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5' C-rich telomeric overhangs are an outcome of rapid telomere truncation events

Liana Oganessian¹ and Jan Karlseder^{1,2}

¹The Salk Institute for Biological Studies, Molecular and Cellular Biology Department, 10010 North Torrey Pines Rd., La Jolla, CA92037, USA

Abstract

A subset of human tumors ensures indefinite telomere length maintenance by activating a telomerase-independent mechanism known as Alternative Lengthening of Telomeres (ALT). Most tumor cells of ALT origin share a constellation of unique characteristics, which include large stores of extra-chromosomal telomeric material, chronic telomere dysfunction and a peculiar enrichment in chromosome ends with 5' C-rich overhangs. Here we demonstrate that acute telomere de-protection and the subsequent DNA damage signal are not sufficient to facilitate formation of 5' C-overhangs at the chromosome end. Rather chromosome ends bearing 5' C-overhangs are a by-product of rapid cleavage events, processing of which occurs independently of the DNA damage response and is partly mediated through the XRCC3 endonuclease.

Keywords

Telomeres; C-overhangs; T-loops

1. Introduction

Telomeres are universal nucleoprotein caps that mark the end of linear chromosomes across species. Tumor cells acquire mechanisms to sustain telomere length indefinitely, which is sufficient to evade replicative aging and attain immortality. The majority of human cancers rely on activation of telomerase [1] as their means of telomere length maintenance. However, a distinct telomerase-independent mechanism, termed ALT, is active in a subset of tumors [2]. ALT is based on deregulated homologous recombination (HR) events occurring specifically at telomeres, sparing other regions of the genome [3–5]. Prominent telomere length heterogeneity, abundance of linear and circular extra-chromosomal telomeric repeats (ECTR) and chronic telomere dysfunction [6], manifested as the constitutive presence of DNA damage sensor/repair proteins at chromosome ends, are some of the features collectively peculiar to ALT tumors.

The precise inner-workings of the ALT pathway have been challenging to decipher. A 3' single-stranded overhang is a prerequisite for any given recombination event. Such a

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²To whom correspondence should be addressed: Karlseder@salk.edu, phone: 858 453 4100 x1867, fax: 858 457 4765.

Conflict of Interest

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structural motif, known as the 3' G-rich overhang, due to its 5'-3' orientation and G-rich composition, is thought to be a signature hallmark of all telomeres [7]. The G-overhang is of essence to telomere integrity as it is the structural motif that facilitates the formation of a protective higher-order structure referred to as the T-loop [8], postulated to sequester the overhang and thus hinder unwanted DNA repair and nuclease activities at the chromosome end. While the 3' G-overhang is documented to be a universally conserved structural feature at the chromosome end of most species [9], recently the presence of its 5' C-rich counterpart was also described in the nematode *C. elegans* [10]. In this organism single stranded G- and C-rich telomeric tails are equally abundant and are sequence specifically bound by two distinct proteins. Curiously, loss of the protein with specific affinity for the C-overhang in worms causes severe telomere length heterogeneity and enrichment in ECTR. This implicates the telomeric C-overhang in HR-driven telomere maintenance, raising the possibility that it may represent a precursor and/or a byproduct of aberrant homologous recombination at worm and, conceivably, human telomeres. In agreement, human tumors engaged in ALT exhibit elevated levels of 5' C-rich telomeric overhangs, which occur at a frequency comparable to that of G-overhangs [11].

This study aims to delineate the conditions necessary for the formation of 5' C-overhangs at the chromosome end of tumor cells engaged in the ALT pathway of telomere maintenance. More specifically to address whether a constitutive DNA damage signal at the chromosome end is a prerequisite for 5' C-overhang formation in ALT or other contexts. Thus we examined C-overhang dynamics in response to induced telomere truncation events, which were or were not accompanied by telomere de-protection and a subsequent DNA damage response.

2. Materials and methods

2.1. Cell lines and retroviral transduction

Cells were cultured in Glutamax-DMEM (Gibco) supplemented with 0.1 mM non-essential amino acids and either 10 % (for HT1080) or 15 % (for IMR90 and WRN syndrome fibroblasts) fetal bovine serum or 10 % bovine growth serum (for KMST6). WRN syndrome fibroblasts (AG05229C, Coriell Cell Repositories) and young (PD 25) IMR90 fibroblasts were infected with HPV16 E6 and E7 vectors as described [12, 13]. HT1080 cells expressing pBabePuroU3hTR or pBabePuro empty vector at PD 10 and 250 [14] were a kind gift from the Reddel Laboratory (Sydney, Australia).

2.2. Antibodies and Western Blotting

Whole cell lysates (20–30 µl) equivalent to 200,000–300,000 cells were loaded per well of an SDS-PAGE gel. Rabbit anti-TRF2 (in-house), mouse anti-XRCC3 (Santa Cruz sc-53471, 1:250), mouse anti-tubulin (Sigma T6557, 1:20,000) were used for immunoblotting.

2.3. 1D/2D gel electrophoresis

DNA preparation, electrophoresis, hybridization and exposure were performed as described [11].

2.4. T-circle amplification assays

The protocol for T-circle amplification was modified from [15]. Genomic DNA was prepared as described for 1D/2D gel analysis. The solubilized DNA was phenol/chloroform purified, precipitated, resuspended in water, denatured at 96°C for 5 min and annealed to 50 pmol of the telomere-specific primer (AATCCC)₅ for 30–60 min at RT in a buffer containing 200 mM Tris-HCl pH 7.5, 200 mM KCl and 1 mM EDTA in a total volume of 20 µl. Half (10 µl) of the annealed reaction was incubated at 30°C for 12 hours in the presence

of dNTPs (0.2 mM each), BSA (NEB; 0.2 mg/ml), ϕ 29 buffer (NEB) and ϕ 29 DNA polymerase (NEB; 7.5 U) in a total volume of 20 μ l, and the remaining half was incubated under identical conditions with one exception; the ϕ 29 DNA polymerase was excluded from the reaction. Both sets of reactions that did or did not include ϕ 29 were further incubated at 65°C for 20 min. The samples were then electrophoresed on 0.5 % agarose gels at 120 V for 1 hour then 38–40 V overnight in 0.5 x TBE buffer in the presence of ethidium bromide. After electrophoresis the gels were processed in the same manner as that described for denatured 1D/2D gels. Radiolabeled (TTAGGG)₄ oligonucleotide probe was used for in-gel hybridizations.

2.5. IF-FISH

Cell preparation and staining were conducted as described [6].

2.6. siRNA treatments

IMR90 E6/E7 cells stably expressing either TRF2^{ΔB} or pLPC vector control on day 2 post selection were seeded at a low confluency (700,000 cells in a 10 cm dish) in antibiotic free medium one day prior to siRNA treatment. ON-TARGET^{plus} SMART pool of four siRNAs (Dharmacon; AAACUGAAAUCGGUAAAGG, UGUUGGAGUGUGUGAAUAA, GGACCUGAAUCCCAGAAUU, GGACCAGACUUGAAGAGAC) against XRCC3 or a control non-targeting pool (catalogue # D-001810-10-20) were transfected into cells at 50 nM final concentration using DharmaFect1 (Dharmacon) transfection reagent (10 μ l per 10 cm dish). The cells were collected at 72 hours post treatment and processed for protein (Western Blotting) and DNA (1D gel) analyses.

2.7. RecJF treatment

RecJF exonuclease digestion was performed as described previously [11].

3. Results and Discussion

3.1. De-protected telomeres do not bear 5' C-overhangs

Telomeres of tumor cells engaged in the ALT pathway of telomere maintenance are highly recombinogenic, undergoing stochastic gains and losses of telomeric DNA [16], a phenomenon, which manifests itself as pronounced telomere length heterogeneity, an abundance of extra-chromosomal telomeric material [17, 18], a constitutive DNA damage signal [6], and an enrichment of 5' C-rich telomeric overhangs [11]. It has been postulated that loss of telomeric DNA in the context of ALT may occur upon T-loop cleavage events releasing, so called, T-circles, extra-chromosomal circular double-stranded DNA of telomeric sequence, highly abundant in the large majority of ALT tumors [19, 20]. In untransformed cells drastic losses of telomeric DNA in the form of T-loop-sized deletions and the ensuing telomere dysfunction have been attributed to compromised shelterin function [20]. Shelterin is comprised of six telomere-specific proteins, which tightly regulate all transactions that occur at the chromosome end [21], particularly those, which facilitate the formation of the protective T-loop structure. TRF2 is the most significant member of the shelterin complex in dictating T-loop integrity, since introducing a deletion mutant of TRF2 lacking its amino-terminal basic (B) domain (TRF2^{ΔB}), has been shown to result in rapid telomere deletion events, involving formation of extra-chromosomal T-circles in otherwise normal cells [20]. In addition to T-circles these cells exhibit severely truncated telomeres, modest telomere dysfunction and signs of senescence. Conversely, the dominant negative mutant of TRF2, lacking both its basic and carboxy-terminal DNA binding Myb domain (TRF2^{ΔBΔM}) does not result in telomere loss, nor T-circle formation, rather it is reported to precipitate severe telomere dysfunction and a partial loss of 3' G-overhangs accompanied by telomeric fusions [22]. Therefore, expression of these TRF2 deletion mutants that on one

hand cause rapid telomere deletion and on the other hand induce severe telomere dysfunction without telomere loss in primary human cells can be a useful tool to study aspects of ALT metabolism in an otherwise normal setting, especially in light of the knowledge that introducing exogenous TRF2 in ALT cells dampens the frequency of spontaneously occurring DNA damage associated foci [6]. Thus, introducing one or the other mutant can help to determine whether it is the persistent DNA damage signal, as demonstrated by the continual presence of DNA damage markers, or the frequent shedding of telomeric DNA in the form of T-circles that promotes the formation of 5' C-rich telomeric overhangs at the chromosome end.

To this end ΔB and $\Delta B\Delta M$ deletion mutants of TRF2 were individually overexpressed in young primary human fibroblasts (IMR90 PD 23; Fig. 1A). Both mutants yielded the expected degree of DNA damage specifically at telomeres (Supplementary Fig. 1A) as measured by the number of telomere dysfunction-induced foci (TIFs) [23], whereby chromosomal telomeric DNA co-localizes with DNA damage associated proteins, such as $\gamma H2AX$, a phosphorylated form of histone variant H2AX, which normally accumulates at double-stranded DNA breaks. To confirm that the TRF2 ΔB expression gives rise to circular ECTR, T-circle amplification assays (TCA) were performed, which make use of $\phi 29$, a phage DNA polymerase with high strand-displacement activity, which efficiently utilizes a circular template via rolling circle amplification to produce a linear DNA product [15]. As expected, TRF2 ΔB expression resulted in free double-stranded DNA circles, judging from a clear product in TCA assays (Fig. 1B, see arrow), which was close to undetectable in empty vector treated IMR90 cells. Template DNA from an established ALT cell line (KMST6) gave rise to a robust signal, which served as a positive control (Fig. 1B). To determine whether introduction of this mutant indeed causes rapid telomere deletion events resulting in dramatically shorter telomeres, consistent with the presence of circular ECTR, digested genomic DNA from either the ΔB or $\Delta B\Delta M$ or empty vector expressing primary fibroblasts was analyzed on telomere restriction fragment (TRF) gels under native and denatured conditions. As expected, $\Delta B\Delta M$ but not ΔB expressing cells suffered from a partial loss (≈ 20 to 70 %) of 3' G-overhangs (Fig. 1C, upper left panel). Strikingly, native gels revealed the robust appearance of single-stranded (ss) C-rich telomeric DNA in response to the ΔB but not the $\Delta B\Delta M$ TRF2 mutant as early as 3 days and up to 11 days of expression (Fig. 1C, upper right panel). The levels of C-rich DNA waned with time in culture, presumably because cells with shorter telomeres would present a growth disadvantage. No detectable signal for ss C-rich DNA could be detected for DNA from vector only expressing cells. The induction of ss C-rich DNA was concurrent with the appearance of dramatically shortened telomeres in ΔB expressing cells (Fig. 1C, lower panels). The ss C-rich DNA induced as a result of ΔB mutant expression was sensitive to RecJf (5'-3' exonuclease) treatment, suggesting that it is terminal and 5'-3' in orientation, likely corresponding to a 5' C-rich overhang (Supplementary Fig. 1B). Indeed, when analyzed by two-dimensional (2D) gel electrophoresis under native and denatured conditions, the migratory path of at least a part of this ss C-rich telomeric DNA on a native gel corresponded to that observed for a 5' C-rich overhang (Fig. 1D, upper panels). A proportion of this DNA also migrated as linear ss ECTR, which is normally very abundant in tumor cells with activated ALT. Denatured 2D gels reinforced enriched levels of circular double-stranded ECTR (T-circles) in the context of TRF2 ΔB but not TRF2 $\Delta B\Delta M$ expression (Fig. 1D, lower panels). Thus DNA damage alone is not sufficient to allow for processing events leading to 5' C-overhang formation.

3.2. Telomere dysfunction is not required for 5' C-overhang formation

Our findings indicate that formation of 5' C-rich telomeric overhangs is linked with the production of T-circles, which in turn, are a by-product of telomere trimming. This is in agreement with recent findings indicating that T-loop shedding in response to telomerase

upregulation in normal human lymphocytes and germs cells yields telomeres with 5' C-overhangs [24]. Since both ΔB and $\Delta B\Delta M$ mutants of TRF2 give rise to telomere dysfunction, although to a varying degree (Supplemental Fig. 1A), the contribution of DNA damage at telomeres to 5' C-overhang formation is unclear. Nevertheless, the fact that $\Delta B\Delta M$ mutant expression does not induce C-overhangs suggests that telomere dysfunction is not required for generation of telomeric C-overhangs. To test this idea in a setting, in which telomere trimming and release of T-loop-sized DNA occur in the absence of telomere dysfunction, the presence of 5' C-overhangs was probed in an established telomerase positive tumor cell line, which exogenously expresses the telomerase RNA component (HT1080-hTR) [14]. These cells have 'overlengthened' telomeres [14] (Fig. 2A, right panels) and have been shown to shed telomeric DNA in the form of T-circles in the absence of a detectable DNA damage response at the chromosome end [14]. The shedding is postulated to occur as an attempt to negatively regulate telomere length to a homeostatic level. DNA species, corresponding to T-circles is readily detectable on denatured 2D gels in HT1080-hTR cells (Fig. 2B, lower panels). Curiously, hTR overexpression in these cells clearly induces formation of ss C-rich telomeric DNA (Fig. 2A, upper left panel), and since the migration pattern of this DNA is comparable to that of the 3' G-overhang (Fig. 2B, upper panels) and the bulk of the double-stranded telomeric DNA (Fig. 2B, lower panels) on 2D gels then it likely represents the 5' C-rich telomeric overhang. The presence of ss C-rich telomeric DNA in the context of telomerase overexpression was noted previously [14]. This DNA species was speculated to be of extra-chromosomal origin or represent chromosome ends bearing 5' C-overhangs. Although the two propositions are not mutually exclusive, the present study provides credence to the latter speculation.

Collectively, these data strongly suggest that C-overhangs are most likely an outcome of processing events that follow T-loop resolution at the chromosome end and that telomere dysfunction plays at most a minor role in this process.

This conclusion is further supported by the presence of 5' C-rich telomeric overhangs in Werner syndrome (WS) fibroblasts (Fig. 3A). It has been reported that WS protein WRN represses formation of spontaneous telomeric circles, as demonstrated by the elevated levels of T-circles in WS fibroblasts [25] (Fig. 3B). TCA assays performed on DNA from WS fibroblasts concur with this proposition (Fig. 3B).

Thus, the connection between T-loop resolution and 5' C-overhang formation holds true in three distinct settings: in normal human fibroblasts expressing a deletion mutant of TRF2, in telomerase positive cells overexpressing hTR and in WS fibroblasts.

3.3. 5' C-overhang generation at telomeres is partly dependent on XRCC3

In the context of TRF2 ΔB expression it has been shown that T-loop resolution events giving rise to catastrophic telomere deletions, are driven through the unchecked activity of the HR endonuclease XRCC3 [20]. This endonuclease has also been reported to be involved in T-loop cleavage activity as a way of negatively regulating telomere length to prevent excessive telomere elongation by telomerase [24]. Importantly, it has also been shown to be required for T-circle production in ALT cells [26]. To test whether 5' C-rich telomeric overhang formation is dependent on XRCC3, the endonuclease was transiently depleted via RNA interference in normal human fibroblasts stably expressing the ΔB mutant of TRF2 (Fig. 4A). Native TRF analysis showed measurable ($\approx 30\%$) reduction in the levels of ss C-rich telomeric DNA upon XRCC3 depletion in ΔB expressing cells (Fig. 4B upper left panel; 4C), suggesting that XRCC3 is at least partly responsible for generating the C-overhang in the context of T-loop resolution. XRCC3 depletion also resulted in lower levels of G-overhang signal with an approximately 20% drop in this signal (Fig. 4B upper right panel; 4C). This indicates that XRCC3 plays a role in both C- and, to a lesser extent, G-overhang

generation. It remains difficult to speculate whether it is the frequency or length of overhangs, which is being affected.

4. Conclusions

Overall these findings support a model, in which the potential resolution of the T-loop structure has two distinct consequences: release of free circular double-stranded telomeric DNA and chromosome end processing, which yields telomeres bearing 5' C-rich overhangs (Fig. 4C). Our data suggest that at least part of this processing is mediated by XRCC3. Whether T-loops are resolved aberrantly with an accompanying DNA damage response or deliberately as an active mechanism to regulate telomere length without triggering a damage response, appears to have little bearing on C-overhang generation. Thus T-loop release as an attempt to shorten excessively long telomeres is probably the key event, which leads to a high frequency of 5' C-rich telomeric overhangs in ALT tumor cells.

Although the fate of chromosome ends bearing such overhangs in ALT or other contexts remains unknown, it is clear that 5' C-rich overhangs do not necessarily trigger a canonical DNA damage response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. A DNA damage signal is not required for the formation of 5' C-rich overhangs at the chromosome end
2. 5' C-overhangs do not necessarily trigger a DNA damage response
3. 5' C-overhangs are an outcome of telomere truncation events, which give rise to circular extra-chromosomal telomeric repeats
4. De novo formation of telomeric C-overhangs is partly XRCC3 dependent

Figure 1. Telomere rapid deletions but not telomere dysfunction induce C-overhang formation in IMR90 cells

A. Western analysis of IMR90 cells expressing an empty vector or TRF2 deletion mutants. γ -tubulin serves as a loading control. A longer exposure to demonstrate the level of expression of endogenous TRF2 is shown in the lower panel. **B.** T-circle assays for 0.25, 0.5 and 1.0 μ g of digested genomic DNA from IMR90 cells expressing the TRF2 Δ B mutant or vector control. KMST6 DNA was included as a positive control. The presence or absence of ϕ 29 DNA polymerase is indicated. The gel was probed using radiolabeled (TTAGGG)₄ under denaturing conditions. **C.** 1D gels for digested genomic DNA (4 μ g) from IMR90 cells stably expressing the indicated TRF2 mutants or vector control on days 3, 5, 8 and 11 of selection. The gels were probed for G- and C-rich telomeric DNA with radiolabeled (AATCCC)₅ or (TTAGGG)₄ probes respectively, under native (top) and denaturing (bottom) conditions. **D.** 2D gel analysis of digested genomic DNA (20 μ g) from cells expressing the indicated TRF2 mutants on day 3 of selection. A radiolabeled (TTAGGG)₄ probe was used under native (top) and denaturing (bottom) conditions. Schematic diagrams of DNA species are shown on the left.

Figure 2. T-loop deletion events at overlengthened telomeres give rise to telomeric C-overhangs
A. 1D gels for restriction digested genomic DNA (4 µg) from cells overexpressing hTR or vector control at population doublings (PD) 10 or 250. The gels were probed for C-rich (top panels) versus G-rich (bottom panels) telomeric DNA under native (left panels) or denaturing (right panels) conditions as described in Figure 1. The amount of single stranded telomeric DNA in native gels was normalized against the total amount of telomeric DNA in denatured gels and expressed as a ratio indicated at the bottom. **B.** 2D gel analysis of genomic DNA (15 µg) from control or hTR overexpressing cells at PD 250. Gels were probed for both C- and G-strand specific telomeric DNA under native (top) and denaturing (bottom) conditions. The arrows indicate circular double-stranded telomeric DNA (see diagrams in Figure 1D).

Figure 3. T-circles and C-overhangs are enriched in Werner syndrome fibroblasts
A. 2D gel analysis of restriction digested genomic DNA (25 μ g) from E6 and E7 expressing IMR90 or WRN syndrome fibroblasts. Gels were probed for both C- and G-strand specific telomeric DNA under native (top) and denaturing (bottom) conditions. Ethidium bromide staining of digested genomic DNA electrophoresed in the first dimension is indicated to demonstrate equivalent loading. **B.** T-circle assays for increasing concentrations (0.25, 0.5 and 1.0 μ g) of digested genomic DNA from IMR90 or WRN syndrome fibroblasts. Digested DNA from KMST6 cells was included as a positive control. The presence or absence of ϕ 29 DNA polymerase is indicated. The gel was probed as described in Figure 1B.

Figure 4. C-overhang formation is partially XRCC3-dependent

A. Immunoblots of whole cell lysates from E6 and E7 expressing IMR90 cells transduced with TRF2 Δ B or vector (pLPC) control retroviruses (day 4 of selection), and each further treated with either control or XRCC3 siRNAs for 72 hr. The blots were probed for TRF2 (top) or XRCC3 (bottom) expression. γ -tubulin serves as a loading control. **B.** Representative example of 1D gels for digested genomic DNA derived from the cells described in (A). Gels were probed under native (top) and denaturing (bottom) conditions with C- (left panels) or G-strand (right panels) specific telomeric oligonucleotide probes as described for Figures 1–3. **C.** Quantitation of three independent experiments as described in B. **D.** Proposed model for the generation of C-overhangs at the chromosome end. T-loop

release partially mediated by XRCC3 and resulting in free circular ECTR and a shortened telomere is one requirement for C-overhang formation. Chromosome ends bearing a C-rich overhang do not necessarily bear a DNA damage signal.