

Telomere recombination and alternative telomere lengthening mechanisms

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1. ABSTRACT

Telomeres are nucleoprotein structures at the ends of linear chromosomes that protect them from being recognized as DNA double stranded breaks. Telomeres shorten with every cell division and in the absence of the checkpoint mechanisms critical telomere shortening leads to chromosome end fusions and genomic instability. Cancer cells achieve immortality by engaging in one of the two known mechanisms for telomere maintenance: elongation by telomerase or through recombination. Recombination based elongation of telomeres, also known as alternative lengthening of telomeres or ALT, is prevalent among cancers of mesenchymal origin. However, the conditions favoring ALT emergence are not known. Here we will discuss possible players in ALT mechanisms, including recruitment of telomeres to recombination centers, alterations of telomere associated proteins and modifications at the level of chromatin that could generate recombination permissive conditions at telomeres.

2. INTRODUCTION

Telomeres are nucleoprotein structures of fundamental importance for genome stability since they guard linear chromosome ends from DNA damage responses, thereby preventing chromosome fusions and loss of genetic material. In mammalian cells, telomeres are composed of double-stranded tandem repeat sequences, ending in a short single-stranded 3'overhang, and telomere associated proteins (reviewed by (1, 2)). Telomeres must contain a minimum number of tandem repeats in order to bind the sufficient amount of telomere associated proteins thus allowing the assembly of a protective nucleoprotein structure. Healthy telomeres that can completely block the DNA damage responses are referred to as being properly “capped”. However, if the number of tandem repeats is compromised or the protein capping function is perturbed, a strong DNA damage response is elicited at telomeres leading to cell cycle arrest, senescence and/or apoptosis (for a review see (3)). Telomeres therefore control cell

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proliferation, inducing cell cycle arrest whenever there is a risk of genome instability due to uncapping.

Paradoxically, telomeres are intrinsically unstable, prone to constant changes in the number of telomeric repeats. The two most important factors contributing to the dynamic nature of telomeres are the gradual telomere shortening with every replication cycle due both to the “end replication problem”, affecting the strand replicated by lagging mechanisms, and to the controlled 5’ erosion undergone by the strand replicated by leading mechanisms in order to produce a functional 3’ overhang, essential to adopt a protective structure. The processing of the telomere ends created by the leading-strand DNA synthesis machinery is, in mouse embryonic fibroblasts, at least partly mediated by Apollo (which is recruited through its interaction with TRF2, see below) (4, 5) and requires, in humans, the presence of MRE11 (6). The resulting telomere shortening can be counteracted by the active addition of telomeric repeats by telomerase, a dedicated reverse transcriptase mostly present in the stem cell compartments of highly proliferative tissues. In the absence of telomere maintenance mechanisms, cells can divide only for a limited number of divisions (7) until the so-called Hayflick limit is reached due to shortened telomeres thus leading cells to replicative senescence. An average human telomere contains close to two thousand telomeric repeats, enough material to efficiently buffer against telomere erosion for about 50-90 replication cycles in the absence of telomere maintenance mechanisms, with an estimated loss of about 50-150 nucleotides per division (8, 9), depending on the cell type and culture conditions.

In addition to the gradual shortening of telomeres during cell aging, telomeres can undergo recombination-mediated rapid deletion events resulting in a shortened telomere and extra-chromosomal telomeric DNA, either circular or linear (reviewed by (10)). In *Kluyveromyces lactis*, these telomere rapid deletions (TRD), that may result in extremely short telomeres, depend on RAD52 (11). Unlike these dangerous rapid deletion events, TRD events were shown to contribute to the normal telomere length homeostasis in human and yeast cells, by trimming abnormally long telomeres (11-13). When telomeres reach a critical length where the capping function is compromised, or if uncapping occurs despite the normal telomere length, a DNA damage response is triggered and repair mechanisms are activated. In most cases, unprotected telomeres fuse through the non-homologous end-joining (NHEJ) DNA repair pathway. While the classical NHEJ appears to be responsible for fusions implicating unprotected telomeres with normal lengths, the sequence pattern of human telomere fusions after critical shortening is compatible with an alternative NHEJ pathway that uses microhomology (14, 15). Strikingly, NHEJ may be suppressed at breaks too close to telomeric repeats, likely because of the proximity of telomere capping proteins which may negatively influence the reaction (16). Dividing cells carrying fused telomeres enter breakage-fusion-bridge (BFB) cycles leading to genomic instability. Eventually, a broken chromosome without telomeric repeats may be stabilized by the addition of new telomeric repeats by

telomerase in a reaction called chromosome healing (reviewed by (17)). In yeast, this reaction is suppressed by the helicase Pif1 (18).

The dynamic nature of telomeres thus allows for adjustments of chromosome end function during cell aging. In particular, replication-associated shortening of telomeres limits the proliferation capacity of cells and therefore functions as a tumor suppressive mechanism (19). In addition, telomere dynamics may have an impact on the stability of subtelomeric regions, which are hotspots for recombination in higher primates (20), thus contributing to the makeup of genomes at an evolutionary scale.

2.1. Telomere structure

The TTAGGG tandem repeated sequence is bound by a complex of specialized proteins called shelterin (reviewed by (1, 2)). Three of these proteins, TRF1, TRF2 and POT1, are DNA binding proteins. Telomere repeat binding factors TRF1 and TRF2 are high affinity binders of double stranded telomeric repeats (21-23), while POT1 (protection of telomeres) recognizes the single stranded G-rich telomeric repeats found at the end of telomeres (24). In addition to the direct DNA binders, TPP1, TIN2 and RAP1 are also part of shelterin. They function as adaptor proteins, mediating interactions among the telomere DNA binding proteins. Besides shelterin, mammalian telomeres contain nucleosomes, although not as densely packed as in the rest of the genome (25), and numerous other proteins involved in DNA damage and repair. The presence of the latter is intriguing since the main role of shelterin is to prevent DNA damage responses. These DNA damage responses may also be blocked by a lasso-like structure called the T-loop, which forms by the looping back of the telomeric double-stranded repeats and the insertion of the single-stranded overhang into the double helix, thus hiding the extremity and avoiding its recognition as a double stranded DNA break (26). The formation of the T-loop is facilitated by the telomere-bound proteins, in particular TRF2 which was shown *in vitro* to stimulate the invasion of the single-stranded overhang into the double-stranded repeats thus forming a three-stranded DNA displacement loop (D-loop) (27, 28). However, definite proof of the *in vivo* existence of T-loops is still missing since there is a formal possibility that the T-loop structures visualized under electron microscopy are artifacts due to crosslinking (26).

2.2. Sources of telomere instability

Telomere instability is partially due to the intrinsic instability of TTAGGG repeats (Figure 1). When single stranded, TTAGGG repeats may organize into higher-order DNA structures called G-quadruplexes due to the repeated tracts of guanines, which can be organized into hydrogen bond reinforced tetrads (29). Even though their existence *in vivo* is still controversial, those structures may form in the G-rich overhang, in the displacement loop, and during lagging strand replication. During replication, G quadruplexes may pose physical barriers for the replication machinery. The formation of secondary structures by the G-rich lagging strand, and in particular G quadruplexes, is counteracted by the members of the RecQ-type helicases, all of which can efficiently unwind G quadruplexes *in vitro*

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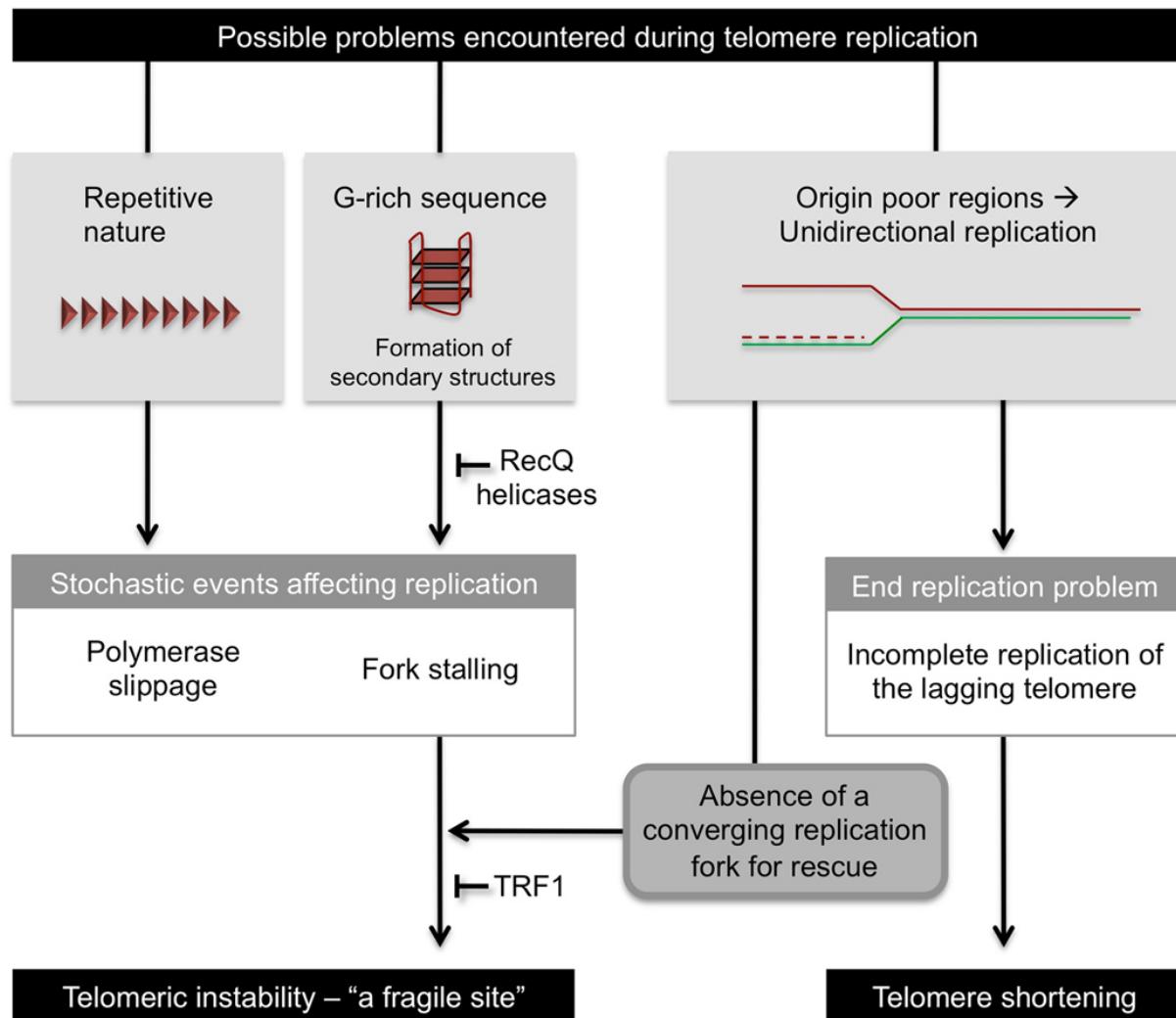


Figure 1. Telomeres are inherently unstable structures. TTAGGG repeated regions are reputedly difficult to replicate perhaps due to, among other factors, the formation of G quadruplexes, which can lead to lagging strand replication defects or complete fork stalling. Shelterin proteins recruit ancillary factors such as RecQ helicases to facilitate fork progression. Stalled replication forks cannot be rescued by replication from a converging fork and in the absence of DNA repair mechanisms, this results in telomere shortening. In addition, telomeres naturally shorten with every replication cycle due to both incomplete replication on the lagging strand and controlled processing of the leading strand.

(reviewed by (30)). Two of the five known RecQ helicases in humans, Werner (WRN) and Bloom (BLM), have been shown to be important for telomere stability and replication (30). Another helicase, PIF1, can bind *in vitro* and unwind DNA structures resembling stalled replication forks (31) and was recently shown to promote replication through G-rich sequences in yeast (32). In addition, the shelterin protein POT1 present at the G-rich single-stranded sequences was shown to promote G quadruplex unfolding in single molecule studies (33) and became essential for the progression of lagging strand replication at telomeres in the absence of WRN (34).

Telomeric shortening is counteracted by the enzyme telomerase (reviewed by (35)). Tissues with high

turn over, such as hematopoietic cells, skin cells and cells from the gastrointestinal epithelium, express telomerase which to a certain extent counteract replication-related telomere shortening. Telomerase is also present at high levels during embryonic development and in many stem cells. Cancer cells acquire the capacity to divide indefinitely through reactivation of telomerase expression or by implementing an alternative mechanism based on recombination. Alternative lengthening of telomeres or ALT was initially described as a capacity to maintain telomeres in the absence of telomerase (36, 37). It was later shown that these alternative mechanisms utilize recombination reactions to maintain telomeres (38). ALT is the mechanism of choice for telomere maintenance in tumors from mesenchymal origin (39-42) and can be

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spontaneously activated in cultured (mostly fibroblast) cells that have been allowed to enter chromosome instability because of telomere shortening (37). Whether ALT also exists under normal physiological conditions remains to be determined. Indeed, it has been suggested that telomeres may elongate through recombination-based mechanisms in mouse embryos during the early cleavage steps following fertilization and before telomerase is re-expressed (43). Regardless of whether ALT is a normal physiological mechanism or a deregulation of telomere homeostasis permitting recombination, understanding ALT is of great importance in the clinics since about 10% of all tumors depend on ALT for telomere maintenance and because anti-telomerase based strategies may result in the selection of ALT-based resistance. However, our current understanding of ALT is quite limited. In particular, we lack ways of testing mechanistic models and our knowledge of environmental and genetic factors that favor ALT is close to nil, thus hampering any effort in designing effective therapies that will specifically target ALT cells. In this review, we will focus on the particular features of ALT cells that in our view constitute promising leads for future investigation toward the answer "Why ALT?".

3. TELOMERE RECOMBINATION IN ALT

ALT telomeres are unique, in the sense that besides showing the intrinsic instability and fragility characteristic of all telomeres, they are prone to recombination. The reasons why ALT telomeres become recombinogenic remain unknown. On the other hand, ALT cells display several characteristics and it is based on these hallmarks that we can distinguish ALT from telomerase-positive cells (for a review see (44-46)). ALT cells lack telomerase, have on average very long but otherwise highly heterogeneous telomeres and contain in most cases special nuclear bodies found only in ALT cells called APBs (ALT-associated PML bodies). In addition, an unusually high telomere instability is observed in ALT cells, manifested as rapid deletion and elongation events accompanied by the accumulation of extra-chromosomal telomeric repeats (ECTR). This extra-chromosomal material is present in the form of double stranded T-circles (47), partially single-stranded (CCCTAA)n DNA circles (C-circles) (48) and also linear DNA, which increases following DNA damage (49). The recently discovered C-circles seem to be a promising clinical marker for ALT since they appear to be present in all ALT-positive tumors tested including in the blood of osteosarcoma patients but also in those rare ALT cell lines that do not have APBs (48).

How the increased instability of telomeres in ALT is linked to recombination is not known, but a model has been proposed, according to which cleavage at the base of the T-loop leads both to the excision of circular ECTR and abrupt telomere shortening. On the other hand, a G-rich overhang may invade another double stranded telomere, thus creating a substrate for a replication reaction (similar

to what is observed during break induced replication). How this overhang becomes available for recombination is also not known but it is conceivable that this happens during replication (through resolution of the T-loop) or at any time during the cell cycle if the telomere is too short to form a T-loop. Indeed, it has been suggested that short telomeres are the preferential substrate for recombination in ALT cells. In the following two subsections we will first discuss the possible recombination mechanisms present in ALT cells together with putative crucial regulation steps in those mechanisms. In the second subsection we will discuss unique features of ALT cells that may contribute to the activation of ALT mechanisms.

3.1. Recombination at telomeres – Is it different from other homologous recombination reactions?

In most cells, a DSB can be repaired by any of two pathways: by NHEJ, which is prone to error, and by homologous recombination, also referred to as homology-directed repair (HDR), which is error free. HDR begins with the processing of the broken end to create a 3' overhang which then "invades" an identical (or almost identical) intact DNA molecule. After strand invasion, elongation of the 3' end by replication followed by branch migration allows capturing the second 3' end to create a double Holliday junction. Depending on how these junctions are incised by resolvases, HDR results (or not) in a chromosomal crossover. HDR appears to be highly regulated, and in most organisms limited to the S/G2 phase of the cell cycle when identical sister chromatids are available to carry out an error-free repair. In addition, homologous recombination is finely tuned through the regulation of individual steps of the reaction: 3' overhang formation, synthesis of a recombinogenic filament, 3' invasion, DNA synthesis, branch migration and resolution of the Holliday junction (for a review see (50)).

Given the nature of telomere sequences, telomeres do not require a sister chromatid for perfect error-free recombination and instead may use various types of recombination substrates. The possibilities formally include recombination between two heterologous chromosomes, recombination between two sister telomeres and recombination with ECTR. Since recombination was originally demonstrated in ALT cells by the spread of a telomeric tag between chromosomes during *in vitro* passage (38), direct interchromosome recombination is certainly possible in ALT cells. In further support of a direct intertelomeric recombination, our group reported the existence of telomere bridges in metaphase spreads of ALT cells. Those bridges were composed of telomeric sequences that had incorporated a base substitute on either the C-rich or the G-rich strand of the same chromatid and were therefore visualized as intercalated green/red signals using differentially labeled C-rich and G-rich probes. Such pattern was interpreted as interchromosome recombination

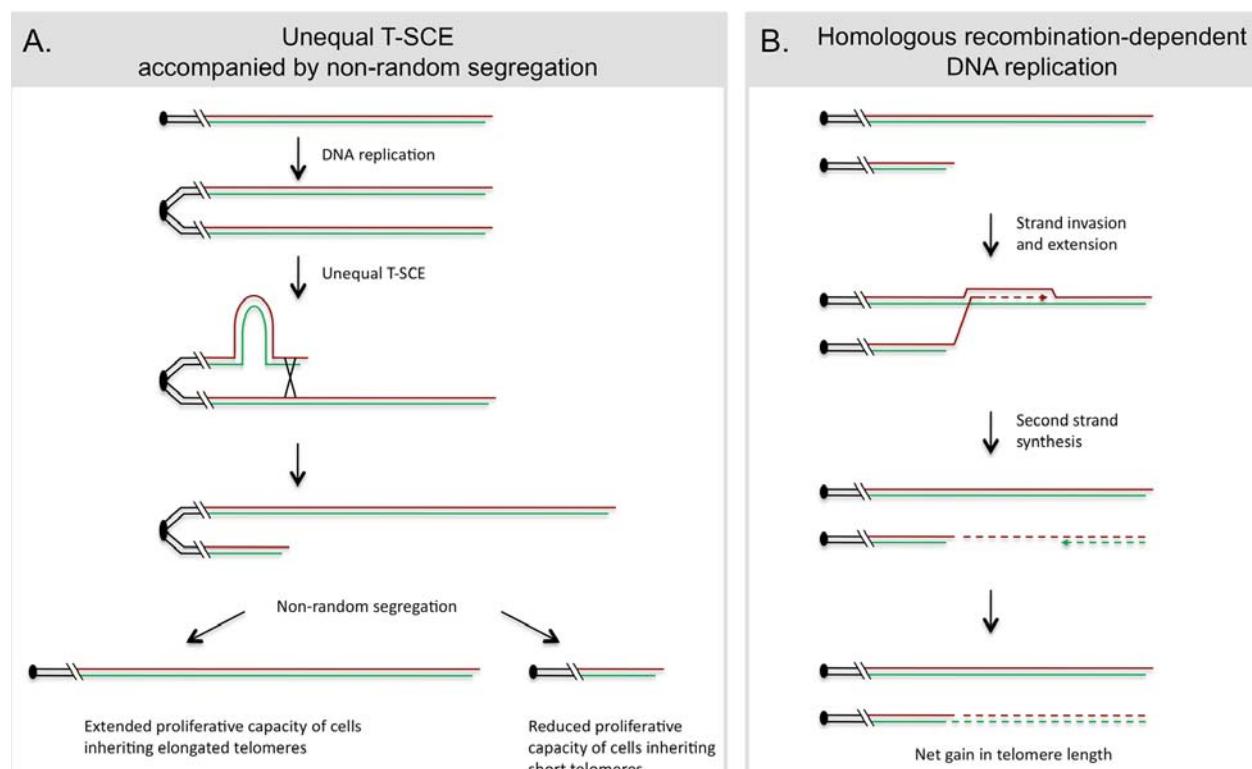


Figure 2. Two mechanistic models for the alternative lengthening of telomeres. A. An unequal telomere sister chromatid exchange (T-SCE) leads to telomere lengthening of one sister chromatid at the expense of the other. To be efficient, this model must assume the non-random segregation of sister chromatids since a daughter cell will have increased mean telomere length, and therefore increased proliferation capacity, only if it inherits mostly chromatides with long telomeres. B. A 3' overhang from a short telomere invades the double strand portion of a longer telomere on another chromosome. The extension reaction creates the substrate for the synthesis of the second strand by lagging mechanisms after resolution of the heteroduplex. Alternatively, the invasion/extension reaction may lead to the formation of a *bona fide* fork on the target telomere, with simultaneous leading and lagging replication progressing till the end of the chromosome, thus resembling break-induced replication (BIR) (not shown).

intermediates which could only arise after replication (51). By perturbing the structure of APBs (through infiltration by a viral protein), the frequency of telomere bridges was significantly increased, while there was no increase in the rate of anaphase bridges, thus suggesting that cells can resolve such telomere-recombination intermediates prior to chromosome segregation (51).

It is very likely that intertelomere recombination also takes place between two sister telomeres in ALT cells as it has been suggested by the detection of telomere sister chromatid exchange (T-SCE) (52), another hallmark of ALT activity. Whether T-SCE is merely a reflection of an increased recombinogenic potential of ALT cells, the result of perturbed replication of very long telomeres or actually leads to telomere elongation (Figure 2) remains to be determined. Furthermore, in yeast it has been shown that telomeres can be elongated using ECTR as a template in a reaction that may resemble rolling-circle amplification (53). Although theoretically possible, this mechanism has not yet been validated in mammalian cells. In all, telomere-telomere

recombination can be initiated using numerous template sequences. Determining whether this process is stochastic or regulated constitutes one major challenge in the field.

Although telomeric recombination could theoretically take place any time during the cell cycle, the presence of post-replicative telomere bridges suggest that replication may favor such reactions. The passage of the replication fork at telomeres may facilitate access of the recombination machinery and certainly should help in exposing the single-stranded 3' overhang. To our knowledge, the size of 3' overhangs has not been measured in ALT cells, but it is possible that the natural overhang length of 100-400 bp (54) is sufficient to form a recombination substrate with no additional extensive end processing by MRE11 and EXO1. The obvious control step in this process becomes the nucleation of a RAD51 filament. RAD51D, one of the five RAD51 paralogs in mammalian cells, has been shown to localize at telomeres in mice and to be important for telomere maintenance in ALT cells, as RAD51D depleted ALT

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cells showed telomere erosion and chromosome fusions (55). In the classic recombination context, the presence of RPA on the 3' single strand represents the major obstacle to the formation of a RAD51 filament. In that context, mediator proteins, such as RAD52, promote RPA displacement by RAD51 (56). In the telomeric context, on the other hand, the 3' overhang is normally coated by POT1. How this POT1-coated overhang may be converted into a recombination filament is unknown. We can envision a situation in which POT1 is relatively deficient in ALT cells. In this case, the accumulation of replicative G-rich single strands during replication may provide an opportunity for RPA to compete favorably with POT1 for the 3' overhang thus providing a classic substrate to initiate a nucleation by RAD51. It is not clear what the relative affinities of POT1 and RPA for single stranded telomere repeats are. For instance, in one study, POT1 was reported to have a higher binding affinity than RPA for a single-stranded telomeric oligonucleotide of 21 bp (34), while in another study RPA bound more tightly to an 18 bp telomeric nucleotide when compared to a TPP1-POT1 complex (57). It has to be stressed that these assays used substrates considered to be suboptimal for RPA, which requires a 30 nucleotide binding site to form a stable, extended conformation complex (reviewed by (58)). However, it has been shown that RPA can readily form more compacted complexes with smaller substrates, in particular telomeric oligonucleotides able to form secondary structures such as G-quadruplexes (59). Regardless of the individual affinities of those two proteins for telomeric single-stranded repeats, a mediator protein is most likely required to replace POT1 by either RPA or perhaps directly by RAD51. Alternatively, POT1 may be displaced during the unfolding of the T-loop due to replication fork passage (which likely promotes environment enriched for RPA) thus creating another opportunity for filament nucleation.

Once formed, the recombination filament invades another double stranded telomeric sequence and may engage in any of the known HDR pathways. The choice of the repair pathway outside a telomeric context is influenced by the extent of the homology of the processed ends. If both ends carry homology to the intact DNA molecule, recombination can proceed through a double Holliday junction (dHJ) or through synthesis-dependent strand annealing (SDSA). When only one end of the resected DSB is available for pairing, break-induced replication (BIR) is likely triggered. In the context of telomeres, telomere elongation following recombination most likely resembles BIR, during which a semi-conservative DNA replication fork with both leading and lagging strand synthesis is engaged and may continue for many tens of kilobases leading to extensive copying of sequences from the intact DNA molecule (Figure 2). It is not known if telomere recombination in mammalian cells depends on all three major

replicative polymerases, including the delta subunit Pol32, as it is the case in yeast (60, 61).

The final step in recombination, the resolution of the Holliday junction, is probably another critical step for ALT cells. There appears to be a plethora of structure-specific endonucleases that can resolve these structures resulting in the completion of DNA repair by homologous recombination. Enzymes, such as GEN1 (Yen1 in yeast) incise Holliday junctions producing directly ligatable crossover and non-crossover products (62). Alternatively, a DNA helicase, BLM, in combination with a type I topoisomerase, can resolve Holliday junctions exclusively in a non-crossover mode (63). Branched DNA intermediates in HR can also be acted upon by evolutionarily conserved MUS81/EME1 (EME1 being the noncatalytic partner of the resolvase) and in mammals also by SLX1/SLX4, the latter potentially interacting with TRF2/RAP1 (64). In yeast, Mus81 and Yen1 were shown to promote non telomeric reciprocal exchange during mitotic recombination and in the absence of those two genes, all intermediates were channeled into Pol32 dependent BIR (65). In humans, a knock down of MUS81 led to a decrease in T-SCE and cell growth arrest, suggesting that this endonuclease is essential for ALT-associated telomere recombination (66). Whether this protein is implicated in recombination reactions that effectively lead to telomere elongation remains to be demonstrated.

Finally, the timing for telomere replication may have some implications for recombination. In yeast, all telomeres are replicated at the end of the S phase (67), and telomerase and recombination are believed to act on replicated, unfolded telomeres with exposed 3' overhangs in late S and G2 phases of the cell cycle. However, in mammals, telomeres replicate throughout the S phase (68-70) raising the question whether telomere recombination in mammals, if it is tightly linked to replication, could also occur throughout the S phase. It is likely that initiation of recombination early in the S phase may interfere with replication if the target telomere has not been replicated yet. On the other hand, the replication timing of individual telomeres is influenced by nuclear position and heterochromatic properties (68), although this remains to be shown in the context of ALT. In any case, telomeres with particular heterochromatic marks or nuclear positions may bear specific replication timings during which accessibility of HDR factors may be either increased or limited, thus suggesting another source of heterogeneity in the recombinogenic behavior of ALT telomeres.

3.2. Why are telomeres in ALT cells recombinogenic?

Recombination in mitotic cells is highly regulated and the repair of DSB using this pathway is limited to S/G2 phases of the cell cycle. At the same time, normal telomeres actively repress

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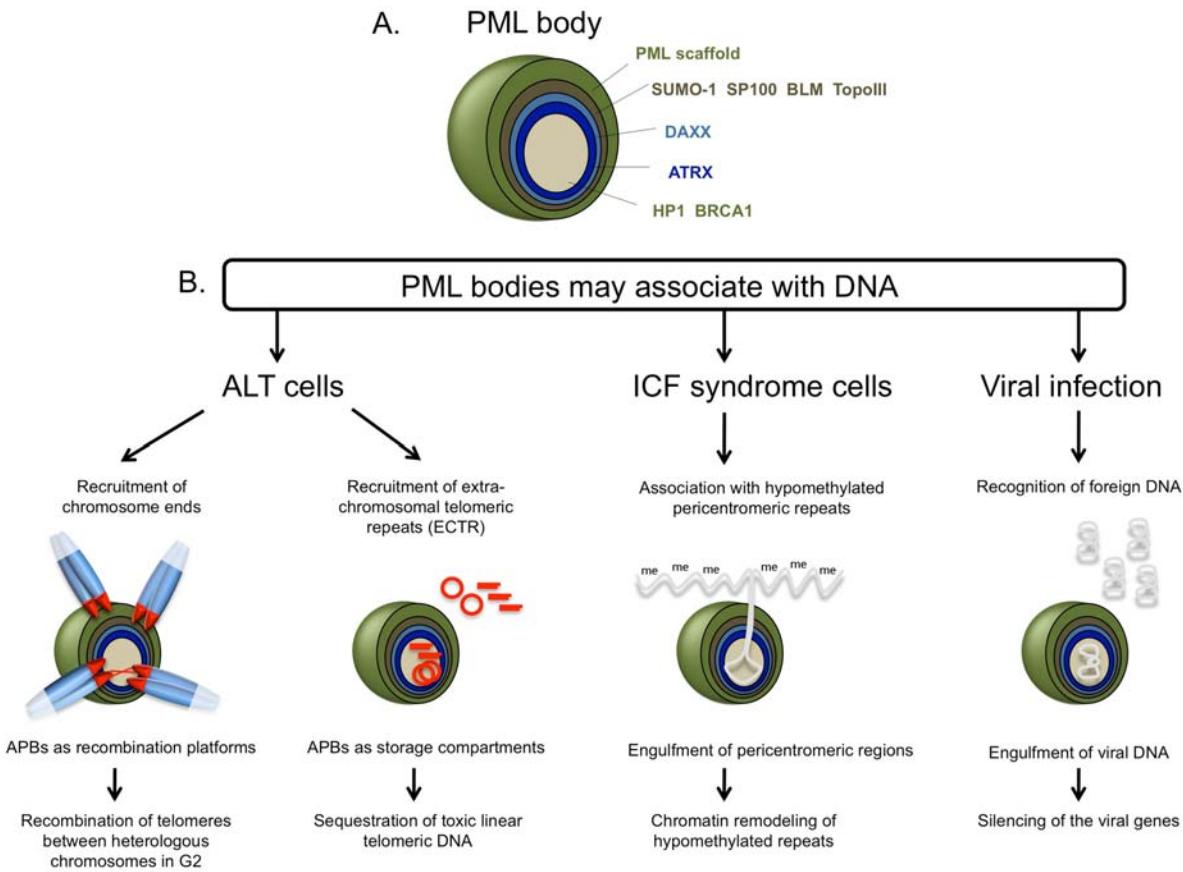


Figure 3. PML bodies can associate with DNA. A. The outer borders of PML bodies are defined by the scaffold protein PML while numerous other proteins are packed in the core of those structures in onion-like concentric rings (76, 137). B. In ALT cells, telomeric repeats with associated proteins accumulate in PML bodies forming ALT-associated PML bodies or APBs. Telomeric material found in association with PML bodies derives either from chromosome ends recruited to PML bodies for recombination (51) or from extrachromosome double stranded telomeric sequences (49). In ICF (Immunodeficiency, Centromeric region instability, Facial anomalies) syndrome patients, defects in the DNA methylation at pericentromeric repeats provoke the engulfment of those hypomethylated regions by PML bodies (76). PML bodies also associate with viral DNA in latent stages (81).

recombination *via* the shelterin complex (discussed in section 3.2.2.) and it is not known how telomeres repair stalled replication forks in this recombination repressive environment. It is possible that telomerase positive cells do not bother repairing telomeric DSBs since the enzyme can rapidly add *de novo* telomeric repeats to shortened telomeres. Cells without telomerase, however, cannot repair drastically shortened telomeres and enter into senescence or apoptosis. The presence of one or few critically shortened telomeres is enough to trigger a permanent cell cycle arrest (71). On the other hand, recombination at telomeres in ALT cells is not only possible, but is significantly elevated in comparison to other genomic regions (52, 72). A general deregulation of recombination is therefore not a basis to develop a telomere recombinogenic behavior. In the light of recent data, it appears that several factors may contribute to the activation of ALT: 1. Recruitment of telomeres to PML bodies and creation of recombination

platforms, 2. Aberrant composition and/or post-translational modification of shelterin, 3. Presence of telomere-bound helicases creating recombination permissive conditions, and 4. Chromatin modifications at telomeric and subtelomeric regions permitting recombination. The following sections will address these points.

3.2.1. Role of ALT-associated PML bodies (APBs)

APBs are a special class of PML (promyelocytic leukemia) nuclear bodies found exclusively in ALT cells that in addition to the proteins normally associated with PML nuclear bodies, contain telomeric repeat DNA, telomere-specific proteins and recombination and repair proteins (73). However, recombination and repair factors are not unique to APBs and may be present in regular PML bodies found in non-ALT cells, as reported for RAD51 and NBS1 (74-76). Therefore, the only recognizable difference between

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APBs and PML bodies is the presence of telomeric material.

In ALT cells, there is a clear association between the presence of APBs and the utilization of ALT for telomere maintenance. In studies where APBs have been successfully disrupted, ALT has also been suppressed. For instance, the over-expression of SP100, an abundant protein in PML bodies, simultaneously led to sequestration of the MRE11/RAD50/NBS1 (MRN) complex, to suppression of APBs and to progressive shortening of telomeres (77). Furthermore, in knockdown experiments, every protein in the MRN complex proved to be essential for the formation of APBs and for the maintenance of telomere length (78). It is not known why and how telomeric material associates with APBs but based on the nature of the observed telomeric material in APBs two hypotheses have been advanced (Figure 3). A first interpretation contends that since APB-associated telomeric material corresponds, at least in part, to *bona fide* chromosome ends (51, 79) and since perturbation of APB structure leads to the accumulation of telomere bridges, APBs may function as telomere recombination platforms that recruit chromosome ends for recombination (51). On the other hand, it has been shown that APBs accumulate linear, low-molecular weight extra-chromosomal telomeric repeat (ECTR) DNA following DNA damage, suggesting that APBs prevent inappropriate DNA damage responses by sequestering free DNA with unrepaired ends (49). However, DNA damage itself is not always required for the association of DNA with APBs. For instance, PML bodies can also associate with genomic DNA in the absence of damage and to foreign DNA such as viruses, which leads to sequestration of viral DNA and suppression of lytic infections (see below and Figure 3).

Numerous recombination factors have been found associated with APBs; however, of all these factors, only RAD51 and RPA32 have been demonstrated to colocalize with telomeric material but only with a limited number of chromosome ends associated with APBs (51). It remains to be determined how telomeres are targeted by these proteins and whether this association is inevitably followed by recombination. Since APBs can be viewed as specialized PML bodies that in addition to the normal PML body functions sequester telomeres we can learn a great deal about those bodies from the existing literature concerning PML bodies.

Unlike APBs, which are only present in ALT cells, PML nuclear bodies are present in most cells and have been extensively studied due to their versatile functions (reviewed by (80, 81)). PML nuclear bodies are spherical, matrix associated structures ranging in size from 0.1-1 μ m and varying in number from 5 to 30, depending on the cell type and the cell cycle. The PML protein is essential for

the formation of PML bodies and provides the structural scaffold to which other proteins bind (82). Over 60 additional proteins have been localized to PML bodies partially or temporally (see the information on PML bodies from the Nuclear Protein Database, (83)), and as a consequence, PML bodies have been implicated in the regulation of virtually every biological function including DNA damage responses. PML bodies respond dynamically to the cell cycle and to environmental signals such as interferon, viral infections, heat shock and heavy metals. Upon environmental stress, PML body components relocate to the nucleoplasm (84), while entry into mitosis requires entire PML body disassembly (85). Despite the impressive list of cellular processes in which they are involved, PML bodies are not essential for cell viability. In patients with acute promyelocytic leukemia, PML bodies are disrupted due to a fusion of the PML protein with the retinoic acid receptor alpha (86). Regular PML bodies do not specifically bind DNA, however they can make extensive contacts with chromatin domains and respond dynamically to DNA damage by sequestering or releasing protein factors participating in signaling and repair (for a review see (87)). It is not known whether PML bodies make direct contact with DNA under normal conditions but it has been suggested that such bodies function as chromatin remodeling factories involved in the response to improperly folded chromatin or DNA damage. They have been reported to interact with foreign DNA, such as viral DNA (80, 81), with single stranded DNA upon exogenous damage (88) and with subcentromeric satellite DNA in G2, in patients with immunodeficiency, centromeric instability and facial dysmorphia (ICF) syndrome (76).

Taking into account that PML bodies can interact with chromatin, with modified DNA due to damage and with foreign DNA, it is clear that APBs are not unique in their DNA recruitment capacity. It is likely then that in ALT cells telomeres have acquired particular modifications that contribute to their interactions with PML bodies. Alternatively, PML bodies may have acquired the capacity to modify the telomeric chromatin (see below) so that telomeres become stably associated with PML bodies. Whatever the case, PML associated telomeres may take the advantage of high local concentrations of repair factors to initiate recombination reaction.

Finally, it has been shown that APB formation is not essential for ALT (42, 89-91). However, in at least a few cases, the structure of telomeres in these cells has undergone modifications by the insertion/amplification of non-telomeric sequences, suggesting the existence, like in yeast (92), of different types of ALT in human cells. However, there is no documentation on whether or how mechanisms of homologous recombination differ between these ALT types.

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3.2.2. Shelterin and telomere recombination

If mechanisms of telomere capping and damage signaling are conserved from rodents to humans, their understanding has immediate implications for the comprehension of ALT mechanisms in human cancers. In fact, the functions of individual shelterin proteins in telomere capping have been extensively studied in mice. Thus, TRF1 protects telomeres against fragility during DNA replication (93), TPP1/POT1 are required for the repression of ATR signaling (94) and TRF2 was found to be the predominant repressor of both ATM signaling and NHEJ, independently of its binding to RAP1 (94). RAP1, on the other hand, was found to be an important repressor of (T-SCE) (95). Interestingly, loss of RAP1 in mice induces telomere sister recombination in the absence of DNA damage signal (95), a situation somewhat different from humans where ALT-associated T-SCEs occur in cells bearing elevated levels of telomere-induced foci (TIFs) (79). Nevertheless, it is not known whether in human cells the formation of TIFs is mechanistically connected to T-SCEs. Ku70/80 has been also shown to inhibit telomere recombination but only in a context where shelterin is already dysfunctional (96, 97).

Given the available data, it is reasonable to think that compromised or altered function of one or several sheltering proteins involved in repressing recombination may be coupled to the activation of ALT during tumorigenesis. All six shelterin components have been shown to be present at human ALT telomeres *in vivo*, either through immunofluorescence or chromatin immunoprecipitation (98). In addition, there is some evidence that shelterin may be involved in creating the permissive conditions at telomeres for recombination. For instance, studies in which the expressions of four shelterin components, namely TRF1, TRF2, TIN2 and RAP1, were independently inhibited using siRNA, showed that these proteins were essential both for the formation of APBs and for telomere maintenance by recombination (99). Nevertheless, since disruption of APBs was achieved by modulating the expression of shelterin components that are also essential for recombination-independent telomere maintenance, it has been difficult to determine the extent to which the observed telomere shortening is a consequence of a perturbed ALT mechanism as opposed to telomere dysfunction. As mentioned above, elevated levels of TIFs are detected at ALT telomeres, and such foci can be partially suppressed by the overexpression of TRF2 (79). It has been therefore suggested that recombination at telomeres could be promoted, at least in part, by the reduced levels of TRF2 at telomeres. Consistent with this interpretation is the observation that ALT cells usually express low levels of TRF2 (79). Based on these results, a new state for telomere capping unique to ALT has been proposed following which the telomere is neither fully capped nor uncapped, but bears an intermediate state that still efficiently prevents NHEJ but

fails to block homologous recombination. This is dramatically illustrated by one of the hallmarks of ALT cells, which is the persistence of chromosome ends practically devoid of telomere repeats; presumably, such extremities are able to escape repair by NHEJ but are available for recombination-mediated elongation. It remains to be determined how and when telomeres acquire this intermediate-capping state.

In addition to altered levels of shelterin components at ALT telomeres, it is possible, although admittedly hard to prove, that the composition and distribution of shelterin sub-complexes differ all along the telomeric tracts, specifically in ALT cells. Alternatively, it has been shown that some shelterin proteins may be modified post-translationally and that these modifications may alter their affinities for telomeric repeats or their association with APBs. Consistent with this idea is the observation that sumoylation of TRF1 and TRF2 is required both for the recruitment of shelterins to APBs, for the formation of APBs and for telomere length maintenance (100). The complex responsible for the sumoylation of these proteins is SMC5/SMC6/MMS2, which was shown to localize to APBs (100). Therefore, SUMO-modification of shelterin components was suggested to either play a role in the recruitment and/or retention of telomeres to/in APBs since the PML body scaffold contains numerous SUMO binding domains. Yet, it is not known if SUMO modification of shelterin is unique to ALT cells or whether SUMOylation of these proteins exist in other non-ALT contexts.

In conclusion, we are in need of a comprehensive comparative analysis of shelterin components in ALT versus non-ALT cells to further understand the role of these proteins in telomere HDR and recruitment to PML bodies.

3.2.3. Telomere-associated helicases

3.2.3.1. BLM (mutated in Bloom syndrome)

BLM encodes a RecQ DNA helicase, whose absence results in genomic instability characterized by elevated levels of sister-chromatid exchanges (SCEs). BLM was shown to promote D-loop unwinding (101) and is believed to counteract recombination at stalled replication forks. ALT cells are characterized by elevated levels of T-SCEs and intuition would suggest that these effects may be explained by low local concentrations of BLM at ALT telomeres. However, cells deficient in BLM do not show elevated levels of T-SCE, suggesting that lack of BLM at telomeres is not enough to allow recombination. On the other hand, BLM overexpression in ALT cells promotes telomere replication in APBs and an increase of telomere material associated with these bodies (102). Using FRET and co-immunoprecipitation, BLM was shown to interact *in vivo* with the telomeric protein TRF2 in ALT cells (102). In another study, BLM was reported to colocalize with TRF2 in telomere replicating foci

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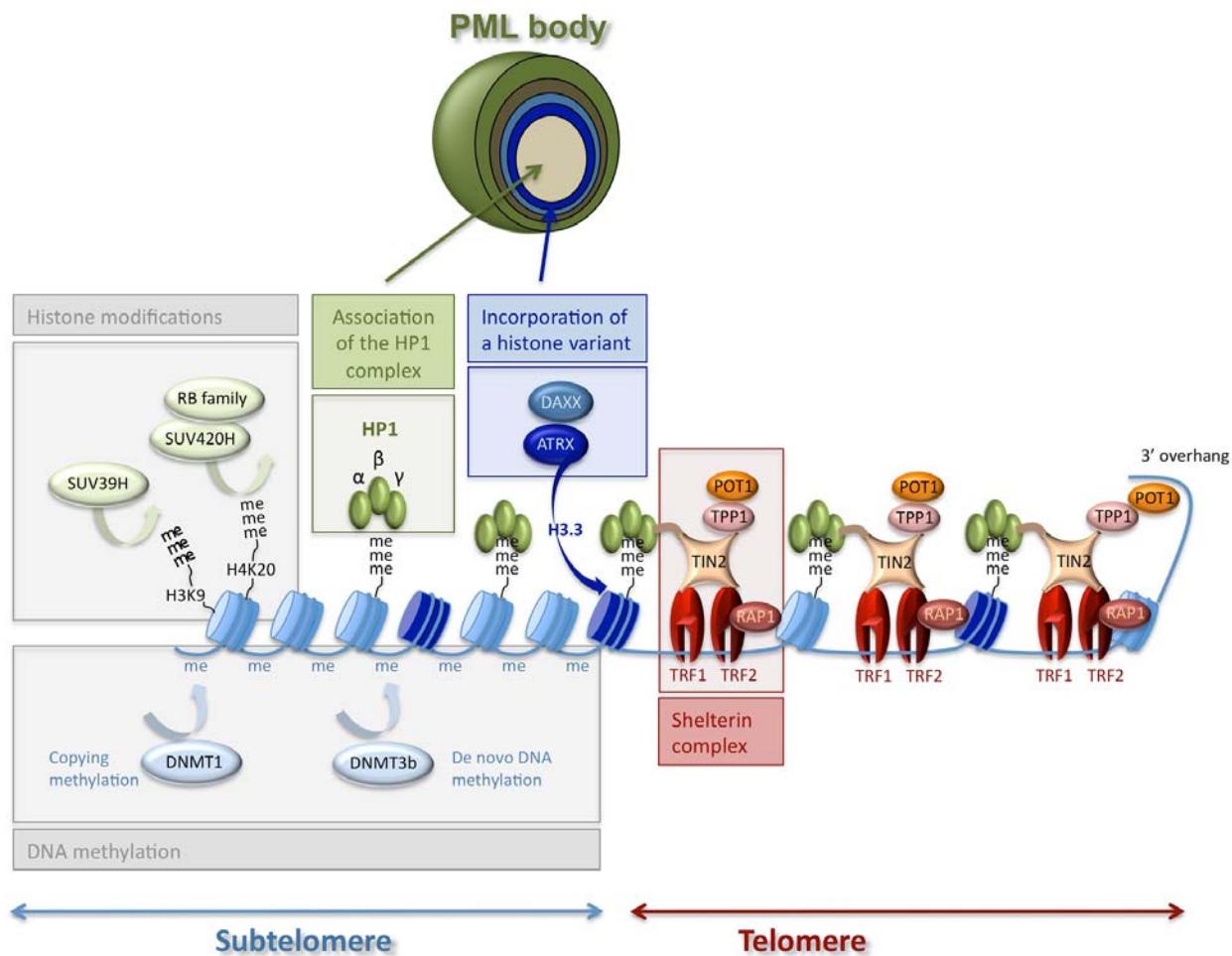


Figure 4. Potential chromatin modifications at ALT telomeres. Telomeres and adjacent subtelomeric regions carry common marks of heterochromatin. Subtelomeric regions are subjected to DNA methylation (mediated by DNMT enzymes). There is no apparent association between levels of DNA methylation and ALT mechanisms. In addition, the relative enrichments on ALT telomeres of tri-methylation of lysine 9 of histone H3 and lysine 20 of histone H4, heterochromatic marks found at normal telomeres and mediated by enzymes SUV39H and SUV420H, respectively, and regulated by the RB pathway, have not been reported. These marks may contribute to the recruitment of heterochromatin proteins HP1 alpha, beta and gamma at telomeres, which are found in ALT and non-ALT telomeres. TIN2, a Shelterin component bridging TRF1 to TRF2 and those to POT1/TPP1, serves also as an adaptor protein for HP1 gamma. In particular contexts, mammalian telomeres are enriched in histone variant H3.3, and its recruitment requires the chaperone ATRX, a component of PML bodies. However, the presence of this histone variant at human ALT telomeres has not been shown. On the other hand, the loss of ATRX-DAXX may be associated with a high prevalence of ALT in human tumors (see text).

during late S and G2/M (103). However, colocalization of BLM with large TRF2 foci or, for that matter, its association to APBs is hardly a direct proof of BLM binding to ALT telomeres. In fact, BLM is an endogenous component of PML bodies (104) and although it largely colocalizes with the telomere material found in APBs, most, if not all, of the BLM protein leaves these bodies when APBs are infiltrated by the Herpes virus protein ICP0 (51). This behavior is in striking contrast to that of all shelterin components (as well as some recombination proteins), which, under the same conditions, not only remain in APBs, but perfectly colocalize with individualized telomeres, indicating a strong

association with these structures. Perhaps more relevant is the fact that TRF1 and TRF2 regulate BLM unwinding activity, at least *in vitro*. Whereas TRF2 stimulates BLM unwinding of telomeric and non-telomeric substrates, TRF1 inhibits BLM unwinding of telomeric substrates only (103). Since BLM interacts with shelterin, its role at telomeres may be very general in promoting unwinding of G quadruplexes and blocking HDR at stalled recombination forks. In addition to this general function, BLM may have a more specific role at ALT telomeres in promoting dissolution of post-replicative recombination intermediates. In favor of this hypothesis is the increase in the frequency of

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telomere bridges following enlargement of (and BLM exclusion from) APBs (51). Interestingly, a SUMO modification of budding yeast BLM ortholog Sgs1 was shown to promote telomere-telomere recombination in that organism (105). In human cells, BLM is also sumoylated and lack of this modification reduces both BLM activity and its association with PML bodies (106). It will be important to evaluate the impact of these modifications (or lack thereof) on ALT telomere metabolism.

3.2.3.2. WRN (mutated in Werner syndrome)

Cells lacking a functional WRN helicase have an increased sister telomere loss that affects telomeres replicated by a lagging mechanism (107). Furthermore, it has been shown that WRN is absolutely required for the complete replication of every lagging telomere (34). Unlike BLM, WRN does not appear to be necessary for ALT since immortalized Werner syndrome cell lines may use recombination pathways to maintain telomeres (91, 108). In a study, using tagged proteins in U2OS cells, WRN colocalized with TRF1-PCNA foci (109). However, we failed to detect WRN in APBs using antibodies (51) and WRN was not detected by Mass spectrometry at telomeres purified from an ALT cell line U2OS (98).

3.2.3.3. RTEL1 (regulator of telomere length 1)

RTEL1 was initially discovered as a regulator of telomeres in mice (110). *In vitro* studies showed that RTEL1 is able to actively disrupt D-loops. *In vivo*, *C. elegans* RTEL1 is required to suppress hyper-recombination and crossovers in meiosis. In spite of its supposed role at maintaining long telomeres, evidence for a direct association of RTEL1 with telomeres is lacking. If RTEL1 can indeed function as an anti-recombinase at telomeres, ALT cells may need to tightly regulate its expression or recruitment to telomeres to allow efficient recombination. Indeed, while telomerase-positive cancer cells contain significant amounts of RTEL1, the protein is barely detectable by immunofluorescence and Western blotting in ALT cancer cell lines (our unpublished results). We are currently investigating the consequences of RTEL1 overexpression in ALT cells.

3.2.4. Chromatin and telomere dynamics

In mammalian cells, the telomeres and adjacent subtelomeric regions are packed as constitutive heterochromatin, perhaps facilitating the stabilization and capping of chromosome ends. Modifications at the level of protein and DNA that are associated with heterochromatin are well characterized and comprise post-translation modifications of histone tails, DNA hypermethylation and incorporation of variant histones (Figure 4). Heterochromatin, as opposed to the open transcriptionally active chromatin, is considered to be transcriptionally silent. However, telomeres were recently shown to be actively transcribed and levels of this transcription varied significantly between cell lines and tissues (111, 112). Since telomeres are transcribed from promoters located in the subtelomeric regions (113), this suggests that there are differences in the

heterochromatic nature of telomeres between cell types. Whether these differences are implicated in the activation of ALT is discussed next.

3.2.4.1. DNA methylation

DNA methylation is ensured by a class of DNA methyltransferases (DNMTs). In mammalian cells, DNMT1 is responsible for copying of the methylation marks during replication, while DNMT3A and DNMT3B are responsible for *de novo* methylation. Telomeres in mammalian cells are devoid of CpG dinucleotides and therefore do not undergo DNA methylation. However, the subtelomeric regions are rich in CpG islands and are heavily methylated in mouse somatic cell in contrast to sperm and oocytes, where they are hypomethylated. Mouse subtelomeric repetitive sequences, like other repetitive sequences in the human genome (human satellite 2 and NBL2), undergo *de novo* methylation during development, facilitated mainly by the DNMT3B, the enzyme involved in *de novo* methylation of repetitive sequences. While repetitive sequences in somatic cells are heavily methylated, this hypermethylation phenotype is lost in some cancer cells, although the way these changes affect tumor progression remains largely uncharacterized. In particular, it is not known how these changes in DNA methylation affect the activation of telomere maintenance mechanisms.

Based on evidence in mice, it has been suggested that DNA methylation of subtelomeric regions plays a role in telomere length regulation. Mice lacking Dnmt1 or both Dnmt3A and Dnmt3B have dramatically elongated telomeres and show signs of ALT, such as elevated rate of T-SCEs and presence of APBs (114). However, these mice retain histone modifications of heterochromatin, such as H3K9 tri-methylated and H4K20 tri-methylated at telomeric and subtelomeric sequences. Similarly, Dicer 1 deficient mice with lower expression of all three Dnmts (Dnmt1, Dnmt3a and Dnmt3b) show increased telomere recombination and telomere elongation (115). Although ALT associated marks are detected in these genetic backgrounds, telomere elongation appears to completely depend on telomerase activity, suggesting only partial de-repression of ALT-related recombinogenic activities.

Although mouse models suggest that the ALT mechanism of telomere maintenance is facilitated by a loss of CpG methylation in subtelomeric regions (114), there is little evidence for the conservation of this model in humans. In the latter, mutations in the DNMT3B gene results in ICF, a rare autosomal-recessive inherited disease linked to the hypomethylation of repetitive sequences including subtelomeric regions. Cells from ICF patients show advanced telomere replication timing and elevated levels of TERRA; however, unlike Dnmt3b-/- mice, they do not display increased T-SCEs (116). Although the majority of ICF

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mutations are single amino acid substitutions in the conserved catalytic domain of DNMT3B, CpG methylation defects in ICF syndrome may also result from impaired stimulation of DNMT3B activity by DNMT3L (117). A carrier of a rare DNMT3L variant (R271Q) with impaired capacity to recruit DNMT3A presented subtelomeric hypomethylation and shorter than average telomeres (118), though it is not known whether any other telomere abnormalities accompanied this telomere shortening. In conclusion, while human genetic variants of DNMT3B and L lead to telomere shortening, there is no evidence that telomeric hypomethylation stimulates telomere recombination in human, non-cancer cells.

There is no clear link between subtelomeric methylation and the type or telomere maintenance mechanism activated in cancer cells. Ng and coworkers have analyzed subtelomeric CpG islands within 2 kb to the telomere on chromosomes 2p, 4p and 18p and reported that telomerase-positive cells invariably show denser methylation than normal cells, while four different ALT cells showed highly heterogeneous patterns in those three loci (119). The authors proposed that there is no methylation requirement for ALT development but rather an imposition on high methylation for telomere elongation by telomerase. They further propose that ALT telomeres, in the absence of a selection pressure, may stochastically lose marks of heterochromatin prior to immortalization, which in turn could increase TERRA production and prevent the action of telomerase. In contrast to the proposed stochastic loss of subtelomeric methylation marks in ALT cells, we have found that there is a pronounced difference in these marks between tumor derived and *in vitro* immortalized ALT cells. While tumor derived ALT cells showed lower than normal subtelomeric CpG methylation levels, *in vitro* immortalized cells showed increased levels (120). Thus, we suggest that methylation in ALT subtelomeric regions is not stochastic but rather influenced by the environment in which ALT mechanisms were activated.

3.2.4.2 Histone modifications

Telomeres are heterochromatic regions displaying all major heterochromatic marks, including increased histone trimethylation and reduced acetylation and the presence of the heterochromatic protein HP1. Even though telomeres are considered heterochromatic, nucleosomes may not be as tightly bound, as suggested by *in vitro* nucleosome assembly studies which showed that telomeric DNA does not properly bend around histones (25), thus explaining, at least in part, the reduced density of nucleosomes at mammalian telomeres. Heterochromatic marks at telomeres have been extensively studied in mice, confirming that telomeres indeed contain classic heterochromatic marks including the trimethylation of H3K9 and H4K20 along with low levels of acetylated H3 (AcH3) and H4 (AcH4) (114). The enzymes

SUV420H1 and SUV420H2 were identified as being responsible for the methylation of telomeric H4K20 in mice (121) and the retinoblastoma family of tumor suppressors (RB, p107 and p130) were shown to be required for the maintenance of these trimethylated marks at both telomeric and pericentric chromatin (121). In humans, it is very difficult to find a consensus between the published studies and as a consequence, histone modifications at telomeres remain poorly defined. Nevertheless, a recent, thorough study including primary human fibroblasts IMR90 confirmed the presence at human telomeres of the same type of heterochromatic marks found present in mouse (122). Regarding ALT, there is no currently available evidence that specific histone modifications are associated with an increased recombinogenic potential of telomeres.

In addition to nucleosome remodeling and covalent modifications, eukaryotic cells generate variations in chromatin by the introduction of variant histone proteins. Mammalian cells express three major types of non-centromeric histone variants, H3.1, H3.2 and H3.3 (123). The H3.3 variant was recently discovered to be incorporated at human and mouse telomeres (124, 125). In other genomic regions, H3.3 is incorporated by the help of a histone chaperone HIRA, and the presence of HIRA-H3.3 was associated with either transcriptional repression or activation (for a review see (126)). Telomeric H3.3 on the other hand, is incorporated by a different chaperone called ATRX and the presence of ATRX-H3.3 at telomeres is associated with the repression of telomeric RNA or TERRA (124, 125). ATRX also strongly interacts with HP1 alpha, DAXX, and MECP2, all of which can associate with PML bodies (127, 128), suggesting that in ALT cells telomeric chromatin remodeling may occur in APBs. Recent observations suggest that loss of ATRX-DAXX complex function may impair the heterochromatic state of the telomeres, perhaps due to reduced levels of H3.3 incorporation, leading to telomere destabilization and increased homologous recombination at telomeres, thereby facilitating the development of ALT (129).

Mammalian telomeres are also enriched in non-histone HP1 family proteins, which are recruited to chromatin through their affinity for trimethylated H3K9 residues and are important for chromatin compaction. In addition, shelterin may contribute to the further enrichment of the heterochromatic marks at telomeres, for instance TIN2 was shown to directly interact with HP1 (130) and TRF1 with an HP1-interacting protein SALL1 (131). In ALT cells, all three HP1 proteins, alpha, beta and gamma, were found in APBs following the activation of the p53/p21 pathway (132) in a HIRA-dependent manner (133). The authors suggested that HP1 proteins mediate chromatin compaction required for APB formation. However these observations were made in

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the context of an active p53 pathway inducing senescence, while in most ALT cells p53 is inactivated.

In conclusion, telomeres are densely packed regions of the chromatin carrying many heterochromatic marks. Yet, telomeres do not belong to the constitutively silent heterochromatin and can be actively transcribed into telomeric RNA. Furthermore, TERRA levels are regulated during development (134) and all along the cell cycle (135) suggesting that telomeres undergo dynamic changes in the chromatin structure. Remodeling of the telomeric chromatin has also been documented during telomere shortening in human and mouse cells leading to a decreased density of heterochromatic histone marks, such as trimethylated H3K9 and H4K20, and more evidently in mouse cells where there is an increase in the density of open chromatin markers such as H3 and H4 acetylation (122, 136). In human cells approaching senescence, histone modification were accompanied by a boost of telomere-associated DNA damage signaling (122), thus creating a particular context perhaps favorable to the emergence of HDR-driven telomere maintenance, as suggested by studies showing an accumulation of short telomeres immediately prior to senescence (9). Nevertheless, more (genetic or epigenetic?) changes are clearly required in order to convert this incipient recombinogenic situation into a telomere-mediated immortalization mechanism.

4. PERSPECTIVE

In spite of exciting discoveries, especially regarding the role of homologous recombination in ALT, the intimate molecular mechanisms that lead to telomere elongation in the absence of telomerase remain poorly understood. Whether the choice of chromosome ends that will undergo recombination is a stochastic or regulated phenomenon is a central question, together with the identification of key players in the recombination initiation and resolution steps, which could provide us with much needed potentially druggable targets. Finally, the opportunity of a therapeutic intervention raises the crucial, but so far not addressed possibility of the existence of a physiological counterpart to ALT in humans, as it has been suggested in mouse embryonic development (43).

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Abbreviations: ALT: alternative lengthening of telomeres; APB: ALT-associated PML body; BFB: breakage-fusion-bridge; BIR: break-induced replication; C-circle: partially single-stranded (CCCTAA)n DNA circle; D-loop: displacement loop; ECTR: extra-chromosomal telomeric repeats; dHJ: double Holliday junction; HDR: homology-directed repair; ICF: centromeric instability and facial dysmorphia; NHEJ: non-homologous end-joining; SCE: sister-chromatid exchange; SDSA: synthesis-dependent strand annealing; T-circle: telomeric circle; T-loop: telomeric loop; TERRA: telomeric repeat-containing RNA; TIF: telomere-induced focus; T-SCE: telomere sister-chromatid exchange; TRD: telomere rapid deletion

Key Words: Alternative-Lengthening Of Telomeres, ALT, APB, Shelterin, Chromatin, DNA Methylation, Histone Modifications, Review

Why ALT?

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