C-rich/G-rich read ratio. Alternatively, they could separate high-molecular-weight (HMW) DNA from low-molecular-weight DNA in ALT cells and repeat the S1-END-SEQ in the HMW fraction.*

The reviewer is absolutely correct. Our calculation did not exclude the possibility of extrachromosomal DNA as a source of telomeric ssDNA. We have now addressed this point in our discussion.

> *(4) What is the authors' perspective on the presence of ssDNA at ALT telomeres? Do they attribute this to replication stress? It would be helpful for the authors to repeat the S1-END-SEQ in telomerase-expressing cells with very long telomeres, such as HeLa1.3 cells, to determine if ssDNA is a specific feature of ALT cells or a result of replication stress. The increased abundance of G4 structures at telomeres in HeLa1.3 cells (as shown in J. Wong's lab) may indicate that replication stress is a factor. Similar to Wong's work, it would be valuable to compare the C-rich/G-rich read ratios in HeLa1.3 cells to those in ALT cells with similar telomeric DNA content.*

The reviewer is correct in pointing out that we still do not know what causes ssDNA at telomeres in ALT cells. Replication stress seems the most logical explanation based on the work of many labs in the field. However, our data did not reveal any significant difference in the levels of ssDNA at telomeres in non-ALT cells based on telomere length. We used the HeLa1.2.11 cell line (now clarified in the Materials section), which is the parental line of HeLa1.3 and has similarly long telomeres (~20 kb vs. ~23 kb). Despite their long telomeres and potential for replication-associated challenges such as G-quadruplex formation, HeLa1.2.11 cells did not exhibit the elevated levels of telomeric ssDNA that we observed in ALT cells (Figure 4B). Additional experiments are needed to map the occurrence of ssDNA at telomeres in relation to progression toward ALT.

Finally, Reviewer #3 raises a list of minor points:

(1) The Y-axes of Figure 4 have been relabeled to account for the G-strand reads.

(2) Statistical analyses have been added to the figures where applicable.

(3) The manuscript has been carefully proofread to improve clarity and consistency throughout the text and figure legends

(4) We have revised the text to address issues related to the lack of cross-referencing between the supplementary figures and their corresponding legends.

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