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Review

Assaying and investigating Alternative Lengthening of Telomeres activity in human cells and cancers

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ABSTRACT

Alternative Lengthening of Telomeres (ALT) activity can be deduced from the presence of telomere length maintenance in the absence of telomerase activity. More convenient assays for ALT utilize phenotypic markers of ALT activity, but only a few of these assays are potentially definitive. Here we assess each of the current ALT assays and their implications for understanding the ALT mechanism. We also review the clinical situations where availability of an ALT activity assay would be advantageous. The prevalence of ALT ranges from 25% to 60% in sarcomas and 5% to 15% in carcinomas. Patients with many of these types of ALT[+] tumors have a poor prognosis.

1. Introduction

All immortalized human cell lines analyzed to date have a telomere length maintenance mechanism (TMM) to compensate for the telomere shortening that accompanies cellular proliferation [1]. In many cell lines, the mechanism involves a ribonucleoprotein enzyme complex, telomerase, that utilizes its integral RNA molecule as a template for reverse transcription of new telomeric DNA [2]. Other cell lines use a non-telomerase mechanism, referred to as Alternative Lengthening of Telomeres (ALT) [3]. Although the definition of ALT encompasses any non-telomerase TMM [4,5], so far there is no clear evidence that there is more than one ALT mechanism in human cells. In contrast to RNA-templated DNA synthesis by telomerase, synthesis of new telomeric DNA by ALT involves the use of a DNA template [6]. As these TMMs are either undetectable or have low levels of activity in normal somatic cells and are relied upon by the vast majority of cancers [7,8], they provide important targets for detection and treatment of cancer cells. ALT is being found in an increasing number of cancer types, including some that have a very poor prognosis with currently available treatments (as discussed below).

Telomerase-independent telomere length maintenance was first described in yeast, where genetic analyses demonstrated its dependence on homologous recombination (HR) [9]. The existence of an ALT mechanism in mammalian cells was deduced from the observation that telomere lengths were maintained for hundreds of population doublings (PD) in telomerase-negative immortalized human cell lines [3,10]. Showing that a cell line can maintain the length of its telomeres in the absence of telomerase for a suitable number of PD (e.g., 20–30) is still the only definitive test for ALT activity, but in a number of regards it is an unsatisfactory assay. First, it is not suitable for detecting ALT activity in human tumors, where it is usually not practicable or ethical to obtain serial samples. Second, the need to demonstrate that the cells or tumors are telomerase-negative means that this assay is not suitable for detecting ALT in situations where both ALT and telomerase may be present. Although there are no known examples of ALT and telomerase being activated spontaneously in the same cells, it has been shown experimentally that both mechanisms can co-exist in cells [11–14]. Third, it takes a long time – 20 PD may take more than a month – and, fourth, this test can only determine whether ALT activity is present and is unable to measure the amount of activity; both of these aspects make it very unsuitable as an assay to screen large numbers of chemical compounds to find ALT inhibitors, and thereby find anticancer treatments that target this mechanism. Finally, the need to demonstrate that telomere length is maintained excludes the possibility of detecting ALT activity levels that are insufficient to completely prevent telomere shortening. The ability to do this will be essential for understanding the role of ALT in normal biology and the potential side-effects of anti-ALT treatments.

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In contrast, there are activity assays for telomerase (the most widely used being the TRAP assay [15]), which was originally detected by its enzymatic activity [16]. This means that it can be detected in tumor samples and cell lines regardless of whether ALT is also present. It can also be detected in some types of normal cells, where the level of telomerase activity is insufficient to completely prevent telomere shortening. Telomerase activity assays are also rapid and can be quantitative. Ideally, assays for ALT activity would be able to provide the same type of information. Telomerase activity assays, however, simply indicate the presence and amount of active enzyme and yield no information about its activity at telomeres [17]. The activity of telomerase in vivo appears to be regulated by the accessibility of telomere termini and by *cis*-acting telomeric factors [18]. In this important regard, analyses of telomerase activity are currently no more advanced than analyses of ALT, because the only way of assessing telomerase activity at human telomeres in vivo is to do medium- to long-term studies of cultured cells to analyze telomere lengths for a suitable number of PD.

Without the availability of an ALT activity assay, the presence of ALT in tumors has been inferred from phenotypic features that are characteristic of ALT[+] cell lines. There is a growing list of these phenotypic characteristics, but in most cases the nature of their relationship to ALT activity is unclear, which means that their use as a surrogate for ALT activity when telomere-related factors are perturbed experimentally is fraught with difficulties of interpretation. However, a few ALT markers may directly assay an aspect of the ALT mechanism or could be based on an intermediary molecule, and hence might be able to be used as a definitive assay for ALT.

Here we review these phenotypic characteristics of ALT-positive cell lines and tumors, the extent to which they can be relied on for detecting the presence of ALT activity, and progress towards an ALT activity assay. We also briefly reflect upon the implications these ALT characteristics have for understanding the mechanism of ALT, models of which have been presented in a recent review [19]. Finally, we summarize the increasing number of cancer types in which ALT activity has been detected, and clinical situations where availability of an ALT activity assay would be advantageous.

2. ALT assays

2.1. The "gold standard": maintenance of telomeres in the absence of telomerase activity

The only test for ALT that can be regarded as definitive at present is to determine whether telomere length is maintained in the absence of telomerase activity [3], although as a practical assay this has the drawbacks listed above. It can only be used for cell lines (or animal model systems) where telomerase is absent, and it is a medium- to long-term assay that is essentially non-quantitative. Even in clonally derived cell lines, there can be minor fluctuations in telomere length from passage to passage, so in practice it is necessary to culture cells for at least 20–30 PD in order to be confident that telomere length is being maintained, and ideally the absence of telomerase activity should be demonstrated at multiple PD levels. Because maintenance of telomere length in the absence of telomerase has never been demonstrated in non-immortalized human cells, a useful confirmatory test is to demonstrate that the cells being studied are in fact immortalized. This is usually done by determining whether the cells are able to grow in culture for >100 PD.

Limited quantitative information can be extracted from this assay in a carefully controlled context. In an ALT[+] cell line, inhibition of ALT can be detected by demonstrating that telomeres shorten steadily with increasing PD [20–22]. For example, in an ALT[+] fibroblast cell line in which the MRE11/RAD50/NBS1 (MRN) complex was sequestered following expression of high levels of SP100 protein, telomere length declined linearly (over several time-points) at a rate of approximately 120 base pairs (bp)/PD. This is within the range of telomere attrition rates in normal human fibroblasts that do not have any detectable telomere maintenance mechanism, and is consistent with ALT having been almost completely inhibited in these cells [20]. The rate of telomere attrition following treatment with a candidate ALT inhibitor can yield quantitative information about the extent of inhibition. However, the length of time required for this assay results in the possibility of mutations or other adaptations occurring in the cultured cells that confound the results. Moreover, it may not be possible to use this assay to demonstrate that a candidate has ALT inhibitor activity if it also has effects that result in cell death or senescence within that time period, which may explain the difficulty demonstrating telomere shortening in some circumstances [23–25]. A more practical assay is clearly required.

2.2. Telomere length heterogeneity

One of the first hallmarks found in immortalized human cell lines that use the ALT TMM was a characteristic pattern of telomere lengths (as analyzed by terminal restriction fragment [TRF] Southern blots), ranging from very short to extremely long, and with a modal length approximately twice that in comparable telomerase[+] or normal cells [3,26]. The telomere length heterogeneity can also be visualized in metaphase spreads by telomere fluorescence in situ hybridization (FISH) [14]. Telomere lengths in ALT cells are not normally distributed, in contrast to telomerase[+] cells, most of which regulate their telomere lengths about a mean of 5–10 kb [27,28]. Analyses of single telomeres in ALT[+] cells revealed sporadic, unsynchronized increases and decreases in telomere lengths of a variable amount (that are sometimes very large – up to ~20 kb) on a background of gradual erosion (~50 bp/PD) [29], which appear to be responsible for the broad distribution of ALT telomere lengths [28]. This pattern is established within one PD (equivalent to approximately 17 cell generations, which are required for one clone to overgrow a culture that is in crisis) of ALT being activated during immortalization in vitro [30].

The long heterogeneous telomere length pattern remains the best-established marker for ALT in human cells, including tumors archived under conditions where it is possible to extract high-quality genomic DNA [5,31]. In tumor specimens the distinction between ALT[+] and ALT[−] is sometimes less obvious due to tumor heterogeneity and non-tumor cells in the specimen [31]. Tumor length heterogeneity needs to be interpreted cautiously, especially under experimentally perturbed conditions, as this characteristic may not be completely ALT-specific. Long and/or heterogeneous telomeres are seen in telomerase[+] cell lines when expression of hTR or hTERT at supra-physiological levels challenges the telomere length regulatory mechanism with excessive telomere lengthening [32,33]. Conversely, it may not be necessary for cells using the ALT mechanism to have long telomeres [34].

2.3. Telomere length fluctuation

Because rapid, unsynchronized changes in telomere length cause telomere length heterogeneity [29], detection of these length changes may be regarded as closely related to detection of length heterogeneity. However, detection of ongoing fluctuations in individual telomere lengths may be useful in the situation where length heterogeneity has already been established by ALT activity, and it is necessary to determine whether an experimental manipulation has subsequently repressed ALT [14]. This could potentially be done by subcloning followed by Southern blot analysis of individual telomere lengths using a probe for a DNA marker inserted in

a subtelomeric location [29]. Alternatively, it can be done by treating an ALT[+] cell line with a candidate ALT repressor, subcloning, preparing metaphase spreads, and then determining the extent of telomere length fluctuation by measuring the variation in the ratio of p:q-arm telomere FISH signal intensity on an easily identifiable individual chromosome (e.g., the Y chromosome, or a marker chromosome, if present as a single copy), every example of which within the cell population is the progeny of a single chromosome [14,20]. If the unsynchronized changes in length generated by ALT activity have been eliminated by the treatment, then every example of this chromosome will have the same p:q ratio, even though the telomere lengths within the cell may remain highly heterogeneous because of previous ALT activity. If the treatment has had no effect on ALT activity, then the variability of the p:q ratio will be similar to the variability on the same chromosome in subclones of untreated cells. The utility of this technique is limited by the need to obtain metaphase spreads, and the sources of inaccuracy in quantitation of telomere lengths in a relatively small number of cells by FISH, the consequences of which become more problematic when telomeres are short. If an individual chromosome is not readily identifiable by karyotypic analysis, it may be possible to measure telomere length fluctuations using single telomere length analysis (STELA) [28]. It needs to be kept in mind that fluctuations in telomere length may not be specific for ALT because they potentially may also be caused by unsynchronized telomeric deletions in the absence of length maintenance.

2.4. ALT-associated PML bodies (APBs)

Like telomere length heterogeneity, the APB assay [30] is a well-established test for ALT, and has been especially useful for determining the ALT status of tumors as it can be used on paraffin-embedded specimens, and it is not affected by intra-tumoral heterogeneity in TMM because it assesses individual cells [31]. APBs are subsets of the ubiquitous PML nuclear bodies, which are foci of PML protein that bring many other nuclear proteins close together at one location [35]. PML bodies contribute to many diverse nuclear processes, including DNA repair and replication. PML bodies can function as active sites for nuclear processes requiring macromolecular interactions, as storage depots, or as sites involved in degradation of macromolecules [36]. These bodies can be statically bound to the nuclear matrix or mobile, large or small, and composed of several different isoforms of PML [37–39]. The subset of PML bodies that contain telomeric chromatin and DNA homology-directed repair and replication proteins are indicative of an active ALT mechanism [23,30,40–43] and PML bodies containing telomeric chromatin are defined as APBs [30]. Localization of telomeres to PML bodies requires the presence of the PML isoform, PML3 [44], the SUMOylation of telomeric chromatin by the SMC5/6 complex [21] and a functional MRN complex [40,45]. The MRN complex is involved in most aspects of DNA repair including HR [46].

Frequently occurring APB[+] nuclei have only been documented in ALT[+] cell lines and tumors [30,47,48]. APBs with telomeric signal intensities greater than those arising at the telomeres usually occur in around 10% of nuclei in an exponentially growing ALT[+] cell line [30], but smaller colocalizations of PML and telomeric DNA can be seen in most if not all ALT[+] nuclei under optimal conditions (A. Muntoni, H. Pickett, R. Reddel, unpublished results). The APBs with telomeric signals greater than those of the telomeres could arise from aggregations of telomeres [49–51] in the PML bodies and/or extrachromosomal telomeric repeats [52]. These large APBs usually increase in frequency with any cellular stress that causes cell cycle arrest, including senescence [43,45,52].

APBs could be an exaggeration of a transient event in G2 when PML bodies are proposed to be the sites for resolution and condensation of newly replicated tandem repeat DNA. Regarding juxtacentromeric satellite DNA, there is some evidence that BLM and TopIIIα in PML bodies may help resolve inappropriate recombination products formed during replication of repetitive DNA, and then BRCA1 and HP1 proteins (as well as ATRX and DAXX) in the same PML bodies may help condensation and reestablishment of the heterochromatin state [53]. Although this process is normally only transient, in the immunodeficiency, centromeric instability and facial dysmorphy (ICF) syndrome, defective DNA methylation of the juxtacentromeric repetitive DNA causes much larger and longer colocalizations of the DNA and repair proteins inside the PML bodies. In ALT[+] cells, BLM and TopIIIα are present in APBs and are required for cell viability [24,54], possibly by preventing unresolved, post-replicative, recombination intermediates [24]. BRCA1 and HP1 proteins are also present in APBs [40,43] and HP1α and HP1γ are required for formation of large APBs [43]. APBs can rarely be seen in non-ALT cells (A. Muntoni and R. Reddel, unpublished results), so it would be interesting to determine if this is related to early G2. Moreover, analogous to the effects of defective pericentromeric methylation in ICF syndrome, defective subtelomeric methylation induces APBs (long-lasting telomeric DNA in PML nuclear bodies) in mouse cells [55]. Although not ubiquitous, subtelomeric hypomethylation does occur on some chromosomes of human ALT cells, so it seems possible that this may contribute to APB formation [56,57].

A question regarding APBs that remains unanswered is whether their association with ALT is due to the abnormal telomeres in ALT cells and/or to a direct involvement in the ALT mechanism. If the telomeric DNA inside APBs does contain inappropriate replication or recombination structures, these could facilitate telomere repeat amplification by HR-primed replication in ALT cells before being resolved. APBs are sites of active DNA synthesis [41,47], although this DNA synthesis may be limited to the repair of aberrant replication or recombination structures at ALT telomeres. There is strong circumstantial evidence that ALT activity can occur in APBs. The MRN and SMC5/6 complexes, which are needed for the ALT mechanism, are present in APBs [20,21]. Furthermore, APBs disappear or decrease when ALT is inhibited by sequestration or knockdown of these complexes [20–22,45] and APBs are seen soon after activation of ALT [30]. PML bodies often seem to be redundant for their many functions [58], so even if APBs are sites of ALT activity it would not be surprising if they are not essential for ALT. There are two unusual ALT cell lines that do not have APBs (29/31 ALT[+] cell lines tested have APBs; Refs. [30,48] and J. Henson and R. Reddel, unpublished results). One of these, AG11395, still has aggregates of telomeric DNA and HR proteins – but without PML protein [59,60]. The second ALT cell line without APBs also lacks long heterogeneous telomeres and t-circles (a type of telomeric circular DNA associated with ALT, described below) [34]. This could indicate an association between these three ALT markers, which is also supported by the observation that telomerase-positive cell lines which had excessively elongated telomeres from telomerase overexpression were found to have t-circles and a low frequency of APBs [33]. Occasional APBs could be a marker of long telomeres and increased trimming rather than a direct marker of the ALT mechanism.

A modification has been made to the APB assay to use it as a test for inhibition of APB formation and, potentially, inhibitors of the ALT mechanism. Reduction of methionine in the growth medium for the IIICF/c ALT cell line increases the percentage of cells containing large APBs from 5% to >50% [45]. This makes measuring changes in APB levels a more sensitive test for changes in ALT activity and a more rapid test for inhibition of ALT, because even if APBs persist for a while after inhibition of ALT [61], an induction in the number of APBs should only be seen if ALT activity is still present. This test may not be directly related to ALT activity, so sec-

ondary testing is required to determine if the factors identified as inhibiting APB formation also inhibit ALT activity. Although reasonably rapid, this test is dependent on the cells remaining viable for three to four days after treatment. Methionine restriction has been useful in identifying proteins required for APB formation and demonstrating that APBs can be induced in G0/G1-arrested ALT[+] cells [45], in contrast to the usual association of APBs with G2-arrested ALT[+] cells [47,48,59]. The induction of APBs by methionine restriction is likely to result from the activation of the signaling pathways that lead to cell cycle arrest [43]. It has been suggested that methionine depletion might also lead to a methylation defect in the telomeric chromatin [45], which may also influence APB formation.

2.5. Copying of telomeric tags from one telomere to another

ALT amplification of specifically designed telomeric tags has been used to show that a telomere can template the ALT-mediated extension of either itself [62] or another telomere [6]. Because amplification of these tags indicates the synthesis of new telomeric DNA from a DNA template, testing for telomere tag amplification is a specific test for ALT. The tag designed to demonstrate inter-telomeric copying has been used to confirm ALT status [33,59]. These techniques are time-consuming to set up as they involve generation of clones with telomerically integrated DNA tags. Since the two different tags separately test for inter-allelic or intra-allelic copying (Fig. 1), it is possible that ALT activity could be missed if the ALT mechanism can operate either without any inter-allelic or without any intra-allelic copying, or without both by using untagged extrachromosomal telomeric DNA as the copy template.

2.6. Telomeric t-circles

An increase in the level of t-circles has been used as a marker for ALT activity [25,34,59,63–65], however t-circles do not appear to be essential for, or confined to the ALT mechanism. Of the variety of types of circular telomeric DNA in ALT cells, t-circles refer to double stranded (ds)DNA telomeric circles that are relaxed (not supercoiled) due to nicks in both strands [64,66] (Fig. 2). These circles are thought to be formed when the branch point of the telomeric t-loop migrates, forming a Holliday junction that is then resolved with XRCC3 [64]. One reason for a general increase in t-circles in ALT cells could be as a by-product of trimming long telomeres [33]. The only ALT cell line without long telomeres is also the only known ALT cell line with low levels of t-circles [34]. T-circles can be formed independently of ALT in response to DNA damage [67,68]. They may also be generated following depletion of Ku [65,69] or over-expression of mutant TRF2 lacking its basic domain

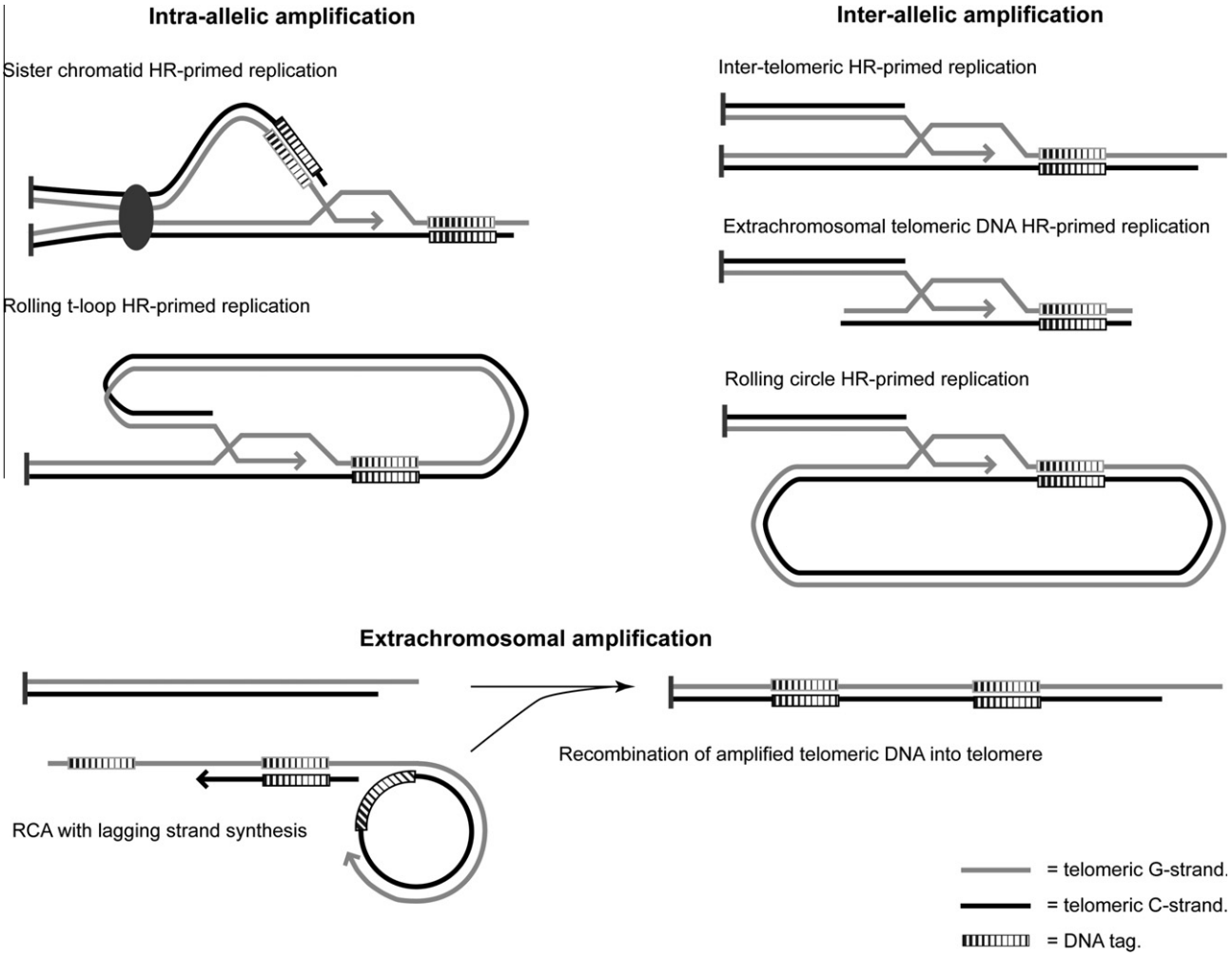

Fig. 1. Mechanisms for ALT telomere elongation. The ALT mechanism involves both intra- and inter-allelic amplification of telomeric DNA. DNA tags inserted into single telomeres (as illustrated) have been used to demonstrate inter-allelic amplification [6] and specifically designed tags have been used to distinguish intra-allelic amplification [62]. This mechanism most likely involves HR-primed replication. However, extrachromosomal amplification of telomeric DNA by rolling circle amplification (RCA), followed by recombination of the amplified DNA with a telomere could also be involved.

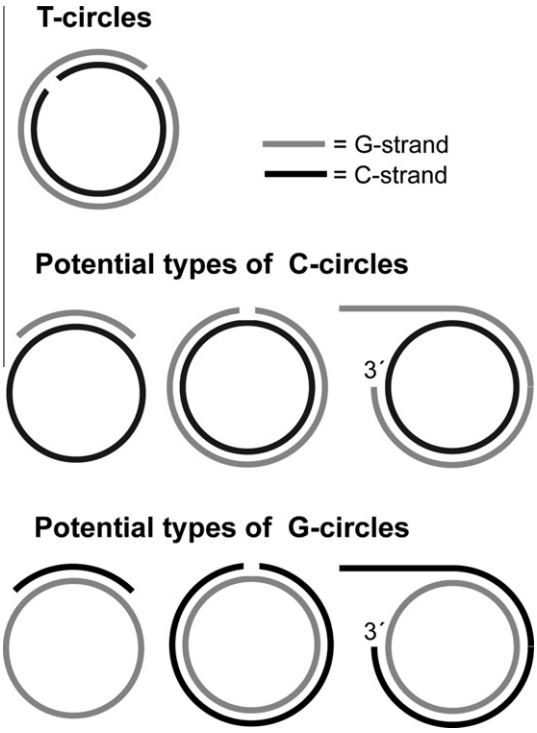

Fig. 2. Telomeric circles. Telomeric t-circles have nicks in both strands [64] and are unable to sustain RCA reactions [66]. Telomeric C-circles and G-circles are defined by their ability to self-prime RCA of telomeric C-strand and G-strand templates, respectively. While this definition of C-circles could include a variety of different structures, it requires that C-circles must have at least one 3′end available on an uninterrupted template C-strand. Similarly, different possible structures for G-circles are shown.

[64]; Ku and TRF2 both appear to protect the telomere from inappropriate HR [70,71]. Furthermore, t-circles may form following depletion of either ORC2 [68] or WRN [72], possibly because these proteins are required to avoid replication difficulties in the leading [73] or the lagging [74] strand, respectively. The long telomeres in ALT cells may be especially dependent on these proteins. Further evidence that increased t-circles may not be directly related to the ALT mechanism comes from the observation that although both ALT and t-circles depend on NBS1 [22,64], t-circle formation appears to be more sensitive than the ALT mechanism to decreased NBS1 levels [75].

2.7. Minisatellite instability

Tandem repeat instability at telomeres and the MS32 minisatellite has been used to investigate ALT activity [28,76]. Telomere variant repeat mapping of the proximal telomere (where variant repeats are common) indicates that intra- and inter-allelic recombination are increased in ALT telomeres [28,77], although HR is not generally elevated in ALT[+] cells [78]. Telomeric tandem repeats are a type of minisatellite tandem repeat, and ALT[+] cells have increased tandem repeat instability at the MS32 minisatellite locus [76] and at minisatellites detected by the 33.15 and 33.6 multi-locus probes [79], but not at five other minisatellite loci [76]. Minisatellites are tandem repeats of 6–100 bp units that for technical reasons were traditionally defined to have array lengths of >500 bp [80]. The increased tandem repeat variability associated with ALT was not found in microsatellites, which usually have shorter repeat units and a shorter array length than minisatellite repeats [76]. The frequency of MS32 mutation varied among 15 ALT[+] cell lines, but was on average 55-fold greater than in ALT[−] cell lines. The changes in ALT were different to both the simple deletions seen in normal somatic cells and the typical germ-line inter-allelic recombinations that are clustered near a meiotic cross-over hotspot, and included complex internal changes in the sequence of variant repeat units, suggestive of multiple events [76]. The tandem repeats known to have length instability in ALT have G and C segregated into one G-rich and one C-rich strand. Although G:C strand segregation is common to most minisatellite repeats, such as the MS1 minisatellite that is not unstable in ALT cells [76], it may contribute to the instability because it can cause mispairing, formation of intrahelical pseudoknots and interhelical associations in vitro [81]. This could promote HR and interfere with DNA replication. G:C segregation-facilitated instability could be related to increased ALT activation in WRN null mouse cells [82] and possibly cancers and cell lines from WRN patients (Refs. [59,60] and J. Henson and R, Reddel, unpublished results). WRN helicase is presumably involved in unwinding G-quartets that can form in the G-strand telomeric template for lagging strand DNA synthesis, potentially causing large excisions of the lagging strand replicated telomere [74]. Because general tandem repeat length plasticity is thought to be caused via tandem repeat excision of circular intermediates and reintegration of their rolling circle amplification (RCA) products [83], it will be interesting to determine whether there are DNA circles of these unstable tandem repeats in ALT cells.

2.8. Telomere-sister chromatid exchange (T-SCE)

T-SCE is increased in ALT cells [84,85] and has been used as an assay for ALT [25,86,87], although it may not be an ALT marker that can be depended on in isolation to reflect ALT activity. T-SCE can be detected by Chromosome Orientation (CO)-FISH [88], which involves complete destruction of the newly synthesized DNA strand, leaving the template strand intact for FISH with telomere strand-specific probes that can indicate if a cross-over event occurred after replication between a leading and lagging strand telomere [84]. If probes for both C-rich (leading strand template) and G-rich (lagging strand template) sequences are used, then it can be confirmed that the cross-over event involved the sister chromatid (i.e., was a T-SCE). T-SCE reflects increased recombination at the telomere rather than ALT activity per se, although this characteristic does appear to be tightly linked to ALT activity. A marked increase in T-SCEs is generally only seen in ALT[+] cells [84,85], although a recent report identified four telomerase[+] cell lines with T-SCE levels similar to those found in ALT[+] cells [89]. The value of T-SCE as a marker for ALT was demonstrated in an ALT[+] clone that lost other ALT markers (long heterogeneous telomere lengths, APBs and t-circles) but retained elevated T-SCEs [34]. This suggests that T-SCE may be more tightly linked to the ALT mechanism than most other markers. On the other hand, an ALT[+] cell line depleted for MUS81 had reduced T-SCE without any significant reduction in telomere length maintenance over 2 weeks (although limited ALT cell viability resulting from MUS81 depletion may not have allowed sufficient time to observe telomere shortening) [25]. In mouse cells, a small increase in T-SCEs was seen following DNA damage [90] or severe telomere shortening [91]. In general, short telomeres are thought to be more recombinogenic because they are more mobile [51], have a more open chromatin structure [92], and elicit a DNA damage response (DDR) [49]. Thus the increased T-SCE seen in ALT may partly reflect the high proportion of critically short telomeres that are a feature of ALT cells [30], rather than being directly related to the ALT mechanism. Up to 30% of chromosome ends in ALT cells have been reported as having undetectable telomeres [51] and even some easily detectable ALT telomeres may be “functionally short” due to an increased number of variant telomeric repeats [28,66,77,93].

T-SCE could be directly related to one possible replication template for the ALT mechanism. If ALT-mediated elongation of a telomere occurs after priming replication by recombination with the sister chromatid telomere, and if the resultant structure is resolved by a cross-over event, then this would result in a T-SCE. The newly elongated section of the telomere would not be visible, however, as both of its strands would be destroyed in the CO-FISH technique. Sister chromatid-primed elongation is consistent with the observation that intra-allelic tag amplification occurs in ALT cells [62], although rolling t-loop replication is another possible explanation of this observation (Fig. 1). Telomere CO-FISH may not detect rolling t-loop elongation, elongation primed by inter-telomeric recombination, or telomere elongation by integration of telomeric repeats from RCA.

It has been suggested that ALT could occur without amplification of telomeric DNA, by a mechanism involving unequal T-SCE and selective segregation of all sister chromatids with the longer telomeres (on both the p and q arms, presumably) into the same daughter cell, leaving the other sacrificial daughter cell with mostly short telomeres [94]. However, there is no evidence for unequal T-SCE (resulting in one short and one long telomere) being common in ALT, or for selective segregation of the chromatids with longer telomeres into one of the daughter cells. Furthermore, amplification of telomeric DNA is known to occur in ALT [6,62], and commonly from inter-telomeric sources [6,28,77]. Thus unequal T-SCE does not appear to be a likely ALT mechanism although unequal inter-telomeric exchanges could slow down the onset of cellular senescence in mortal cells [95,96].

2.9. Single stranded C-strand telomeric DNA (ss-C-strand)

Ss-C-strand is detectable in ALT[+] cells but not in telomerase[+] or mortal cells [97,98], and is a marker for ALT. Single stranded G-strand telomeric DNA (ss-G-strand) occurs in all cells (normal and immortal) and is bound by POT1 to prevent an RPA/ATR DDR and recombination [99]. The apparent lack of a corresponding ss-C-strand binding protein to prevent ALT-associated ss-C-strand eliciting a DDR and initiating recombination motivated our original interest in looking for ss-C-strand in ALT cells [97]. If some ss-C-strand exists as telomeric interstitial gaps, then this could explain in part why there is an increased DDR at ALT telomeres without increased telomeric fusions [49]. Most of the ss-C-strand in ALT is likely to be in extrachromosomal circular DNA [100] and ss-C-strand can also be found in APBs [97,98]. The precise relationship between ss-C-strand and the ALT mechanism needs to be further investigated, but its detection by PCR could potentially be used as a sensitive assay for ALT.

2.10. C-circles: self-priming circular telomeric C-strand templates

C-circles, defined as self-priming circular telomeric C-strand templates for RCA [66] have been shown to be a specific and useful assay for ALT activity. This is possibly the most convenient and versatile of the ALT assays, but nevertheless needs to be interpreted with caution until it can be determined whether C-circles are an integral part of the ALT mechanism or just another associated characteristic. Our definition of C-circles and G-circles, respectively, as self-priming telomeric C-strand and G-strand templates for RCA distinguishes them from t-circles that have nicks or gaps in both strands [64] and are therefore unable to sustain RCA [66]. While this definition of C-circles could include a variety of different structures, the self-priming component of the definition requires that C-circles must have at least one 3′end available on an uninterrupted C-strand template (Fig. 2).

Both C- and G-circles are specific to ALT, but C-circles appear more numerous in ALT cells and have been more extensively investigated [66]. C-circle levels in non-ALT cells are below background levels of the standard C-circle assay [66] and normal human fibroblasts have levels 1000-fold less than the average ALT[+] cell (J. Henson and R. Reddel, unpublished results). All 19 ALT[+] cell lines tested had raised levels of C-circles, including ALT[+] cell lines lacking t-circles and the established ALT markers of long heterogeneous telomeres and APBs [66]. C-circles were not detected in any mortal or telomerase[+] cell line, even in one induced to have long heterogeneous telomeres, t-circles and APBs. C-circles appeared when ALT was activated and decayed rapidly after inhibition of ALT. Given the specificity of C-circles for ALT and their short half life, it is likely that they are an integral part of the ALT mechanism. The simplest explanation may be that C-circles are ALT RCA intermediates, which is supported by the observation that depletion of the RPA protein (which binds ssDNA) led to an ALT-specific, dramatic increase of ss-G-strand [98].

The C-circle is the first candidate for an ALT specific molecule and an eligible target for a simple and versatile ALT assay. Since C-circles are self-priming RCA templates, they are an ideal substrate for detection by an isothermic polymerase (RCA) reaction that could readily be adapted for use by standard clinical pathology laboratories. The presence of C-circles in the blood of ALT[+] cancer patients means that the C-circle (CC) Assay could be used as routine clinical test for ALT activity [66]. The CC Assay was proven to be a sensitive and specific test for ALT. It was able to detect as few as 100 ALT[+] cells and clearly distinguished ALT[+] from ALT[−] cells. The fact that it was quantitative and responded within 24 h to inhibition of ALT means it is the first ALT assay that could be used to screen for ALT inhibitors. If C-circles can be explicitly demonstrated to be a required intermediate of the ALT mechanism, then the CC Assay could provide a definitive and practical ALT assay that could be used, for example, to determine the exact relationship to the ALT mechanism of important DNA repair proteins such as MUS81 [25], BLM [54], mono-ubiquinated FANCD2 [87], TopIIIa [24], Rad51D [23] and FEN1 [101]. It has been difficult to be certain whether these proteins are required for ALT telomere elongation or for prevention and/or processing of aberrant replication and recombination structures in ALT telomeres. It is possible that ALT may encompass a variety of related mechanisms and the CC Assay might test for only one of these. The C-circle levels were elevated in all 19 ALT[+] cell lines tested that were from a wide range of origins, although they did vary over a 200-fold range. Thus it is theoretically possible that C-circles reflect a form of the ALT mechanism that is ubiquitous but can vary in importance in different ALT cells.

3. Choosing an ALT assay

Detection of ALT often requires choosing the most appropriate ALT assays for the experimental context (Table 1). On the balance of the current evidence, the best frontline tests still include the established ALT markers of long heterogeneous telomere lengths and the presence of APBs. Assays for these characteristics of ALT are relatively convenient, reliable and have been extensively tested. The CC Assay and (if metaphase spreads are obtainable) the T-SCE assay are also convenient, although the latter is not always a reliable indicator of ALT activity. The CC Assay has the added advantage of being quantitative. The only established definitive test for ALT is telomere length maintenance in the absence of telomerase activity, although this is not always practical. Using telomeric tags to detect intra- or inter-molecular telomere amplification directly tests for the ALT mechanism and could also be used as a definitive ALT assay. However, this should be interpreted with caution as it is theoretically possible that it may not test all aspects of the ALT mechanism – especially if only inter-molecular

Table 1
ALT assays.

ALT Assay	Requirements		False +ve	False −ve
	Cell line	Tumor		
Telomerase-independent telomere maintenance	~30 PD	N/A	None known	ALT coexisting with telomerase
Telomere length distribution	~1×10^6 cells (TRF analysis; <1000 cells with STELA)	Frozen specimens	hTR and hTERT overexpression [32,33]	ALT[+] subclone with catalytically dead hTERT [34]
ALT-associated PML bodies (APBs)	~1×10^3 cells	Frozen and paraffin embedded specimens	hTR overexpression [33]; hTERT overexpression [97]	ALT[+] cell line AG11395 [59]; ALT[+] subclone with catalytically dead hTERT [34]
Telomere length fluctuation	Clonal population, marker chromosome	N/A	Not extensively tested	Not extensively tested
T-circles (2D gel)	~1×10^7 cells	Not tested	Hep 3B hepatoma ALT[−] cell line (Ref. [67], A. Englezou unpub. results). DNA Damage [67,68]. hTR overexpression [33]. Depletion or disruption of TRF2 [64], Ku [65,69] WRN [72], ORC2 [68]	ALT[+] subclone with catalytically dead hTERT [34]. Partial NBS1 depletion [75]
Inter- and Intra-telomeric tag amplification	Modified cell line. Isolation of clones	N/A	Not extensively tested	Not extensively tested
Telomeric repeat instability	<1×10^3 cells	Frozen specimens	May detect pre-ALT or non-ALT recombination in tumors [28]	Not extensively tested
MS32 minisatellite repeat instability	<1×10^3 cells	Frozen specimens	May detect pre-ALT or non-ALT recombination in tumors [28]	Five of eight ALT[+] sarcomas negative for MS32 minisatellite instability [76]
Telomeric-sister chromatid exchanges	Mitotic cells	N/A	Four telomerase[+] cell lines [120]; Cells with short [91] or damaged [90] telomeres	Possibly MUS81 depletion [25]
C-circle assay	~1×10^3 cells	Frozen specimens	None known	None known

or only intra-molecular amplification is tested. Testing for telomeric length fluctuations could be considered another ALT mechanism-directed assay, but may not be specific as telomeric deletions in the absence of length maintenance could theoretically cause increased telomeric length fluctuations. The demonstrated specificity of the CC Assay for ALT activity indicates its potential to be used as a definitive assay. However, this needs to be directly proven by showing that C-circles are an intermediate molecule in the ALT mechanism. Even then caution would be required as the CC Assay may only test for RCA and may miss intra- or inter-telomeric amplification. All of the ALT assays described (Table 1) could be used to give a better understanding of the ALT status of the system being investigated and, in situations where the more definitive assays are impractical, it may be necessary to perform several assays.

4. Detection of ALT in human tumors

Partly due to lack of an assay for ALT that is practical for screening large numbers of tumors, the prevalence of ALT is not known for many tumor types, including the most common cancers. So far, a number of areas in oncology have been identified which would benefit from being able to quantitate or inhibit ALT activity (Table 2). ALT is the TMM for approximately 50% of osteosarcomas, 30% of soft tissue sarcomas, 25% of the primary brain tumor, glioblastoma multiforme (GBM), and 10% of neuroblastomas, with the median survival for patients with these ALT[+] cancers ranging from 2 to 5 years (Table 2). Although ALT[+] tumors appear to be less likely to metastasize [31,102], the presence of ALT usually associates with same or worse survival compared to ALT[−] counterparts (Table 2). For example, in liposarcomas the presence of ALT was found to confer a worse prognosis than telomerase activity [103]. The exception is GBM where the presence of ALT conferred a 2–3-fold longer survival [31,104]. ALT was found to be the best prognostic indicator in GBM, for which the median survival is less than 1 year [104]. Detection of ALT in GBM could allow stratification of aggressive management to the patient group where it would be most beneficial to quality of life. Osteosarcoma patients with ALT[+] tumors could benefit from a quantitative blood test for ALT to monitor for success of pre-operative chemotherapy in time to alter it if required [31]. If ALT targeted anticancer therapies eventuate it will be necessary to know the extent of ALT activity in normal tissue. There have been suggestions of ALT activity in activated mouse lymphocytes [86] and mouse embryonal cells [86,105]. Investigation of ALT-like activity in early mouse embryonic cells also indicate that meiosis-specific homologous recombination proteins may be involved [105].

Investigations of ALT in cancer have also had implications for understanding ALT regulation. ALT does appear to be more common in sarcomas and astrocytomas than in carcinomas. The prevalence of ALT in astrocytomas and sarcomas is 25–60%, whereas in most carcinomas it appears to range from 5% to 15% (Table 2). This may reflect tighter regulation of telomerase in the tissue type of origin [27,31], increasing the relative probability that ALT will be activated when there is selective pressure for activation of a TMM. Somatic cell hybridization studies have shown that normal cells contain factors that repress ALT [61], and it is possible that the level and/or mechanism of ALT repression differs in different tissue types. The only ALT[+] breast carcinomas identified so far occurred in the context of either Li-Fraumeni Syndrome (LFS) [5] or Her-2 gene amplification [106]. These data need confirmation in larger clinical studies, but they could suggest regulation of ALT by p53 or the Her-2 pathway, respectively. Inactivation of p53 function by viral oncoproteins or mutation is common in ALT cell lines (Table 3), and p53 appears important for inhibiting HR between repetitive sequences with short homology [107] as may occur in ALT [28]. It is also possible that p53 mutation is merely required for ALT cells to continue to proliferate with their generally high levels of telomeric DNA damage signals [49,108] and chromosomal instability [109–111]. Recently, evidence has emerged suggesting that isocitrate dehydrogenase (IDH) oncogenetic mutations could be involved in the regulation of ALT. IDH oncoge-

Table 2
Prevalence and prognostic significance of ALT in cancer.

Tumor	Total cases	ALT[+] (%)	Survival of ALT[+]		References
			Relative to ALT[−]	Median	
Osteosarcoma	168	59	NSD [31]	5 years [31]	[5,31,121,122]
Gastric carcinoma	42	38			[123]
Soft Tissue Sarcoma	366	30	NSD [31]	4 years [31]	[31,102,110,124–127]
Leiomyosarcoma	21	57			[31,110,124,125]
MFH	78	46	Worse [127] NSD [31]	3 years [31]	[31,110,124,125,127]
Liposarcoma	187	29	Worse [102]		[31,102,110,124–126]
Rhabdomyosarcoma	40	5			[31,110,124,125]
Astrocytoma	117	28			[31,104]
Grades 2 and 3	8	88			[31]
Grade 4 (GBM)	109	24	Better [31,104]	2 years[31]	[31,104]
DMP Mesothelioma	38	26	NSD [128]		[128]
Adrenocortical carcinoma	87	14			[129]
Ovarian carcinoma	15	13			[5]
Melanoma	9	11			[5]
Neuroblastoma	121	9	Worse [130]	4 years [130]	[130]
Breast carcinoma	85	5			[5,106]
Colon carcinoma	22	0			(J.H. and R.R. unpub. results)
Ewings sarcoma	30	0			[111]
Lung carcinoma	7	0[a]			[5]
Papillary thyroid	17	0			[31]

MFH, malignant fibrous histiocytoma; GBM, glioblastoma multiforme; DMP Mesothelioma, diffuse malignant peritoneal mesothelioma; NSD, no significant difference.
[a] Retrospective analysis suggests that telomere lengthening attributed to telomerase in 2/40 Non Small Cell Lung Carcinomas was due to ALT [131].

Table 3
Examples of human ALT cell lines.

Cell Line	Cell type	Transformation	P53 alteration	References
GM847	Fibroblast, skin	SV40	Viral oncogene	[3]
GM0637 (NSV)	Fibroblast, skin	SV40	Viral oncogene	[132]
LM217, KB319	Fibroblast, skin	SV40	Viral oncogene	[29]
HSF E-2B	Fibroblast, foreskin	SV40	Viral oncogene	[133]
JFCF-6/T.1R and 1J series	Fibroblast, jejunal	SV40	Viral oncogene	[30]
WI38-VA13/2RA	Fibroblast, lung	SV40	Viral oncogene	[3]
SW26-I	Fibroblast, lung	SV40	Viral oncogene	[134]
SV/HF-5/39	Fibroblast, bone marrow	SV40	Viral oncogene	[135]
MRC5-V2	Fibroblast, lung	SV40	Viral oncogene	[66]
WHE-7 E7 Cl 1,2,3,4	Fibroblast, embryo	HPV E7	Not known	[136]
WHE-7 E6/E7 Cl 3	Fibroblast, embryo	HPV E6 and E7	Viral oncogene	[136]
82-cycA2	Fibroblast, foreskin	Cyclin A2	Reduced expression	[137]
82-cdk1	Fibroblast, foreskin	CDK1	Reduced expression	[137]
KMST-6	Fibroblast, embryo	Gamma irradiation	Mutated [138]	[139]
OUMS-24F	Fibroblast, embryo	Chemical	Mutated [138]	[140]
SUSM-1	Fibroblast, liver	Chemical	Mutated [138]	[3]
AT1BR44 neo	Fibroblast, skin; AT[a]	SV40	Viral oncogene	[141]
AT13LA(SV)	Fibroblast, skin; AT	SV40	Viral oncogene	[141]
AT18LA(SV)	Fibroblast, skin; AT	SV40	Viral oncogene	[141]
IIICF/a2, /b4, /b5, /c, .2/A1	Fibroblast, breast; LFS[b]	Spontaneous	LOH[d] [10]	[3,10,30]
IIICF-T series	Fibroblast, breast; LFS	SV40	Viral oncogene	[3]
IIICF-E series	Fibroblast, breast; LFS	HPV E6 +/- E7	Viral oncogene	[3]
HMS50-E7	Fibroblast, breast; LFS	HPV E7	LOH[c] [142]	[142]
MDAH087 series	Fibroblast, skin; LFS	Spontaneous	LOH [143]	[26,143]
LCS-AF.1-2, .1-3, .3-1	Fibroblast, skin; LFS	Aflatoxin B1	LOH [144]	[143,144]
LCS-4X2	Fibroblast, skin; LFS	X-Irradiation	LOH [145]	[143,145]
LFS-05F-24	Fibroblast, skin; LFS	Spontaneous	LOH [66]	[66]
W-V	Fibroblast, WS[d]	SV40	Viral oncogene	[59]
AG11395	Fibroblast, WS	SV40	Viral oncogene	[59,60]
MeT-4A	Mesothelial	SV40	Viral oncogene	[3]
H295R	Adrenocortical	Tumor	Not known	[146]
Saos-2	Osteosarcoma	Tumor	Mutated [147]	[5]
G-292	Osteosarcoma	Tumor	Mutated [148]	[5]
U-2 OS	Osteosarcoma	Tumor	Wild-type[e] [149]	[5]
ZK-58	Osteosarcoma	Tumor	Wild-type [109]	[109]
SK-LU-1	Adenocarcinoma, lung	Tumor	Mutated [150]	[5]
DOS16	Leiomyosarcoma; LFS[b]	Tumor	Not known	[66]
BET-3M	Epithelial, bronchial	SV40	Viral oncogene	[3]
HIO107, 117 and 118	Epithelial, ovarian	SV40	Viral oncogene	[48]
OKF6-D1/Δp53	Keratinocyte, oral	Cyclin D1, p53	Dominant-negative	[151]

[a] AT, Ataxia Telangiectasia.
[b] LFS, Li-Fraumeni syndrome.
[c] LOH, loss of heterozygosity for wild-type p53.
[d] WS, Werner syndrome.
[e] Seven-fold increase in MDM2.

netic mutations are stable early mutations in the development of glioma [112,113] which, like ALT, are associated with better survival and other characteristics of secondary GBM and are present in the majority of grades 2 and 3 astrocytomas from which secondary GBM originate [31,112]. IDH oncogenetic mutations alter key metabolite and redox balances in the cell and may activate the cells' hypoxia pathway [114,115]. It is not clear how this could modulate ALT activity, but it would be of interest to confirm the association of ALT and IDH oncogenetic mutations directly in GBM and other cancer types.

5. Considerations regarding the ALT mechanism

Data considered here suggest there may be two types of telomeric recombination activities that are essential for ALT[+] cells. Firstly, there are sporadic elongations of individual telomeres [14,28,29] with the amplified DNA templated from the same [62] or a different telomeric allele [6,28]. These elongations are variable in length and may be large [28,29], suggesting HR-primed replication from either a long linear template or a rolling template. Another explanation of sudden, large increases in length could be RCA of telomeric DNA extra-chromosomally [66,98] and then integration into a telomere. Secondly there may be an ALT recombination activity related to the processing of aberrant replication or recombination structures on ALT telomeres. Long arrays or deficient heterochromatization of repetitive DNA could cause replication difficulties in ALT telomeres. Depletion of the HR protein, RAD51D [116], or several HR-associated proteins diminishes the viability of ALT cells [23–25,54,87,101] without any evidence of inhibiting ALT telomeric elongation. Both processing of aberrant structures and ALT elongation might occur in APBs [23,24,41,47,54]. In mice, defective telomeric heterochromatization leads to increased telomeric HR, APBs and telomere elongation [117–119]. Thus perhaps the unusual instability at ALT telomeres could facilitate ALT telomere elongation by promoting either HR-primed replication or the creation of C-circles. Elucidation of the ALT mechanism and translation of this research to benefit cancer patients will be facilitated by finding ALT-specific proteins or molecules that can be utilized for diagnosis, or monitoring of therapy, or targeted by inhibitors for therapeutic purposes.

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