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Review

Alternative paths to telomere elongation

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ABSTRACT

Overcoming cellular senescence that is induced by telomere shortening is critical in tumorigenesis. A majority of cancers achieve telomere maintenance through telomerase expression. However, a subset of cancers takes an alternate route for elongating telomeres: recombination-based alternative lengthening of telomeres (ALT). Current evidence suggests that break-induced replication (BIR), independent of RAD51, underlies ALT telomere synthesis. However, RAD51-dependent homologous recombination is required for homology search and inter-chromosomal telomere recombination in human ALT cancer cell maintenance. Accumulating evidence suggests that the breakdown of stalled replication forks, the replication stress, induces BIR at telomeres. Nevertheless, ALT research is still in its early stage and a comprehensive view is still unclear. Here, we review the current findings regarding the genesis of ALT, how this recombinant pathway is chosen, the epigenetic regulation of telomeres in ALT, and perspectives for clinical applications with the hope that this overview will generate new questions.

1. Introduction

Due to the manner of DNA replication, linear eukaryotic chromosomes face a problem for replicating their very ends, where RNA primers bind. A unique structure, the telomere, has therefore evolved at the end of the chromosome to protect the genome. Most eukaryotic telomeres are composed of repetitive DNA sequences (TT[A/T]GGG repeats), which do not code for any proteins. The length of these repeats varies from species to species, and the sequences vary slightly as well, but the guanine (G)-rich repeats are conserved from yeast to humans. It is not known how this G-rich conservation affects telomere function. Nevertheless, the emergence of telomerase is responsible for the evolutionary conservation of telomere sequences in eukaryotes [1,2]. Telomerase is a reverse transcriptase that catalyzes the addition of telomere DNA repeats at the 3'-end of a linear chromosome. It is a large multi-subunit ribonucleoprotein, and the essential components are the unique catalytic subunit TERT and the RNA component (TERC or TR). TERC is a long non-coding RNA (lncRNA) of variable length in different species with extensive secondary structure essential for catalysis, holoenzyme assembly and accumulation, as well as serving as a template for telomere synthesis. A telomere template sequence (*CUAACCUAAC*) is located at TERC's 5'-end [3]. DNA synthesis combined with reverse transcription elongates the telomeres and overcomes any shortening of telomeres in proliferating cells. Telomerase is usually inactive in differentiated cells but is active in stem cells, memory T/B cells, and cancer cells.

In tumorigenesis, hTERT reactivation is critical [4] for overcoming senescence and immortalization. Immortalization increases the chance of mutations in the genes crucial for genetic integrity, providing a platform for tumorigenesis. The fact that 85 – 90% of cancers exhibit telomerase activity correlates with this notion. However, there are some exceptions. Instead of expressing telomerase, these cells rely on a telomerase-independent telomere elongation mechanism, known as alternative lengthening of telomeres (ALT) (Fig. 1).

In nature, some species employ ALT for telomere maintenance. Examples are Dipteran insects, such as *Chironomus pallidivittatus* [5] and *Anopheles gambiae* [6], both of which lack telomerase. *Chironomus* has extra-chromosomal RNA-DNA complexes with telomeric repeats, which may be responsible for ALT [7], and the telomeres of *Drosophila melanogaster* utilize retrotransposition for maintenance [8,9]. In early embryogenesis, the telomeres of mouse embryos also display recombination signatures, exhibiting ALT-like features [10].

Ten to fifteen percent of cancers elongate telomeres through ALT, and these ALT-type cancers have been judged by a combination of ALT-related biomarkers: the absence of telomerase activity, heterogeneity in

Abbreviations: ALT, Alternative lengthening of telomeres; TMM, Telomere maintenance mechanism; BIR, Break-induced replication; G4, G-quadruplex DNA; LLPS, Liquid-liquid phase separation.

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telomere length, telomere sister-chromatid exchanges (T-SCEs), extra-chromosomal telomeric repeats (ECTRs) or C-circles, and ALT-associated promyelocytic leukemia protein (PML) bodies (APBs) [11]. Notably, some cancers have neither telomerase activity nor evidence for ALT [12]. Thus, the absence of telomerase alone is not a reliable test for assessing an ALT activity. Evidence also suggests that cancers can switch from telomerase to an ALT mechanism when telomerase is inhibited [13]. The Reddel group has shown that a more robust assay for ALT is the detection of C-circles [14]. More recently, Zhang et al. have developed an advanced assay, ATSA (ALT telomere DNA synthesis in APBs) to precisely define ALT [15].

2. Multiple pathways involved in telomere recombination

During the past years, considerable efforts were made to uncover the underlying mechanisms of ALT. A homologous recombination-mediated repair mechanism was suggested as a telomere maintenance mechanism (TMM), based on the evidence that a telomere tag could be copied to other telomeres [16]. Notably, Cho and colleagues found that RAD51 was required for inter-strand telomere search and synapsis, a process resembling meiotic recombination, for TMM in ALT cancer cells [17].

An important study using *Saccharomyces cerevisiae* indicated that at least two types of ALT mechanisms exist. After telomerase depletion, yeast survivors were initially dependent on *RAD51*. However, when *RAD51* was depleted from these survivors, some yeast cells still survived. *RAD51*-independent survivors required *RAD59*. Therefore, two types of ALT exist in yeast: *RAD51*-dependent type I and *RAD59*-dependent (or *RAD51*-independent) type II [18]. In yeast, RAD59 interacts with RAD52 and acts in the same recombination pathway [19]; however, RAD59 does not exist in mammals. Importantly, RAD59 is involved in both RAD51- and RAD54-independent break-induced replication (BIR) pathways [20], suggesting that type II telomerase-deficient yeast survivors may employ a RAD51-independent BIR pathway for telomere lengthening.

In human ALT cancer cells, Greenberg's group initially reported that RAD51-dependent recombination underlies ALT [17]. However, in a follow-up study, Greenberg's group found that RAD51 was dispensable for ALT telomere synthesis per se, but that PolD3/D4-dependent BIR was instead responsible for telomere maintenance [21]. Numerous studies are in agreement that RAD51 is not essential in ALT [14, 21, 22]. Currently, it is accepted that Rad51-independent BIR pathway may be more central [21]. In mouse fibroblasts deficient in both telomerase and *Brca2* (a tumor suppressor crucial in regulating Rad51 and homologous recombination [23–25]), telomeres were maintained through Rad51-independent recombination [26]. A similar phenomenon was observed in *C. elegans* as well [27]. In an ALT cancer cell line, Dilley et al. showed that telomere damage was recognized by the break-induced replisome, which then directed homology-directed telomere synthesis, the BIR [21]. Because BIR is independent of RAD51, but requires RAD52, it resembles the type II yeast survivors [26,28].

However, there are ALT cells that are still less dependent on RAD52. Zhang and colleagues have shown that there are apparently two different pathways involved in BIR-mediated ALT: the RAD52-dependent and the RAD52-independent [15]. By employing an assay that detected telomere synthesis in APB, ATSA (ALT telomere DNA synthesis in APBs), the authors showed that RAD52-depleted cells eventually recovered ALT activity after three months of passaging [15]. Collectively, three possible mechanisms underlying ALT have been identified: RAD51-dependent recombination, RAD52-dependent BIR, and RAD52-independent BIR. These findings also speak to the fact that ALT may be an evolving process, adopting alternative recombinogenic mechanisms during cancer development.

2.1. Damaged telomeres and break-induced replication

A recombination-dependent DNA replication mechanism, BIR was first identified from break-copy recombination events in bacteriophages [29], but is now recognized as an essential mechanism for double-strand break (DSB) repair in eukaryotes [30]. Essentially, BIR is an error-prone pathway because it repairs DSBs through conservative DNA synthesis [31]. During DNA replication, a progressing replication fork may stall, and this is usually resolved using RAD51-dependent HR (Fig. 2, left). If this mechanism fails, the stalled replication fork may collapse. Breakdown of the stalled fork generates a one-ended DSB, the substrate for BIR [32–34]. BIR fills in the gaps of unfinished DNA replication in G2 and early mitosis [33,34]; thus, BIR uses conservative DNA synthesis (Fig. 2, right). Early findings using yeast showed that DNA Polymerase δ was required for BIR [35]. Congruently, Dilley et al. showed that the PolD3 (Pol32) subunit was crucial for TMM in human ALT cell [21]. Using the MiDAS (mitotic DNA synthesis) assay, Min and colleagues confirmed that BIR during mitosis was responsible for telomere synthesis in ALT cancer cells [28].

Interestingly, telomere synthesis in *Brca2*-deficient; telomerase-null cells was accomplished via conservative replication (Fig. 2). In this

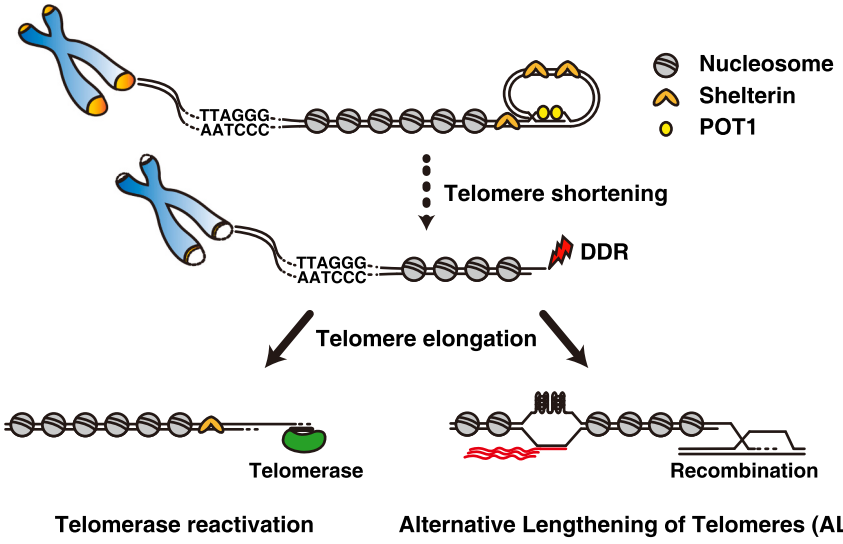

Fig. 1. Two distinct types of telomere elongation. Linear eukaryotic chromosomes experience telomere shortening during continued cell division. Extremely shortened (eroded) telomeres are DNA damage sites that activate DDR, resulting in cell cycle arrest and senescence. To overcome cellular senescence, shortened telomeres must be elongated. One way to achieve this is to reactivate telomerase (left), and an alternative method involves telomeric recombination.

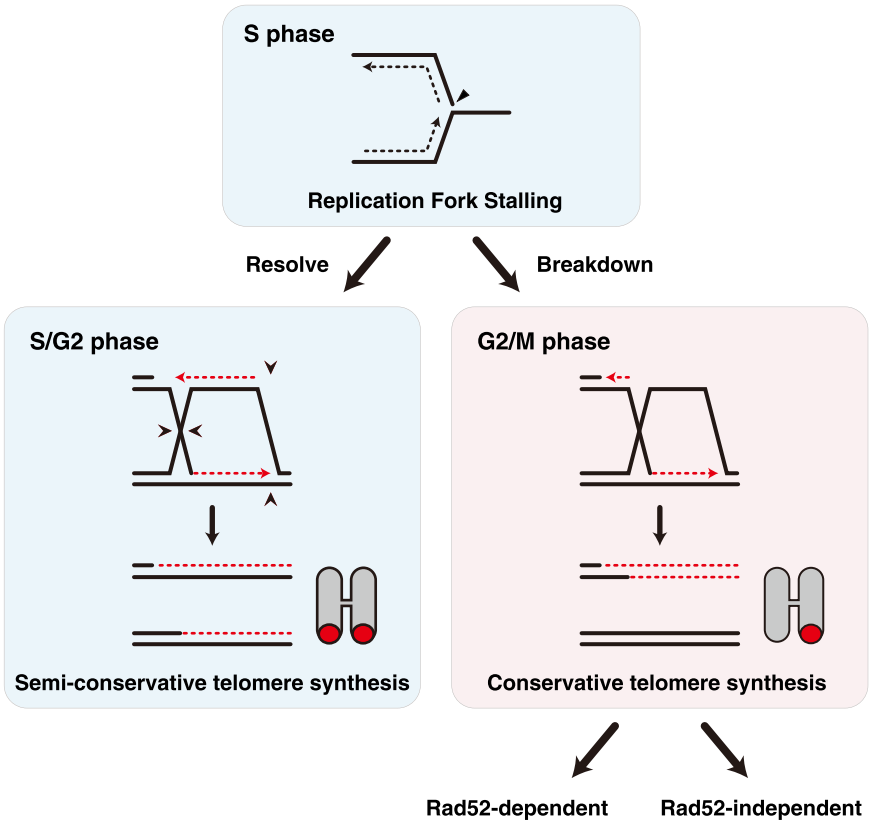

Fig. 2. Alternative paths in telomere synthesis. When a stalled replication fork collapses, cells engage homologous recombination (HR), coupled with semi-conservative DNA synthesis and repair (left). Persistent breakages that have bypassed intra-S checkpoints, and HR, can be filled in by conserved BIR telomere synthesis (right).

case of Brca2 deficiency, Rad51 was dispensable but Rad52 and Mre11 were required for mitotic telomere synthesis [26]. These results suggest that the absence of BRCA2 played a pivotal role in triggering BIR-mediated ALT [26–28]. Currently, it is not yet clear whether BRCA2 deficiency-induced ALT would favor RAD52-dependent BIR over RAD52-independent or vice versa. In addition, it is possible that even more recombination pathways may be involved in *BRCA2*-deficient ALT TMM.

2.2. Molecular basis for replication stress at telomeres

2.2.1. G-quadruplex in telomeres

The so-called fragile sites are chromosomal regions which are most susceptible to replication stress, the breakdown of stalled replication forks [36,37]. Common fragile sites (CFS) tend to be located at conserved loci, and are composed of AT-rich sequences [38], whereas rare fragile sites have repetitive sequences, but not conserved, capable of adopting non-B form structure of DNA [37]. Composed of (TT[A/T]GGG)$_n$ arrays of repeats, telomeres are considered common fragile sites [39,40]. BLM, RTEL1, RECQL4, FANCJ (in mammals), and Pif1 and Dna2 (in yeast) are suggested helicases to resolve telomere replication stress [39–46].

The fragility of telomeres is significantly increased when cells are treated with aphidicolin, a polymerase inhibitor [40]. Notably, its effect is significantly enhanced during lagging-strand synthesis, because the single-stranded G-rich repeats in telomeres challenge the progression of the DNA replication machinery [47]. Interestingly, depletion of Brca2 led to an increase in replication stress, specifically at lagging strands, even without aphidicolin treatment [47], consistent with the finding that BRCA2 protects the stalled replication forks from attack by Mre11 nuclease [48]. What then, is unique about telomere lagging strand synthesis?

Ten years after the identification of Watson-Crick DNA base pairing [49], a variation of hydrogen bonding geometry was found by Karst Hoogsteen [50]. Through Hoogsteen bonding, a four-stranded G-quadruplex can form, which can be found in promoter regions [51]. After the identification of telomere sequences, questions were raised as to whether their guanine repeats could also use Hoogsteen hydrogen bonding to form G-quadruplex secondary structures. Theoretically, four guanines can adopt a flat cyclic arrangement through such hydrogen bonding, called G-tetrads, because guanine can serve as both a donor and an acceptor in hydrogen bonding. When theses G-tetrads stack, it results in G-quadruplex (G4).

Combined biophysical analyses using X-ray crystallography, NMR, and calorimetry revealed that telomere sequences can form G4 in vitro [52–54]. Furthermore, a variety of G4 structures have been shown to coexist [55], but the in vivo evidence for the existence of telomeric G4 was lacking. Using an RNA interference approach in ciliates, Paeschke and colleagues reported that telomeric G4 indeed existed in vivo, which was regulated in a cell cycle-dependent manner [56]. In addition, antibodies generated against G4 also detected telomeres in vivo [56,57].

As secondary DNA structures can act as signals of DNA damage, chemicals that bind and stabilize G4 can target cancer [58]. Such G4 is also thermodynamically more stable and requires special helicases for unwinding. The folding and unfolding of telomeric G4 may be crucial in TMM. FANCJ, PIF1, and BLM have been suggested as potential G4 helicases [44, 59, 60].

However, the biology of G4 has been controversial. Treatment with a G4-stabilizing ligand resulted in an increase of DNA damage, indicating that unresolved G4 is partly responsible for telomere damage [61,62]. On the contrary, a study from *S. cerevisiae* demonstrated that G4 protected telomeres from resection by Exo 1 [63]. Moreover, telomeric G4

interferes with the access of telomerase [64], leading to inhibition of otherwise unlimited proliferation.

During DNA replication, G4 dissociates shelterins from telomeres [65,66]. The packed G4 slows down the replication because the fork stalls [59,67]. When left unresolved, these stalled forks will be degraded, resulting in discontinuous DNA replication, increasing the chances of BIR initiation. Indeed, treatment with a G4 stabilizer resulted in an increase of ALT activity in human U2OS cells [68] and Saos2 cells [28]. Together, this evidence supports the idea that telomeric G4 is a source of replication stress.

2.2.2. Telomere repeat-containing RNA (TERRA) and R-loops

Collisions between the replication and transcription complexes occur during S phase, generating three-stranded RNA:DNA hybrids with displaced single-stranded DNA (R-loops). These R-loops are important drivers of DNA damage [69]. Topoisomerases and helicases normally prevent the formation of R-loops, but if formed, RNase H removes them. ATR-Chk1 sense R-loop-mediated replication impediment [70]. Replication can restart after fork cleavage and re-ligation [71]. After the replication impediment, repriming activity of PRIMPOL restarts the replication in regions forming secondary structures, such as G4 and H-DNA. Thus, PRIMPOL limits the unscheduled R-loop formation [72]. A report showed that PRIMPOL rescues the degradation of nascent strand in BRCA1-deficient cells [73]. BRCA2 has a more direct role that it prevents the accumulation of R-loop via interacting with TREX-2 mRNA export complex [74] or RNA Pol II to prevent unscheduled R-loop synthesis [75].

Several reports have shown that R-loops are relatively abundant in ALT telomeres compared to their level in telomerase-positive cells [76–78]. At telomeres, R-loops are created by the base pairing of the lncRNA TERRA with the complementary telomeric DNA. It has been suggested that TERRA binding and R-loop formation occurs at short telomeres and triggers DNA repair [79]. RNA endonuclease RNase H1, known to regulate the level of R-loops, controls the recombination at ALT telomeres [78], indicating that TERRA and R-loops may be involved in ALT induction.

2.3. Tumor suppressors in suppressing ALT

2.3.1. Tumor suppressor p53

p53 was the first tumor suppressor proposed to suppress ALT. An extensive mutation analysis of *p53* in 110 patients with glioma revealed that 77.8% of the cases with ALT activity also displayed *p53* mutations [80]. In addition, Cesare et al. reported that p53 inactivation is highly associated with spontaneous telomere damages and chromosomal aberrations in ALT cancer cell lines [81].

Mouse genetic studies also supported the idea that p53 may be an ALT suppressor. When WRN helicase was depleted in telomerase knockout mice, MEFs exhibited extremely short telomeres and underwent senescence [82]. These MEFs also displayed hallmarks of ALT, such as chromosomal aberrations, T-SCEs, and APBs [82]. However, for the cells to overcome senescence, immortalize, and finally transform, p53 inactivation was essential [82].

2.3.2. BRCA2

Breast cancer susceptibility gene *BRCA2* is crucial in maintaining telomere homeostasis, and is also involved in suppressing ALT. Two independent reports have shown that BRCA2 deficiency leads to extensive telomere shortening due to problems in telomere replication [47,83]. Badie and colleagues found that BRCA2 loads RAD51 onto damaged sites and facilitates replication [83]. Using a $Brca2^{F11}$ conditional knockout in MEFs, Min and colleagues showed that Brca2 deficiency resulted in breakdown of stalled replication forks, particularly at the lagging strand, leading to telomere shortening [47]. Min et al. also suggested that G4 might form at telomeres during replication, causing replication fork stalling. Without Brca2, these stalled replication forks cannot be protected [47]. Consistent with these findings, treatment with a G4-stabilizing ligand PDS led to a marked increase of telomere fragility in Brca2-deficient cells, particularly at the lagging strand [84].

How then does the absence of BRCA2 lead to tumorigenesis when BRCA2 deficiency induces telomere shortening? When Brca2 was first depleted in telomerase-null MEFs, it led to telomere shortening. However, in later passages, this shortening was diminished and finally telomeres were elongated in immortalized fibroblasts [26], without the requirement of Rad51 or Rad51 paralogues. Instead, BIR was induced at telomeres [26]. Therefore, it is plausible that BRCA2 suppresses ALT in two sequential steps: first, by maintaining telomere replication homeostasis, and then by suppressing BIR. It is known that the majority of *BRCA2*-mutated cancers harbor *p53* mutations [85], and that inactivation of p53 acts synergistically with Brca2-deficiency in neoplastic transformations [86]. Currently, however, there is only limited clinical evidence showing an association between *BRCA2* mutations and ALT cancers, perhaps due to a lack of in-depth genomic examinations. The incidence of ALT in cancers mutated in both *BRCA2* and *p53* is yet to be assessed. Nevertheless, two reports suggested that BRCA2 dysfunction is clinically associated with ALT cancers [87,88].

2.4. Chromatin remodeling in telomere recombination

2.4.1. Chromatin remodelers dealing with replication stress

Telomeres are regions of heterochromatin that are tightly controlled to limit transcription. However, in ALT cells, telomeres are prone to recombination, therefore it is reasonable to think that telomeric chromatin must be remodeled for the access of recombination machinery. Congruently, ATRX (X-linked SWI/SNF family chromatin remodeler), DAXX (H3.3-specific histone chaperone) chromatin remodeler complex, and histone H3.3 are all implicated in ALT [89,90]. A comprehensive analysis of 22 ALT cancer cell lines by ALT Starr Cancer Consortium in 2012 revealed that *ATRX* loss or mutation was associated with ALT [91]. This consortium also reported that ALT cells exhibited a high incidence of genomic rearrangements, altered DNA repair pathways, elevated numbers of micronuclei, and loss of G2/M checkpoints [91]. These findings speak to the fact that genetic instability is profound in ALT cancer cells, and that epigenetic remodeling through ATRX inactivation may be associated with that instability.

Normally, ATRX binds to the tandem repeats, including telomeres [92]. It was shown that ATRX reduces the replication stress, caused by the treatment with a G4-stabilizer [68]. Furthermore, ATRX depletion increases R-loop synthesis [93], suggesting that ATRX counteracts telomeric G4 and R-loops. Thus, it is likely that ATRX suppresses ALT by preventing replication stress at telomeres.

The DNA translocase SMARCAL1 (SWI/SNF-related, matrix-associated, actin-dependent, regulator of chromatin, subfamily A-like protein 1) localizes to stalled replication forks to promote repair and restart the replication [94]. SMARCAL1 specifically localizes to ALT telomeres and resolves replication stress. Its depletion results in excessive DSBs, leading to chromosome fusions [95,96].

Lastly, the TIMELESS-TIPIN complex (orthologues of Swi1-Swi3) is known to be involved in the resolution of replication impediments. This complex helps to maintain stable DNA replication progression through the regulation of the intra-S DNA damage checkpoint [97]. Loss of either TIMELESS or TIPIN leads to significant increases in BIR [28,98].

2.4.2. The FANCM-BLM-TOP3A-RMI complex, SLX4, and SLX4IP in ALT

FANCM, an ATPase/translocase in Fanconi Anemia (FA) complex, has been reported to restrict replication stress at telomeres. Pan et al. showed that depletion of this DNA translocase increases replication stress at ALT telomeres [41]. Following this report, two independent studies have provided evidence that, in several ALT cancer cell lines, FANCM resolves replication stress in ALT telomeres by regulating TERRA and R-loop levels [99,100]. Furthermore, Silva et al. showed that FANCM is responsible for unwinding of telomeric R-loops,

demonstrating that FANCM suppresses R-loop-induced replication stress in ALT telomeres [100].

In the absence of FANCM, both BLM and BRCA1 accumulate at ALT telomeres and promote DNA end resection and recombination [41]. The FANCM-BLM-TOP3A-RMI (BTR) complex has been shown to suppress ALT as well [101]. Such FANCM-mediated ALT suppression requires its DNA translocase activity. Inhibiting the formation of the FANCM-BTR complex is selectively toxic to ALT cells, suggesting that targeting the FANCM-BTR complex can be a promising strategy for treating ALT cancers [101].

BLM and SLX4 have opposing roles in telomere recombination. *BLM* overexpression increased BIR-induced telomere synthesis [102], and was sufficient to induce ALT features in non-ALT cells [103]. In contrast, overexpression of *SLX4*, a scaffold that recruits nucleases to DNA repair sites and resolves Holliday junctions [104,105], suppressed BLM recruitment to telomeres and inhibited ALT [106]. SLX4 interacting protein (SLX4IP) accumulates at ALT telomeres and interacts with SLX4, XPF, and BLM, antagonizing BLM-mediated ALT activity [107]. Collectively, SLX4 and SLX4IP prevent BLM-dependent telomere recombination.

2.4.3. Orphan nuclear receptors and FANCD2

Nuclear receptors participate in ALT by remodeling telomeric chromatin. Due to recombination, repeat variants (e.g., TCAGGG) can accumulate in ALT telomeres [108]. These variant repeats can then bind to NR2C/F-class orphan nuclear receptors, COUP-TF2, and to TR4 [108–110]. These orphan receptors have been shown to be directly recruited to ALT telomeres [110,111] and subsequently recruit NuRD-ZNF827 to telomeres [111]. Interestingly, the localization of the NuRD (nucleosome remodeling and histone deacetylation) complex at telomeres has been shown to promote ALT activity by inducing histone hypoacetylation and telomere compaction [111]. The binding of NR2C/F orphan nuclear receptors to ALT telomeres has been shown to drive telomere clustering via bridging telomeres and promote recombination [110]. Their depletion has been shown to suppress ALT phenotypes [110,111].

FANCD2 has been reported to localize in APBs [112,113], but a precise role in ALT has yet to be determined. Knockdown of FANCD2 in ALT cells resulted in both an increase in telomere damage [112] and a decrease in telomeric sister-chromatid exchanges (T-SCE) frequency [113]. Furthermore, orphan nuclear receptors have been shown to regulate ALT activity through direct binding to FANCD2 [114]. Knockdown of the orphan nuclear receptors COUP-TFII/TR4 or FANCD2 results in a significant reduction of ALT phenotypes. In ALT cells, FANCD2 recruits MUS81 and POLD3 to telomeres [114], suggesting that FANCD2 is involved in BIR. However, a conflicting report showed that FANCD2 knockdown increases telomeric DNA content and the T-SCE rate [115].

2.4.4. The heterochromatin state of ALT telomeres

Telomeric heterochromatin is formed through tight packaging by heterochromatin-specific histones and epigenetic regulators. Mammalian telomeres are marked with constitutive heterochromatin markers, such as K9 trimethylation of histone 3 (H3K9me3) and K20 trimethylation of histone 4 (H4K20me3). Heterochromatin protein-1 (HP1) is enriched at telomeres and the subtelomeres are hypermethylated [116–118]. Mice deficient in the RNA component of telomerase *Terc* exhibit shortened telomeres with a decrease in heterochromatin marks [119], indicating that such telomeric attrition leads to a more 'open state' in its chromatin. Compared to telomerase-positive telomeres in human cells, ALT telomeres display a reduction in H3K9me3, which leads to an increase in telomeric transcription [77]. Collectively, these data suggest that epigenetic changes allow the DNA recombination machinery to access to the damaged telomeres.

Somatic mutations in the chromatin remodeling proteins *ATRX*, *DAXX*, and histone variant *H3.3* have been shown to associate with ALT activity [89–91, 120]. However, the epigenetic markers that specify distinct recombination pathways in ALT have not been elucidated yet. Importantly, the ATRX/DAXX complex has been shown to deposit H3.3 onto repetitive heterochromatin and at telomeres [121,122]. H3.3 is a known substrate of K9 trimethylation [123]. ATRX directly interacts with trimethylated Lys9, and HP1 also recognizes H3K9me3. Cooperation between ATRX and HP1 in chromatin recruitment results in the maintenance of heterochromatin state [122]. Phosphorylation of H3.3 by CHK1 at Serine 31 has been shown to be crucial for survival of ALT cells [124], indicating that the DNA damage response (DDR) opens up the telomeric heterochromatin for ALT activity.

However, some ALT cancers still maintain regular ATRX/DAXX activity. Despite the fact that re-expression of *ATRX* can suppress ALT activity [68,125], knockout or knockdown of *ATRX* alone cannot activate the ALT pathway [125,126]. By comparison, the depletion of histone chaperone ASF1, which participates in nucleosome assembly by shuttling histone H3.1-H4 dimers or H3.3-H4 dimers, resulted in telomeric recombination and ALT features in human cells [126]. Moreover, ASF1 knockdown directly leads to the suppression of telomerase activity [126]. These results suggest that the dislodging of H3.3 from telomeres is correlated with ALT recombination, but it is not the sole determinant for epigenetic changes in telomere recombination (Fig. 3).

The prevailing notion that ALT initiation requires an epigenetic "open" telomeric state has been challenged recently. A common technique for studying epigenetic modifications present in a specific DNA sequence is chromatin immunoprecipitation (ChIP). Due to the tandem repeats found in telomeric sequences, distinguishing between genuine telomere regions and interstitial telomere sequences (ITSs) has not been possible. Cubiles et al. made the distinction by reading the number of perfect telomere repeats: four or more perfect tandem telomere repeats representing telomeres, and less than four representing ITSs [127]. When the histone modifications in five perfect tandem repeats were analyzed, it showed that human telomeres exhibited enrichment of H4K20me1 and H3K27ac marks, suggesting euchromatin [127]. When the telomeres of telomerase-positive H1 ESC cells and ALT-positive U2OS cells were compared, telomeres from U2OS cells exhibited a higher level of H3K9me3 marks [127], challenging the current notion that a heterochromatic state inhibits ALT.

Similarly, by performing telomere-specific quantitative proteomics, Déjardin's group suggested that ALT telomeres do not display open chromatin features but instead contain a high level of H3K9me3 [128]. Knockdown of SETDB1 methyltransferase resulted in a loss of H3K9me3 marks, the disruption of telomeric heterochromatin, and the suppression of recombination [128]. In addition, excessive heterochromatin in telomeres promoted T-SCEs, APB formation, and telomere transcription, arguing that ALT relies on the establishment of a heterochromatic state [128]. Until now, epigenetic regulation and chromatin alterations at telomeres during ALT recombination remain largely elusive (Fig. 3).

2.5. Clustering of telomeres in a specific nuclear compartment

One of the crucial cellular characteristics of ALT cells is the presence of ALT-associated promyelocytic leukemia bodies (APBs) [129]. In ALT cancer cell lines, the long-range directional movement of telomeres for recombination has been observed [17]. Consistently, clustering of 2–5 telomeres in an APB was observed in Brca2-depleted ALT MEFs, indicating that APB is the nuclear compartment for telomere synthesis [26]. BLM of the BTR complex is also crucial for ALT. It is responsible for aggregating telomeres in APBs, consistent with the notion that APB is the site for telomeric recombination [17].

In APB, SMC5/6 complex participates in sumoylation of the telomere-binding proteins, which is a crucial process for maintaining telomere length [22]. Collectively, these results indicate that APBs are not just ALT cell markers, but is a platform for telomere recombination. That knockdown of the promyelocytic leukemia (PML) protein in ALT cells results in telomere shortening, decrease of ECTRs [130], and a dramatic reduction in telomeric DNA synthesis [15] supports this

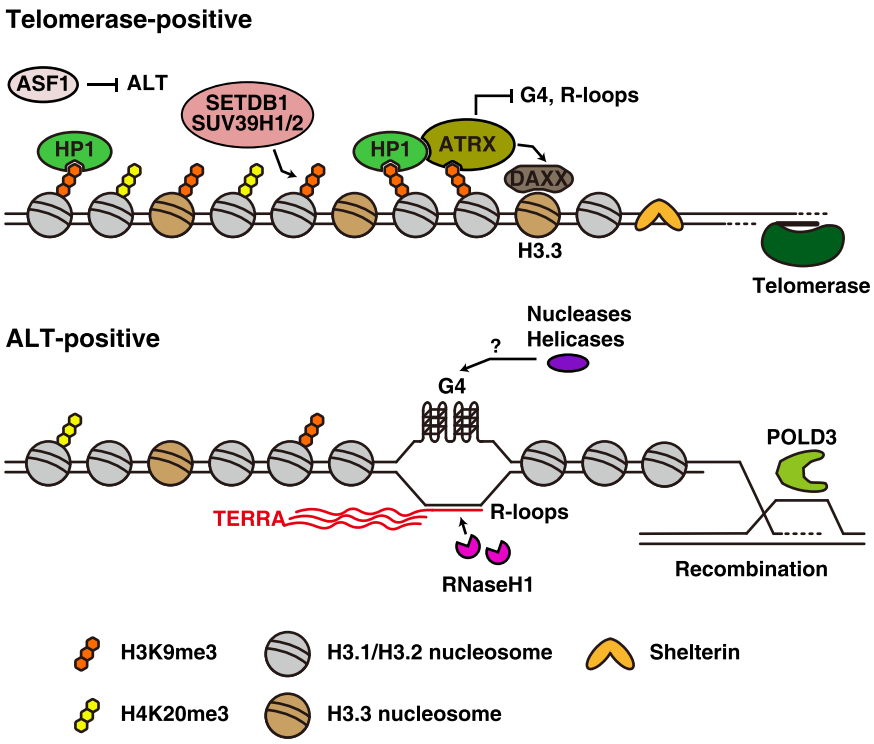

Fig. 3. Comparison of chromatin structures in telomerase-positive and ALT-positive telomeres. In telomerase-positive telomeres, repressive histone posttranslational modifications (H3K9me3 and H4K20me3) and heterochromatin protein 1 (HP1) are abundant. Histone variant H3.3 confers stable telomeric heterochromatin formation. In ALT telomeres, ATRX is mutated or lost, hence H3.3 is reduced in telomeres. This promotes the transcription of TERRA. ALT-positive telomeres display stabilized G-quadruplex (G4) and an increase in R-loops.

notion. However, the precise function of PML in APB is still unclear.

Eukaryotic cells contain membraneless organelles composed of protein complexes and nucleic acids, which can be separated from other organelles by forming specialized droplets through a process called liquid-liquid phase separation (LLPS) [131]. LLPS provides distinct area of highly concentrated proteins and nucleic acids, still dynamically interacting with the exterior cellular space. As PML bodies are membrane-free organelles composed of protein complexes and nucleic acids, gathered by multivalent interactions, PML may be an LLPS.

Utilizing SUMO/SIM (SUMO-interacting motif) interactions, Min and colleagues showed that APB in ALT cells exhibits LLPS property and drives telomere clustering [103]. When *BLM*-expressing construct was overexpressed with a poly-SUMO/poly-SIM-expressing construct, large APBs assembled along with BLM, ssDNA, and RPA [103]. Telomeres clustered in LLPS-induced APBs and RAD52-dependent BIR was instigated [103], demonstrating that LLPS-like property of APB provides a confined space for recombination.

The MRE11/RAD50/NBS1 (MRN) complex is implicated in ALT [132]. The sequestration of MRN by *Sp100* overexpression [132] and the depletion of the MRN complex [133] both resulted in a reduction in ALT activity, suggesting that the function of MRN is closely linked with TMM in APBs. As MRE11 nuclease is responsible for the collapse of stalled replication forks [48], it would be interesting to understand if MRE11 is an essential factor in forming LLPS-APB. Interestingly, ATRX has been shown to interact with, and sequester, the MRN complex from telomeres, consistent with the notion that *ATRX* mutations are frequent in ALT cancers [68]. In the absence of ATRX, MRN relocates to APBs and involves in the ALT pathway [68].

3. Perspectives for clinical applications

As 80–95% of cancers express telomerase, targeting it for cancer therapy was considered effective. A variety of drugs that inactivate telomerase, including G4-stabilizing chemicals, have been used in clinical trials [134]. Despite extensive study, only a limited number of antagonists or antibodies of telomerase are used in the clinic. One reason for this is that only a small amount of telomerase activity may be required for immortalization; hence, the targeting telomerase may be inefficient [135]. A more important reason for a cautious approach to targeting telomerase for anti-cancer therapy may be because cancer cells may have additional options for inducing alternative telomere recombination pathways. Considering that many cancers harbor *p53* mutations, targeting telomerase may instead provoke ALT, and human clinical evidence supports this notion: telomerase inhibition in mismatch repair-deficient human colon cancer cells developed resistance by promoting telomere recombination [136].

G-quadruplex ligands can also block telomerase activity [137]. They can also down-regulate the expression of oncogenes, such as *c-myc* and *her2*, as G4 ligands can target the transcription binding sites [138,139]. When G4-stabilizing ligands were treated to ALT cancer cells, it resulted in cell cycle arrest and apoptosis [140,141], indicating that G4 ligands can be effective in targeting ALT cancers. That Brca2-deficient cells were susceptible to G4 stabilizing ligands is consistent with this notion [84]. Similarly, cancers that exhibit a marked increase in G4 [142] may also be potential targets.

Defining whether tumors are telomerase-dependent or ALT-dependent will be crucial for choosing optimal therapies. Furthermore, determining the specific ALT pathway that is responsible will be essential for choosing the appropriate targets for patient-specific treatments. To facilitate this choice, the identification of specific biomarkers for various TMM is therefore crucial. FANCM-BTR [101], PRIMPOL for treating BRCA-deficiency [73], and the G4 ligands are examples of this endeavor.

As tumorigenesis relies on telomere lengthening, targeting a specific telomere elongation pathway may be useful as an anti-cancer therapy, but there are several roads to telomere elongation. The choice will make all the differences: either effective treatment or resistance followed by the generation of an unexpected secondary ALT cancer development. Therefore, a better understanding of the many different pathways to telomere maintenance is crucial for patient-specific precision medicine.

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