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Online links

DATABASES

Cancer.gov: http://www.cancer.gov/cancer_information/
breast cancer | colorectal cancer | lung cancer | melanoma
LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
erythropoietin-α

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QOLID.com:
http://www.QOLID.com
Access to this interactive links box is free online.

OPINION

Telomere maintenance and cancer — look, no telomerase

Axel A. Neumann and Roger R. Reddel

Activation of a telomere maintenance mechanism seems to be indispensable for the immortalization of human cells. Most cancers and cancer cell lines maintain their telomeres via telomerase. In some cancers, however, telomeres are maintained in the absence of telomerase activity by one or more mechanisms that are known as alternative lengthening of telomeres (ALT). Successful telomere-targeted anticancer therapy might therefore require a combination of telomerase and ALT inhibitors, emphasizing the importance of understanding the molecular details of telomere maintenance mechanisms in immortal cells and their repression in normal cells.

Telomeres are the ends of linear chromosomes, and in most eukaryotes they contain tandem arrays of a GT-rich nucleotide repeat sequence — 5′TTAGGG3′ in vertebrates[1,2]. Telomeric DNA and telomere-specific binding proteins (reviewed in REF. 3), together, have an essential role in stabilizing chromosome ends by forming a cap structure that protects chromosome ends from degradation and terminal fusions. Human telomeres are 5–15 kb long and are predominantly double stranded; however, they end in a 30–200 nucleotide single-stranded GT-rich 3′ overhang[4–6]. This 3′ overhang has been shown to form a lariat structure — referred to as a telomere (T)-loop — by invading the double-stranded region of the telomeric DNA[7] (see FIG. 1).

It was recognized in the early 1970s that the known DNA polymerases would be unable to replicate the very end of linear DNA molecules[8,9] — a phenomenon known as the 'end replication problem'[10]. RNA-primed DNA synthesis of the lagging strand results in a terminal gap after degradation of the most distal primer. In addition to this, the single-stranded GT-rich 3′ overhang at each telomere involves the action of a putative 5′–3′ exonuclease that degrades the 5′ end of the underhanging CA-rich strand[4,5]. Both of these processes lead to shortening of the telomeric DNA template that is available for semiconservative replication in the next round of DNA synthesis. In most normal somatic cells, therefore, telomeres shorten with every cell division[11]. Conversely, for reasons described below, prevention of telomere shortening is crucially important for the development of most human cancers. Cancers might use more than one mechanism to prevent telomere shortening. What are these mechanisms, and how does their elucidation impact on our understanding of cancer biology and our ability to treat cancer?

Cellular immortalization

Many cancers contain cells that have an apparently unlimited capacity to proliferate, and acquisition of this property is referred to as immortalization. This contrasts with the finite proliferative capacity of normal somatic cells[12]. The early evidence that immortalization has a key role in the cancer phenotype came from two main lines of investigation. The first was *in vitro*/*in vivo* studies with hamster cells that were treated with chemical carcinogens, in which it was found that immortality was necessary but not sufficient for malignant transformation[13]. The second was from studies of human cells that had been transduced with the simian virus 40 (SV40) early-region genes, which sometimes results in immortalization. It was found that these cells could undergo malignant transformation by an activated *RAS* oncogene, but only if they were immortalized[14–16] (FIG. 2).

At that time, little was known about the molecular events in immortalization. Shortly after the discovery that normal human cells become senescent after a limited number of cell divisions[12], it was shown that cells infected with the SV40 virus could temporarily evade senescence, but that the culture soon entered a state that is referred to as 'crisis' in which the population stopped expanding[17]. It was subsequently shown that this effect of the SV40 virus could be reproduced by transfecting cells with a plasmid that contains the viral early region[18] and that encodes at least two oncoproteins, which include the SV40 large T antigen that binds to the protein products of the *TP53* and *RB* genes (reviewed in REF. 19). Sometimes, a cell expressing the SV40 early-region genes can bypass or escape from crisis, but this occurs in only 1 in 10^7 cells[20,21]. Although this is a

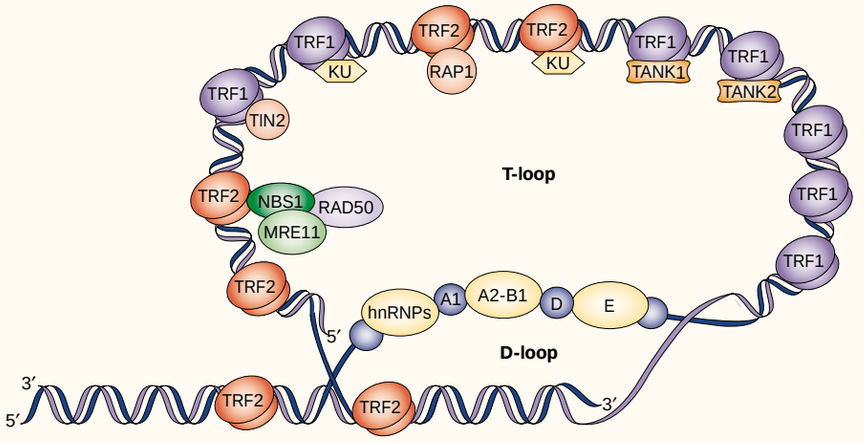

Figure 1 | **Telomeric DNA and protein assembly.** Schematic representation of putative telomere T-loop structure with telomere-specific binding proteins. The single-stranded DNA at the end of the telomere is able to invade and anneal with part of the duplex DNA (thereby forming a displacement (D)-loop) in the same telomere, with the overall result being a telomere (T)-loop[7]. Several proteins bind specifically to telomeric DNA, and these recruit other proteins to the chromosome end.

rare event, it is much less rare than spontaneous immortalization, which is estimated to occur in less than 1 in 10^{12} human cells[22]. It therefore seems clear that expression of the SV40 genes results in increased probability of a change that is responsible for immortalization.

Telomerase and immortalization

The nature of the change that occurs at immortalization was elucidated in a study showing that SV40-transformed cells expressed the enzyme telomerase on emergence from crisis, but not beforehand[23]. This key observation resulted from the convergence of two lines of investigation. The first was the development of the telomere hypothesis of senescence — namely, that senescence is triggered when telomeres become short, and the experimental verification of the prediction that telomeres shorten progressively as normal cells proliferate[11,24,25]. The second was the discovery of the telomerase enzyme, which adds telomeric DNA onto the chromosomes of protozoa[26]. The telomeres of the SV40-transformed cells at crisis were even shorter than at senescence, but their length stabilized post-crisis, after telomerase activity commenced[23]. It was subsequently shown that transducing SV40-transformed cells with the catalytic subunit of telomerase (*TERT*) — which switches on telomerase activity — allows them to bypass crisis completely[27,28] and to be transformed by an activated *RAS* oncogene[29]. These observations showed that expression of telomerase is a key event in the immortalization of SV40-transformed cells.

Alternative lengthening of telomeres

However, some SV40-immortalized cells have been found to be telomerase negative[30–32]. The mean telomere length of telomerase-negative cell lines was much greater than in their pre-crisis counterparts and was greater than in normal cells (FIG. 3), indicating that the telomeres had undergone extensive lengthening[32]. The increased telomere length was maintained over hundreds of population doublings in the persistent absence of detectable telomerase activity[32].

After technical reasons for this apparent lack of telomerase activity were excluded, it was deduced that there must be at least one non-telomerase mechanism of telomere length maintenance[32]; these mechanisms were dubbed alternative lengthening of telomeres (ALT)[33]. Definitive evidence that telomerase was not involved in ALT was provided by a study showing that some ALT cells lack expression of a subunit of telomerase (the RNA template, *TERC*), which is essential for telomerase activity[34]. Mouse cells that are null for the telomerase RNA gene, *Terc*, are also able to undergo immortalization[35]. Although ALT was defined as any telomerase-independent mechanism of telomere length maintenance, so far there is no clear evidence for the existence of more than one ALT mechanism in human cells.

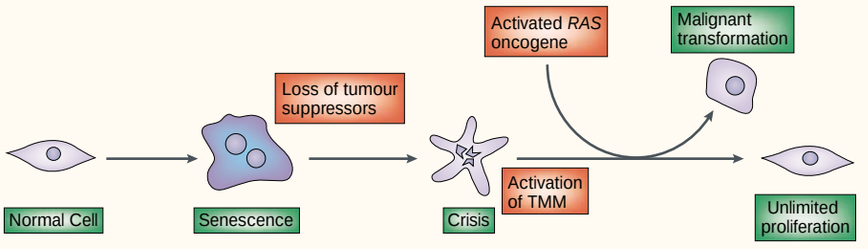

Figure 2 | **Senescence and immortalization.** Normal somatic cells permanently exit from the cell cycle (that is, become senescent) after a limited number of cell divisions. Cells might escape temporarily from the senescence barrier if they lose the function of key tumour-suppressor genes, especially *TP53* and/or *RB*, but most will eventually die, at which stage the cell population is described as being in 'crisis'. Cells might bypass crisis and become immortalized (capable of unlimited proliferation) if a telomere maintenance mechanism — telomerase or ALT — is activated. Immortalized cells, but not their mortal predecessors, might be susceptible to malignant transformation by an activated oncogene such as *RAS*.

ALT and cancer

The original evidence for ALT came from studies of cell lines that were immortalized *in vitro* by SV40 and other means. Its relevance to cancer was indicated by the observation that ALT cell lines that have been transduced with an activated *RAS* oncogene are tumorigenic[36,37]. The presence of ALT in human cancer samples showed most clearly that it is not an artefact of cell culture[38]. Of 57 human tumours, four were telomerase negative and had a significantly increased mean telomere length; the telomere length was also extremely heterogeneous, which is characteristic of ALT cells. Similarly, 4 out of 56 tumour cell lines had ALT. So, in this limited survey, 8 out of 113 (7%) tumours and tumour cell lines had ALT[38], compared with 35% of cell lines that were immortalized *in vitro*[39]. The reason for this discrepancy might be that most of the *in vitro* immortalized cell lines that were examined were of fibroblast (that is, mesenchymal) origin, whereas most human cancers are carcinomas (that is, of epithelial origin; many normal epithelia have low levels of telomerase activity). In support of this explanation, preliminary data indicate that sarcomas (which are of mesenchymal origin) are more likely to use ALT than are carcinomas[38], perhaps because carcinomas are derived from cells that have fewer barriers to upregulation of telomerase activity.

An extensive survey of the published literature on telomerase activity in human cancer concluded that 85% of all cancers are telomerase positive[40]. It cannot be assumed, however, that the remaining 15% must use an ALT mechanism: as argued in more detail elsewhere[41], some malignancies (solid tumours and leukaemias) might not need any telomere maintenance mechanism. It is possible that solid tumours and leukaemias that have a relatively small number of crucial genetic changes, and/or a low cell turnover, can become clinically significant without immortalization[41]. It is important to determine how many of the telomerase-negative tumours use ALT.

To complicate the situation further, however, it seems that some tumours use both telomere maintenance mechanisms[38]. It is not yet known whether ALT and telomerase are sometimes co-expressed spontaneously within individual tumour cells, although cell-culture experiments in which telomerase is artificially switched on in ALT cells have shown, in principle, that this is possible[42–44] and that telomerase activity can sometimes repress or mask the ALT phenotype[45]. One ALT cell line was more likely to become tumorigenic if transduced with expression constructs for both activated *RAS* and *TERT* than if transduced with *RAS* alone[46]. However, it is not yet known whether ALT and telomerase are sometimes co-expressed spontaneously within tumour cells.

Another possible explanation for the presence of both telomere maintenance mechanisms within a tumour is intratumoral heterogeneity. In SV40-transformed fibroblasts, the probability that telomerase or ALT will become activated to allow escape from crisis is quite similar (ALT is activated in at least one-third of SV40-immortalized fibroblast lines)[32]. If this is the case for some types of tumour cell, then it would not be surprising if ALT and telomerase are sometimes turned on in separate areas of the same tumour.

Implications for cancer treatment

When telomerase activity was downregulated in telomerase-positive cancer cells by expression of a dominant-negative *TERT* mutant, the cells became apoptotic[47,48]. This showed, in principle, that telomerase inhibitors could be very useful for treating cancer. Telomerase inhibitors will not be useful, however, for the minority of tumours that use ALT. In addition, in telomerase-positive tumours it would be predicted that effective telomerase inhibitors will exert a very strong selection pressure for the emergence of resistant cells that use the ALT mechanism. Activation of ALT was not observed in cell-culture experiments in which telomerase-positive cell lines were treated with small-molecule inhibitors of telomerase or dominant-negative *TERT* mutants[47–50], indicating that it is not a high frequency event. This might be a problem, however, in clinically significant tumours containing as many as 10^{12} cells. It might, therefore, be necessary to develop ALT inhibitors. For tumours that use both telomere maintenance mechanisms, treatment might need to be initiated with a combination of telomerase and ALT inhibitors. Both telomerase and ALT must access the telomere, but how this might be achieved is, at present, unknown. It would however, be surprising, if the sets of proteins that are involved do not at least partly overlap, so it might be possible to identify molecular targets for simultaneous inhibition of both telomere maintenance mechanisms.

It is expected that telomerase inhibitors will be developed that have far fewer side effects than many of the cancer chemotherapeutic agents that are available at present. The inherited syndrome dyskeratosis congenita (DKC) is caused by a mutation in one of the components of telomerase, so individuals with DKC are deficient for telomerase activity (reviewed in REF. 51). The features of DKC include abnormalities of the skin and nails, and eventual failure of proliferation in the bone marrow, which indicates that telomerase is required for normal proliferative capacity in these somatic tissues. Despite this telomerase deficiency, onset of pancytopaenia in these individuals does not occur until a median age of 10 years[52], which indicates that it might be relatively safe to administer telomerase inhibitors continuously for several years.

The potential toxicity of ALT inhibitors cannot be predicted without knowing the role of ALT in normal cells. It is important to develop an assay that can determine whether an ALT-like mechanism might be active during meiosis, or might modulate telomere length in somatic cells in one or more tissue compartments. Interestingly, there is preliminary evidence indicating that an ALT-like activity might be responsible for lengthening telomeres of lymphocytes in telomerase-null mice[53]. Although this is an entirely speculative idea at present, inherited defects in the putative normal counterpart of ALT might also contribute to features of premature ageing, which is analogous to telomerase deficiency in DKC.

Lessons from yeast

A rational approach to inhibiting ALT requires an understanding of the mechanism. As is often the case in contemporary life sciences research, studies of yeast genetics have pointed the way. When budding yeast — *Saccharomyces cerevisiae* — was made telomerase null by targeting the telomerase RNA gene, it was found that telomeric shortening occurred and the cells eventually died. Survivors were found to maintain their telomeres by a mechanism that was dependent on *RAD52*, which encodes a protein that is involved in recombination[54]. In the period before activation of a survivor pathway, proliferation was enhanced if the DNA mismatch-repair pathway was defective[55]. A genome-wide survey of genes with altered expression after deletion of telomerase activity identified several genes that remain upregulated in survivors, including the putative meiotic recombination/DNA-repair gene *MSC1* (REF. 56).

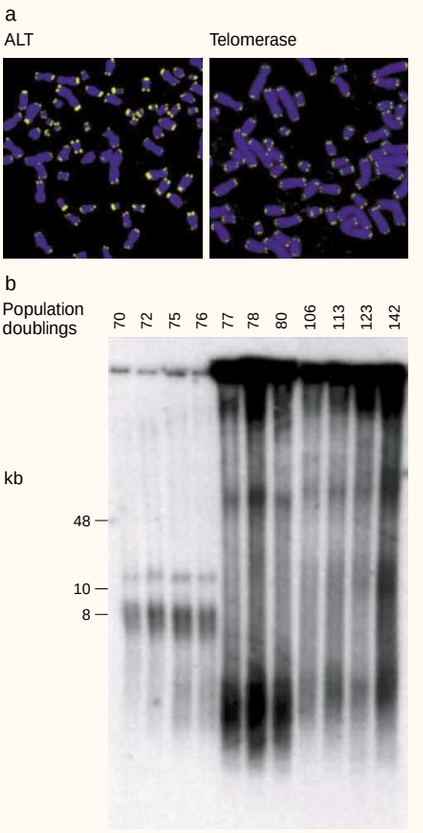

Figure 3 | **Telomere-length phenotype of ALT cells. a** | Telomere-specific fluorescence *in situ* hybridization (FISH) on metaphase chromosomes of ALT and telomerase-positive cells, illustrating the highly heterogeneous telomere lengths within individual ALT cells (yellow, telomere-specific probe; blue, DAPI-stained metaphase chromosomes). **b** | Terminal restriction fragment (TRF) length analysis of an ALT cell line before crisis (at population doubling (PD) 76) and after crisis (at PD 77), showing the temporal correlation between immortalization and occurrence of the ALT characteristic telomere-length pattern. Reproduced with permission from REF. 37 © American Association of Cancer Research.

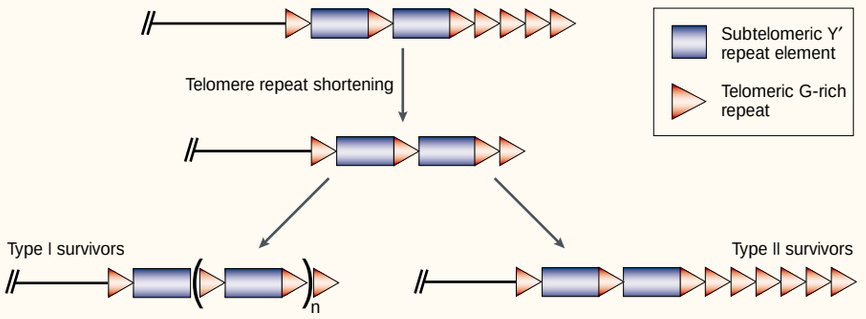

Figure 4 | **Telomerase-null *Saccharomyces cerevisiae* type I and type II survivors.** Schematic representation of the two telomere phenotypes in telomerase-deficient yeast (*S. cerevisiae*) survivors. Shortening of telomeric G-rich repeats results in cell death. Rare survivors either show amplification and dispersal of the subtelomeric Y′ repeat elements (blue rectangles; type I survivors) or show elongation of the terminal G-rich sequence (red triangles; type II survivors) as a result of recombination. Adapted from REF. 83.

Further analyses identified two classes of telomerase-null survivors[57]. Type I survivors undergo amplification of a subtelomeric sequence named Y′, and type II survivors have telomeres with lengths that are very heterogeneous, but are increased overall (FIG. 4). Telomeres of the type II cells therefore resemble those of human ALT cells. At present, it is unknown whether any human cancers use an ALT mechanism that is analogous to that of type I yeast survivors. However, the chromosome ends of telomerase-null mouse embryonic stem cells that underwent spontaneous immortalization in culture contained an amplified non-telomeric sequence[58].

The type I and type II mechanisms are both dependent on *RAD52*, but other gene requirements vary. Type I requires *RAD51*, *RAD54* and *RAD57*, whereas type II requires *RAD50*, *RAD59* and *SGS1* (REFS 59–64). The protein products of all of these *RAD* genes are involved in recombination. Sgs1 is a helicase of the RecQ class and has five human homologues, three of which — *WRN*, *BLM* and *RECQL4* — are known to be mutated in familial syndromes with features of premature ageing and increased cancer incidence (Werner, Bloom, and Rothmund–Thomson syndromes, respectively).

Recombination

Adding to the genetic evidence implicating recombination in the survival of telomerase-null yeast cells, Murnane and colleagues found striking increases and decreases in telomere length in a telomerase-negative human cell line that they attributed to telomeric recombination events[30]. A recombination-based model for ALT was proposed[65] in which one DNA strand of a short telomere anneals to the complementary strand of another telomere and acts as a primer for DNA synthesis (FIG. 5a). The newly synthesized sequence can then be converted to double-stranded DNA, resulting in a net increase of telomeric DNA. This is obviously very different from reciprocal recombination, which results in exchange between chromosomes, but no net increase in total genetic material.

Further indirect evidence that the ALT mechanism might involve recombination came from the finding that some PML bodies in the nuclei of ALT cells are unusual[37,66]. PML bodies are found in the nuclei of most normal cells, and are domains that contain accumulations of proteins that are involved in a wide variety of functions (reviewed in REF. 67). In every ALT cell line that has been examined so far, a proportion of the cells have PML bodies that contain telomeric DNA (at least some of which is extrachromosomal), telomeric binding proteins, and proteins that are involved in DNA replication and recombination (FIG. 6). PML bodies with these contents have not been found in telomerase-positive or mortal cells, so they are referred to as ALT-associated PML bodies (APBs)[37]. Although the function of APBs is unknown at present, the presence of recombination proteins within them — including RAD52, RAD51 and RAD50 — confirmed the importance of testing the recombinational model of ALT.

To test the proposed model, a DNA tag was inserted into telomeres of ALT and telomerase-positive cells[68]. According to the ALT model, when a tagged telomere is used by another telomere as a copy template and when the point of strand invasion is proximal (centromeric) to the tag site, the tag will be copied to the other telomere. In clonal populations of ALT, but not telomerase-positive, cells it was found that the number of tagged telomeres increased during cellular proliferation[68]. This proposed mechanism was also supported by the findings of a study in which a polymerase-chain-reaction-based method was used to sequence the subtelomeric region of specific chromosome ends before and after immortalization of ALT cells[69]. Immediately proximal to the telomere, there is a region of the chromosome that contains a few TTAGGG repeats that are admixed with variant repeats such as TGAGGG, TTGGGG and TCAGGG. According to the model, if severe shortening of the chromosome end occurs before crisis, then all of the telomere and part of the subtelomeric region might be lost; after ALT is activated, annealing of a residual subtelomeric TTAGGG repeat with the complementary sequence of another telomere that still retains some telomeric sequence will allow the synthesis of new telomeric sequence. The net result would be replacement of variant repeats with $(TTAGGG)_n$ sequence; this predicted outcome was observed[69].

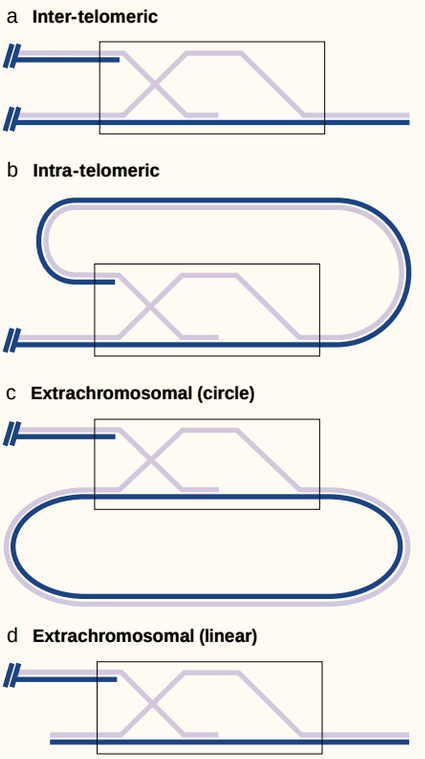

Figure 5 | **Recombination-mediated telomere lengthening in ALT cells.** It is proposed that a terminal telomeric DNA strand within an ALT cell can invade other telomeric DNA and anneal to the complementary strand. Synthesis of new telomeric DNA sequence can be primed on the template to which the invading strand is annealed, and this can be converted to double-stranded DNA (not shown). Potential templates include another telomere (**a**), the proximal (that is the centromeric) region of the same telomere via T-looping (**b**), and extrachromosomal telomeric DNA that is either circular (**c**) or linear (**d**).

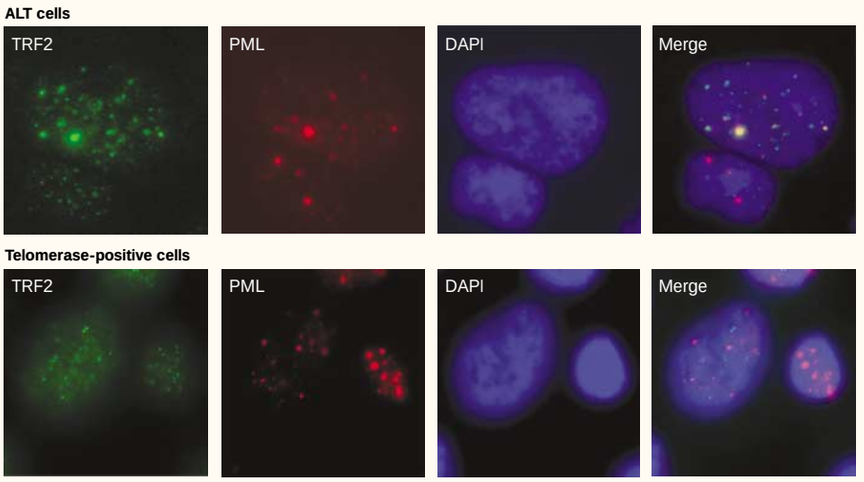

Figure 6 | **ALT-associated PML bodies.** ALT cell lines and tumours contain PML bodies (multiprotein complexes that are found in the nuclei of most normal cells) that have some unusual contents: telomeric DNA and specific telomere-binding proteins, including TRF2. PML protein is found in all PML bodies. ALT-associated PML bodies (APBs; yellow merge) are visualized here by co-localization of PML (red immunostaining) and TRF2 (green). APBs are seen in ALT cells, but not in normal or telomerase-positive cells, and are therefore a useful marker for ALT. With appropriate validation, it is possible that this technique can be used to detect ALT in tumour specimens that have been stored as paraffin blocks, which is how most tumour samples are archived by pathologists.

Although these results strongly support a recombinational mechanism for ALT, they do not exclude the possibility that the telomeres might sometimes use copy templates other than another telomere. It is not known whether telomeres in ALT cells form the T-loops that are observed in telomerase-positive cells (FIG. 1), but if they do it seems possible that a telomere could use itself as a copy template (FIG. 5b). They could also use extrachromosomal telomeric DNA — circular or linear — as a copy template (FIG. 5c,d). A rolling circle can template an essentially unlimited amount of lengthening, and there is evidence that telomerase-negative yeast can use such a mechanism of telomere elongation[70]. Human ALT cells are known to contain extrachromosomal telomeric DNA[37,71,72], as are telomerase-negative insect cells[73].

Repression of ALT in normal cells

ALT-mediated lengthening of telomeres depends on the availability of copy templates (TTAGGG repeats, which are present in every telomere) and proteins that are involved in recombination and DNA synthesis. All of these are present in normal cells, so an obvious question is why the telomeres of normal somatic cells undergo shortening. Presumably, normal cells have a mechanism for repressing telomere length maintenance by ALT. The existence of such a mechanism was shown by fusing normal cells with ALT cells and showing that the ALT mechanism was repressed in the hybrids[74]. Some telomerase-positive cells also contain repressors of ALT[44,74,75]. An understanding of how this repression is achieved might also identify useful molecular targets for cancer treatment.

Why is telomerase needed at all?

The recombinational and DNA synthesis machinery is much more ancient than telomerase. Telomerase is unique to eukaryotes, but conserved recombination proteins are found in bacteria and more primitive organisms. For example, human RAD51 has structural and functional homology to the bacterial RecA protein[76]. Another obvious question that therefore arises is why telomerase is needed at all. There are a few eukaryotes that do not have telomerase, and maintain their telomeres, instead, by recombination (such as mosquitoes and midges[77,78]) or retrotransposition (such as *Drosophila* and related *Dipterans*[79,80]), but the overwhelming majority of eukaryotes do have telomerase. Maybe telomerase is a more readily controlled mechanism for telomere maintenance than ALT. A recent Opinion article[81] reviewed accumulating data from *Terc*-null mice and suggested that telomerase might have functions in addition to telomere maintenance that could provide a selective advantage to organisms (and cancers) that express this enzyme.

Telomerase and ALT: interchangeable?

There is a sense in which the telomere maintenance mechanisms are equivalent: immortalization can be associated with the activation of either telomerase or ALT, and fully malignant tumours can use either mechanism. It seems inadvisable, however, to assume that they are completely equivalent, especially if telomerase does have other functions in the cancer cell. Also, telomeres that are maintained by telomerase or ALT might not be functionally equivalent. Despite their long mean telomere length, most ALT cells contain some very short telomeres; these might be more prone to end-to-end fusion events than telomerase-maintained telomeres and so might be a source of genetic instability[82]. One of the questions that needs to be addressed is whether tumours of the same stage and grade that use either ALT or telomerase have the same prognosis. In addition, the response of such tumours to existing therapies needs to be examined: are there existing cancer chemotherapeutic agents to which tumours using one or other telomere maintenance mechanism are particularly sensitive or resistant? If yes, there might be some tumour types for which it will be necessary to stratify clinical trials according to telomere maintenance mechanism. It will also be important to determine whether an individual patient's tumour has telomerase and/or ALT activity — or neither — when telomere maintenance inhibitors become available for cancer therapy.

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