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JB Review

Alternative lengthening of telomeres pathway: Recombination-mediated telomere maintenance mechanism in human cells

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Unlimitedly proliferating cells need to acquire the telomere DNA maintenance mechanism, to counteract possible shortening through multiple rounds of replication and segregation of linear chromosomes. Most human cancer cells express telomerase whereas the other cells utilize the alternative lengthening of telomeres (ALT) pathway to elongate telomere DNA. It is suggested that ALT depends on the recombination between telomere repetitive DNAs. However, the molecular details remain unknown. Recent studies have provided evidence of special structures of telomere DNA and genes essential for the phenotypes of ALT cells. The molecular models of the ALT pathway should be validated to elucidate recombination-mediated telomere maintenance and promote the applications to anti-cancer therapy.

Keywords: alternative lengthening of telomeres (ALT)/extrachromosomal telomeric repeat (ECTR)/PML body/recombination/telomere.

Abbreviations: ALT, alternative lengthening of telomeres; BIR, break-induced replication; CDK, cyclin-dependent kinase; DDR, DNA damage response; DSB, double-stranded DNA break; DSBR, double-stranded DNA break repair; HR, homologous recombination; RCR, rolling-circle replication.

Telomeres are specific chromatin structures at the linear chromosome ends of eukaryotic cells. Each telomere contains an end of a double-stranded (ds) DNA, which is protected from degradation and repair processes that can cause chromosome instability. This end protection requires functional telomere chromatin that includes specialized DNA (telomere DNA) and its binding proteins (telomere binding proteins) (Fig. 1A and B). The telomere DNA of most eukaryotes is repetitive DNA containing a guanine-rich (in human, 5′-TTAGGG-3′) strand and its complementary strand called G- and C-strands, respectively. The 3′-end of G-strand extends to form a single-stranded (ss) structure called the G-tail (*1*). The G-tail is thought to invade and hybridize with the proximal C-strand to form a special structure called the t-loop (*2*). Shelterin is a conserved protein complex on telomeres, and shelterin in mammalian cells is composed of one ss (POT1) and two ds (TRF1 and TRF2) telomere DNA binding proteins as well as specific proteins to connect those DNA binding proteins (*3*, *4*). This complex serves as the functional framework of telomere chromatin.

Telomere DNA in dividing cells is subject to possible shortening or the so-called end-replication problem (Fig. 1C) (*5*, *6*). Semi-conservative replication cannot complete the syntheses of the very ends of linear DNA. Thus after multiple rounds of cell division, human somatic cells have shortened telomere DNA, which results in irreversible cell growth arrest, namely, replicative senescence (*7*, *8*). On the other hand, cancer cells express certain mechanisms to counteract the shortening of telomere DNA and acquire immortality. Telomerase is a specific reverse transcriptase that elongates the G-strand telomere DNA (*9*). Approximately 90% of cancer cells maintain telomeres in a telomerase-dependent manner; however, some telomerase-negative human cell lines have been established from cancer cells and *in vitro* immortalized cells (*10*). Thus, it is imperative to understand both telomerase- and telomerase-independent pathways to inhibit the immortality of cancer cells.

In yeast and mouse, telomerase-negative cells have been directly isolated from 'survivors' after artificial disruption of the gene encoding the telomerase RNA component (telomere RNA: TR) or the catalytic subunit (telomere reverse transcriptase: TERT). Survivors in fission yeast *Schizosaccharomyces pombe* occasionally develop self-circularized chromosomes to circumvent the end-replication problem (*11*, *12*), whereas survivors in other species generally acquire the lengthening mechanism of telomere DNA at linear chromosome ends (Fig. 1D) (*13*–*15*). Phenotypic variations among telomerase-negative cells gave birth to the idea that there may be more than one telomerase-independent pathway. One of the most significant examples is the alternative lengthening of telomeres (ALT) pathway of human cells. Confusingly, 'ALT' is sometimes used for various telomerase-independent pathways in human and other species. The ALT we describe here is confined to the pathway found in a group of human telomerase-negative cells (hereafter, ALT cells) showing two distinctive telomere

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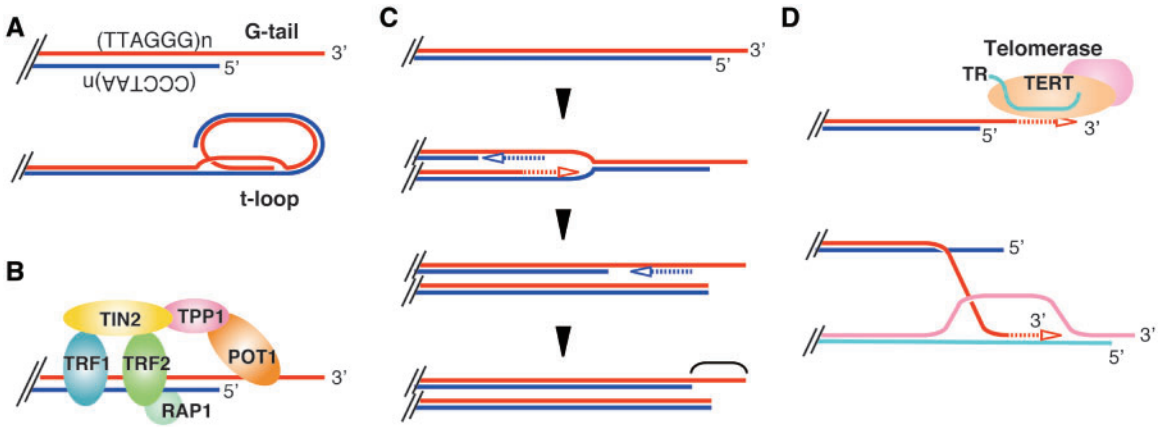

Fig. 1 Telomere structure and lengthening. (A) Vertebrate telomere DNA. (B) Shelterin complex in human cells. (C) End-replication problem. The G-strand (red) is completely replicated to the end. However, the C-strand (blue) of telomere DNA is synthesized as a lagging strand and thus the copy of the very end cannot be accomplished. This causes the gradual shortening of telomere DNA through rounds of cell divisions. (D) Two types of telomere lengthening mechanism. The G-strand is primarily elongated in the telomerase pathway (top). Telomerase-independent pathway (bottom) is generally recombination-mediated.

phenotypes; heterogeneous and long telomere DNA and the formation of ALT-associated promyelocytic leukaemia (PML) body (APB). These phenotypes are widely used as convenient markers for ALT cell lines. In this review, we will summarize the characteristics and the most recent findings of the human ALT pathway. We will also discuss the molecular mechanism of ALT and its relevance to diverse telomerase-independent pathways.

Telomere DNA length and recombination in ALT cells

Telomere DNA length is generally analysed by the Southern hybridization of genomic DNA treated with appropriate restriction enzymes. The mean length of telomere in human telomerase-positive cancer cell lines is usually <10 kb. In contrast, ALT cells have longer and more heterogeneous telomeres; the mean length is ~20 kb (*16*). This suggests that ALT is a distinct lengthening mechanism from the telomerase pathway and telomere length analysis is regarded as one of the vital tests for ALT. A fluorescent *in situ* hybridization (FISH) experiment of metaphase chromosomes also demonstrated the remarkable heterogeneity of telomere lengths in ALT cells. The signal strengths of the telomere foci in ALT cells varied markedly, whereas those in telomerase-positive cells were comparable between chromosome ends (*17*). It is unknown how the heterogeneity of telomere lengths developed; however, it may be related to telomere metabolism specific to ALT cells.

The dynamic behaviour of telomere length in ALT cells was described in a study that used cells with artificially tagged telomeres (*18*). A tag sequence inserted near the telomere repeats of a defined chromosome end was used as a probe to measure the length of this telomere DNA specifically. The telomere in an ALT cell line was drastically elongated during cell division, which differed from that in telomerase-positive cells. The telomeres also showed gradual shortening similar to normal somatic cells and sometimes exhibited rapid deletion. The rapid changes in the telomere DNA length of this ALT cell line suggest homologous recombination (HR) between telomeres. Another study demonstrated that a tag sequence within the telomere repeats of one chromosome was duplicated to other chromosome ends through cell division in ALT cells but not in telomerase-positive cells (*19*). This recombination event may reflect the ALT pathway in which telomeres are elongated by making a copy of the telomere DNAs of different chromosomes.

It is possible that ALT cells represent elevated levels of HR at other loci as well as telomere repeats. The instability of the repetitive sequences of minisatellites was more frequently noted in ALT cell lines than in telomerase-positive cell lines (*20*, *21*). However, ALT-specific hyper-recombination was not observed when the frequencies of intrachromosomal recombination were analysed by using a reporter construct integrated into a certain chromosome locus (*22*). Therefore, ALT cells may have the feature of elevated recombination only in the loci with repetitive sequences, such as telomeres and minisatellites.

To visualize *de novo* synthesized telomeric G- or C-strand specifically, chromosome orientation (CO)-FISH analysis was applied to telomeres (Fig. 2). In metaphase chromosomes, the CO-FISH signal was typically observed at the telomere of either one of the sister chromatids. The frequencies of telomere-sister-chromatid exchange (T-SCE) were calculated as the percentage of chromosome ends positive for CO-FISH signals on both sister telomeres. The high level of T-SCE was generally found in ALT but not in telomerase-positive cells (*23–25*) and therefore, hyper-recombination between sister telomeres was recognized as one of the features of ALT cells. However, T-SCE or the reciprocal exchange of sister telomeres by itself does not accompany massive DNA synthesis and could not account for the net elongation of telomeres or the ALT pathway.

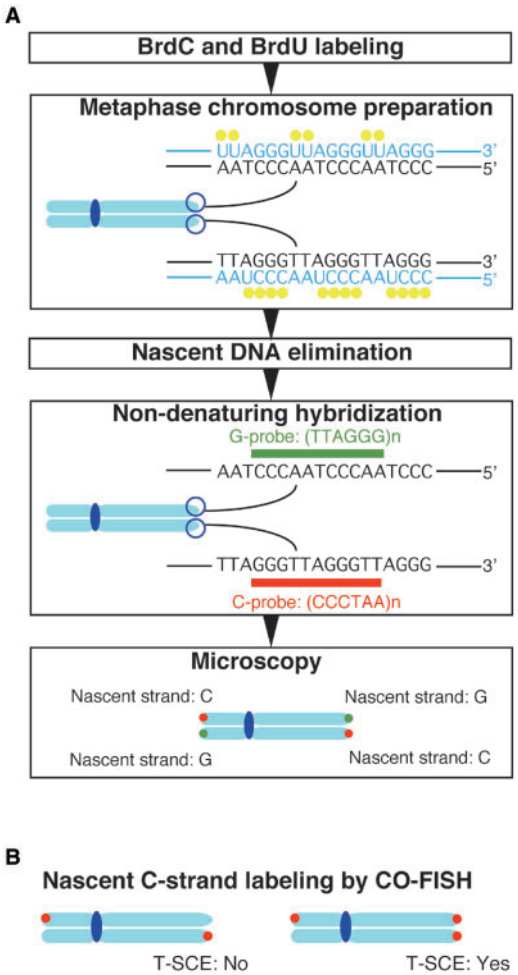

Fig. 2 CO-FISH analysis and T-SCE. (A) Outline of CO-FISH method. The nascent DNA strands (pale blue) are labelled with BrdU and BrdC and they can be specifically degraded. The template strands are exposed and can be hybridized with the florescent G- or C-probe (green or red bar). The signal of each probe can be observed at telomere of each one of sister chromatids.
(B) Observation of T-SCE by CO-FISH. When T-SCE is occurred after DNA replication, the signals of CO-FISH can be observed at the both sister telomeres.

Particular structures of telomere DNA in ALT cells

The first evidence of unusual telomere DNA in ALT cells is the extrachromosomal telomere repeat (ECTR). FISH analyses of metaphase chromosomes of ALT cells suggested the presence of significant telomere DNA repeats other than the chromosome ends (*26*). ALT cells harbour small ds linear telomere DNA in the soluble fractions of the cell extract, which can be separated from bulk chromatin (*27*). Circular DNA molecules were occasionally found in ALT cells by electron microscopy and these are thought to correspond to the circular form of telomere DNA (t-circle) determined by two-dimensional (2D) gel electrophoresis (*28*, *29*). T-circles are now regarded as a marker for ALT cells, although they can be observed in telomerase-positive cells that have a defect in TRF2 or that contain extensively elongated telomere DNA (*29*, *30*). T-circles can be produced by the intrachromosomal recombination of telomere repeats (Fig. 3A). ALT cells may acquire a feature that induces such recombination events.

ALT cells harbour in particular ss structures of telomere DNA (*31*). The average lengths of ss telomere DNA are shorter than those of ds telomere DNA, suggesting that telomere in ALT cells contains nicked and/or gapped structures. Different forms of ss telomere DNA were resolved by 2D gel electrophoresis. The small sized ones of G- and C-strands were separated in the electrophoresis and showed the different sensitivities to structure-specific nucleases: the single G-strand structure (ss-G) was concluded to be linear DNA whereas the single C-strand structure (ss-C) was circular DNA. A sensitive method to detect circular C-strands was developed and applied to examine various cell lines (*32*). Interestingly, ALT cell lines were positive for the circular C-strands whereas telomerase-positive cells were negative. This suggests that this 'C-circle (CC) assay' is useful to determine whether the tested cancer cells are ALT or not. In addition, branched molecules of telomere DNA were found in the ss telomere structures of ALT cells. These remarkable structures of telomere DNA, t-circles and various ss structures, must be specific intermediates or crucial substrates for telomere metabolism in the ALT pathway.

The ALT pathway must be mediated by some HR-based mechanism where the nascent telomere DNA is synthesized efficiently. That is, the ALT pathway could induce pairing of the G- and C-strands in *trans* by HR to form the primer and the template. DNA synthesis should follow to elongate telomere DNA from the primer. One possible mechanism is break-induced replication (BIR). In BIR, the 3′-end of ss DNA invades a region with a homologous sequence at first, and then DNA synthesis is initiated at the 3′-end by using the paired strand as template. It has been suggested that BIR must operate in the telomere lengthening in ALT cells (Fig. 3B). When the 3′-end of the G-tail is paired with the C-strand of other telomeres, a nascent G-strand can be synthesized and elongated up to the end of the template C-strand. BIR readily accounts for the high frequency of copy of a tag in telomere DNA (*19*), although the function of circular telomere DNA specifically found in ALT cells (t-circle and ss-C) is unclear.

The integration of t-circles into the telomere repeats at chromosome ends will result in telomere elongation (Fig. 3C). However, it does not increase the net amount of telomere DNA in the cells and cannot complete telomere maintenance through multiple divisions of ALT cells. Furthermore, circular forms of DNA can be involved in the rapid elongation of DNA ends in rolling-circle replication (RCR) (Fig. 3D). When the 3′-end of the G-tail is paired with ss-C and/or t-circles in ALT cells, the synthesis of the G-strand can be induced on this circular DNA as a template. RCR is fundamentally a continuous process and can accomplish very efficient lengthening even with small templates. Interestingly, the ss-G structure in ALT cells is similar to the linear ss intermediates of RCR in yeast mitochondria DNA (*33*). BIR and RCR are not exclusive of each other and both require invasion

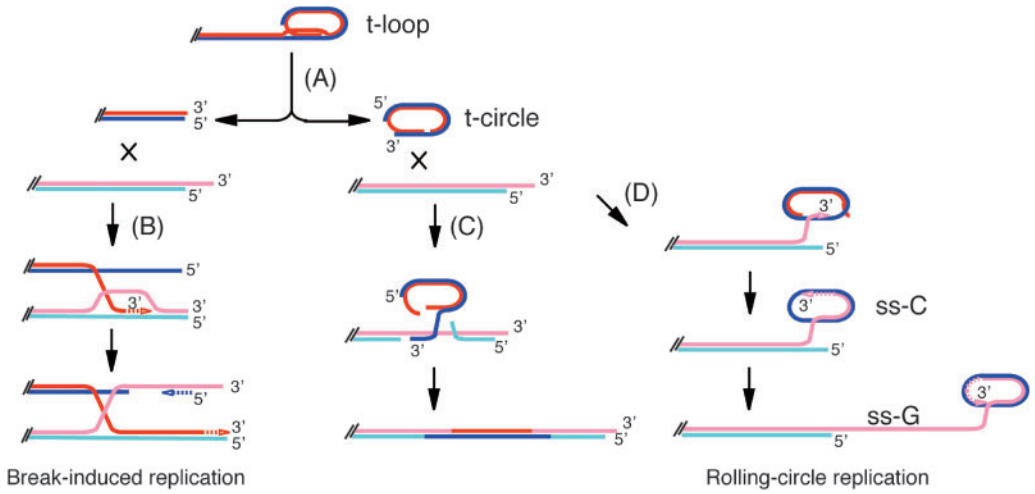

Fig. 3 Telomere DNA metabolism in ALT cells. Four types of recombination events result in particular DNA metabolisms and unique products. (A) Recombination within t-loop causes rapid telomere shortening and t-circle formation. (B) Recombination between telomeres of chromosome ends initiates break-induced replication. (C) Transfer of a strand of t-circle into telomeres at chromosomal DNA initiates elongation of the repetitive DNA. (D) Invasion of t-circle by G-tail triggers rolling-circle replication.

of the 3′-end of the G-tail with the C-strand and the initiation of DNA synthesis at the pairing sites. This implies that both mechanisms may contribute to the ALT pathway and be regulated by common molecular mechanisms.

ALT-associated PML body

PML body is a nuclear aggregate of PML and other proteins and is insensitive to low concentration of non-ionic detergent. PML bodies are present in many types of cells, *e.g.* in both telomerase-positive and ALT cell lines. It has been suggested that PML body functions in various cellular processes, including tumour formation, cellular senescence, stress response, DNA repair, etc. However, its molecular function(s) remains unsolved. Although PML bodies are usually unrelated to telomere, many telomerase-negative cell lines commonly have ALT-associated PML body (APB), a special form of PML body that includes telomere DNA and chromatin (*34*). This feature, namely, the colocalization of both markers for telomere and PML body, can be readily observed and thus, the formation of APB has been used as a marker for ALT cells. Although it is unknown how APBs are related to the ALT pathway, it is noteworthy that APBs possess a variety of proteins working in DNA metabolism and cell growth regulation (Table I). The localization of recombination proteins at APBs supports the idea that HR may be associated with ALT.

The incorporation of the thymidine analogue bromodeoxyuridine (BrdU) was observed in a fraction of APBs (*35*). This nascent DNA synthesis at APBs was suppressed by inhibitors of ATM and ATR (*36*), which are members of phosphoinositide 3-kinase related kinase (PIKK) crucial for the activation of DNA-damage response (DDR). This suggests that the ALT pathway may be regulated by damage signalling. Telomeres of ALT cells are colocalized with the markers for DDR, for example, γ-H2AX, 53BP1, the RAD9-RAD1-HUS1 (9-1-1) complex, and its loader RAD17 (*36*, *37*). However, it is unknown whether DDR of telomere in ALT cells is activated by particular ss structures of telomere DNA. Two reports suggested that APBs have a special form of telomere DNA. The major telomere DNA components in APB are chromosome ends; thus the chromosome ends may cluster at APBs to enhance HR between telomeres (*38*). In contrast, purification study suggests that APBs predominantly contain linear ECTRs (*39*). Therefore, the specific structures of telomere DNA in APBs and their relevance to the ALT pathway have still to be unraveled.

Proteins required by ALT cells

To elucidate the molecular mechanism of the ALT pathway, phenotypes of ALT cells were examined when the function of proteins and/or the expression of their coding genes were suppressed or induced (Table I). The candidate proteins of interest were the components of telomere chromatin, APB and DNA metabolisms including HR. Among the phenotypes, changes in telomere length were particularly notable in the analyses. The erosion of telomere DNA from ALT cells may be due to two reasons: one is a defect in the ALT pathway or the lengthening mechanism of telomere DNA. This will gradually reduce telomere lengths as the end-replication problem emerges. The other is the imperfect protection of telomere. As telomere DNA in ALT cells contains remarkable ss and/or branched structures, ALT cells may be sensitive to even small damages in the telomere DNA caused by certain gene mutations or overexpression. The defect may cause the sudden loss of telomere. In both cases, a shortened telomere DNA is expected to induce defects in cell growth and/or cellular senescence. In addition, ALT-specific phenotypes, for example, the formation of APB and the frequency of T-SCE, were also examined.

Table I. Proteins related to ALT pathway.

Name	Tests[a]	Remarks	References
53BP1	LK	DDR	(*37*, *43*)
ATM	L	DDR; PIKK	(*46*)
ATR	LK	DDR; PIKK	(*57*)
BLM	LK	DSBR; RecQ-like helicase	(*47*, *48*, *70*)
BRCA1	L	HR; breast cancer 1	(*71*)
Cdk2	L	Cell cycle; CDK	(*58*)
COUP-TF1	L	Unknown; orphan nuclear receptor	(*59*)
COUP-TF2	L	Unknown; orphan nuclear receptor	(*59*)
ERCC1	L	Excision repair; endonuclease	(*72*)
FANCA	K	DSBR	(*57*)
FANCD2	LK	DSBR	(*57*)
FANCJ	L	DSBR; structure-specific DNA helicase	(*59*)
FANCL	K	DSBR; E3 ubiquitin ligase	(*57*)
FEN1	K	Replication; flap endonuclease	(*55*)
γ-H2AX	L	DSB marker; phosphorylated H2AX	(*36*, *37*)
hnRNP A2	L	Regulator of splicing; hnRNP	(*73*)
HUS1	L	DDR; 9-1-1 PCNA-like clamp	(*36*)
HP1α	LK	Heterochromatin	(*58*)
HP1β	LK	Heterochromatin	(*58*)
HP1γ	LK	Heterochromatin	(*58*)
HSP90	L	Heat shock protein	(*47*)
MDC1	L	DDR	(*37*)
MMS21	LK	DDR; E3 SUMO ligase in SMC5/6	(*41*)
MRE11	LK	HR; MRN complex subunit	(*42*, *43*, *74*)
MUS81	LK	HR; structure-specific endonuclease	(*53*)
NBS1	LK	HR; MRN complex subunit	(*42*, *43*, *74*)
NXP2	L	PML body	(*59*)
p21	LK	Regulator of growth; CDK inhibitor	(*58*)
p53	LE[b]	Tumour suppression; transcription factor	(*46*, *58*)
PARP2	L	DSBR; poly (ADP-ribose) polymerase	(*75*)
PCNA	LK	Replication; clamp	(*58*)
PML	LK	PML body	(*34*, *43*)
POT1	L	Shelterin; ss telomere DNA binding	(*48*)
RAD1	L	DDR; 9-1-1 PCNA-like clamp	(*36*)
RAD9	L	DDR; 9-1-1 PCNA-like clamp	(*36*)
RAD17	L	DDR; chromatin loader for 9-1-1 clamp	(*36*)
RAD50	LK	HR; MRN complex subunit	(*41–43*, *74*)
RAD51	LK	HR; RecA-like, strand exchange protein	(*34*, *41*)
RAD51D	LK	HR; resolution of Holliday junction	(*54*)
RAD52	L	HR; DNA binding protein	(*34*)
RAP1	LK	Shelterin; TRF2-interacting	(*43*, *71*)
RIF1	L	DDR	(*76*)
RIP140	L	Transcription; co-regulator	(*59*)
RPA32	LK	Replication and HR; ss DNA binding	(*56*)
RPA70	LK	Replication and HR; ss DNA binding	(*56*)
SMC5	LK	DDR; structural maintenance complex	(*41*)
SMC6	L	DDR; structural maintenance complex	(*41*)
Sp-100	LKE	PML body	(*40*, *43*, *71*)
STN1	L	Telomere; ss DNA binding CST complex	(*77*)
TEP1	L	Telomere	(*47*)
TF4	L	Unknown; orphan nuclear receptor	(*59*)
TIN2	LK	Shelterin	(*43*, *48*)
TopoIIα	L	DNA structure; topoisomerase, type II	(*47*)
TopoIIIα	LK	DNA structure; topoisomerase, type IA	(*48*)
TRF1	LK	Shelterin; ds telomere DNA binding	(*34*, *43*)
TRF2	LK	Shelterin; ds telomere DNA binding	(*34*, *43*, *46*)
WRN	LK	HR; RecQ-like helicase and nuclease	(*78*, *79*)
XPF	L	Excision repair; endonuclease	(*72*)
XRCC3	K	HR, resolution of Holliday junction	(*44*)

[a]L, localization at telomeres in ALT cells; K, knockdown of gene; E, ectopic expression of protein.
[b]Activation of p53 by RNAi of SV40 large T-antigen.

MRN complex

The MRN complex, which includes MRE11, RAD50 and NBS1, functions in the early steps of HR and ds DNA break (DSB) repair (DSBR). Interestingly, this complex constitutes APB and the homologous complex in budding yeast plays essential roles in the telomerase-independent telomere lengthening mechanism (see below). Overexpression of Sp-100, a component of PML body that can interact with NBS1, inhibited the proper localization of NBS1 to APBs. This sequestration of NBS1 from APBs or the knockdown of each of MRN gene induced the shortening of telomere DNA in ALT cells (*40–42*). MRN was also required for APB formation (*43*). The knockdown of the gene encoding RAD50 or the overexpression of Sp-100 caused a significant reduction of t-circles (*32*, *44*), suggesting the function of MRN in the maintenance of ALT-specific telomere DNA.

TRF2 and shelterin

TRF2 is a ds telomere DNA binding protein in the shelterin complex, which is essential for end protection. A defect in TRF2 stimulates chromosome fusion between telomeres in telomerase-positive cells (*45*). U2-OS is an ALT cell line established from human osteosarcoma, which is expressing wild-type p53 and Rb, but lacking p16. The phenotypes of U2-OS were examined after inhibition of TRF2 by siRNA or the expression of the dominant-negative form of the protein (*46*). Telomere DNA of TRF2-defecient U2-OS cells was shortened and the cells expressed β-galactosidase activity similar to senescent cells. The significant activation of p53 and p21 observed in those cells was dependent on PML protein, a major component of APB. The formation of APB was not affected in TRF2-deficient cells, although another report showed that four of six shelterin components, TRF1, TRF2, TIN2 and RAP1, were required for APB formation (*43*).

RecQ-like DNA helicases

DNA helicases of the RecQ family act on atypical DNA structures, such as intermediates of HR, stalled

sites of replication forks and the G-quartet structure of telomere DNA. This function is important for genome integrity; mutations in either one of the three genes encoding RecQ-like DNA helicases in human, *BLM* (Bloom syndrome), *WRN* (Werner syndrome) or *RECQL4* (Rothmund–Thomson's syndrome, RTS), cause chromosome abnormality and premature aging. BLM is an APB protein and the knockdown of the gene induces rapid shortening of telomere DNA and inhibition of ALT cell growth (*47*, *48*). BLM forms a functional complex with topoisomerase IIIα (TopoIIIα) to suppress HR (*49*). Knockdown of the TopoIIIα gene in ALT cells also resulted in the loss of the G-tail, chromosome bridges or fusions of sister telomeres at anaphase, and cell growth retardation (*48*). The phenotypes of TopoIIIα-knockdown cells could be due to indirect effects as the amounts of BLM and TRF2 were concurrently decreased.

WRN is also localized at APB, although it seems to be dispensable for ALT cells. When fibroblasts mutated in *WRN* from a Werner syndrome patient were immortalized with SV40, a cell line that utilizes the ALT pathway to maintain the telomere DNA was established (*50*, *51*). In mouse, the survivors of *Wrn* (encodes WRN homologue) *Terc* (TR) double knockout were obtained and they showed elevated levels of T-SCE as in human ALT cells (*52*). These results suggest that ALT (human) and a telomerase-independent lengthening mechanism (mouse) can be activated without the function of WRN.

Resolution of Holliday junction

A striking intermediate of HR is the Holliday junction, a cruciform structure formed by two ds DNAs (Fig. 4). ALT cells show elevated T-SCE and contain branched structures in telomere DNA, so that they may require efficient resolution of the junction. MUS81 and its interacting protein MMS4 together constitute endonuclease specific to unpaired ds DNA, for example, the Holliday junction. The knockdown of *MUS81* in ALT cells caused growth arrest, loss of telomeres on metaphase chromosomes, and reduced frequency of T-SCE (*53*). The ectopic expression of TERT complemented the suppression of cell growth, but not the low T-SCE frequency. Interestingly, the length of telomere DNA and the amount of t-circles from Southern analyses were not altered in *MUS81* knockdown cells. It is unknown how telomere loss from chromosome ends is induced without a significant reduction in telomere length.

XRCC3, a RAD51-paralog that is presumably involved in the resolution of the Holliday junction, is required for the maintenance of t-circles (*44*). RAD51D, another RAD51-paralogue for the resolution, is an APB protein and its inhibition led to shortened telomere DNA and chromosome bridges (*54*). These RAD51-paralogues must be required for HR of telomere DNA in ALT cells. RAD51, which plays a central role in strand transfer in HR rather than the resolution of the Holliday junction, was not crucial for APB formation (*41*). It remains unknown whether RAD51 functions in the HR of telomere DNA and the ALT pathway.

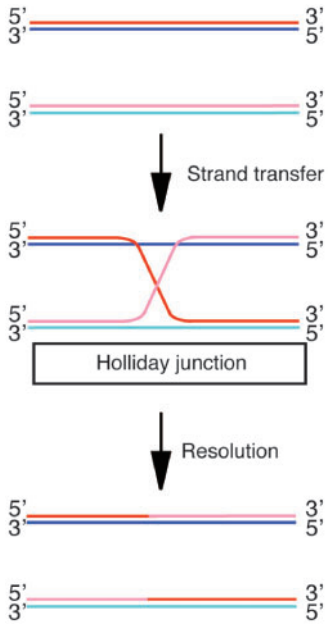

Fig. 4 Formation and resolution of the Holliday junction. The reciprocal exchange between two ds DNA (top, red/blue and pink/pale blue) is shown. The Holliday junction (middle) is a cruciform DNA formed in HR, and the resolution (bottom) requires cut and ligation at the junction.

Other proteins in DNA metabolism

FEN1 is a flap endonuclease that processes the 5′-end of Okazaki fragment to assist the completion of the lagging-strand synthesis. The homologue of FEN1 in budding yeast plays a role in the processing of the C-strand of telomere DNA. FEN1-deficient ALT cells showed elevated levels of DDR at telomeres (*55*). This response was suppressed by the ectopic expression of TERT. RPA is a conserved trimeric protein complex essential for replication and recombination. Two subunits of RPA, RPA32 and RPA70, are localized at APB, and the knockdown of either one of them in ALT cells resulted in ss G-strand accumulation and cell growth arrest (*56*). It needs to be clarified how crucial these replication proteins are for the telomere maintenance in ALT cells.

Sumoylation and ubiqutination

The PML body generally concentrates small ubiquitin-like modifier (SUMO) and proteins required for sumoylation. The first evidence of the function of sumoylation in ALT and APB was provided by a work on the SMC5/6 complex (*41*). SMC5/6, which is one of the structural maintenance of chromosomes (SMC) complexes in eukaryotes, plays important roles in DNA repair and DDR. The complex includes MMS21, a specific E3 SUMO ligase, as a non-SMC subunit. SMC5/6 and MMS21 are APB components and the knockdown of each of the genes caused reduced numbers of APBs in a cell. The knockdown of *SMC5* or *MMS21* in ALT cells induced telomere DNA shortening, telomere DNA loss at chromosome termini, cell growth suppression and β-galactosidase activity expression as in senescent cells. Meanwhile,

telomere DNA shortening and cell growth inhibition were not observed in SMC5- or MMS21-deficient telomerase-positive cells, suggesting their specific function in telomere maintenance and cell growth in ALT cells. MMS21 was able to sumoylate at least four shelterin proteins, TRF1, TRF2, TIN2 and RAP1. Mutated TRF1 at its sumoylated site abolished the localization at APB, suggesting that the sumoylation of the telomere binding proteins regulates APB formation.

FANC proteins were originally identified from mutated genes in patients with the genetic disease Fanconi anemia, whose cells commonly show chromosomal abnormality. The localization of FANCD2 at APBs required the function of FANCA and FANCL, both of which are essential for the monoubiquitination of FANCD2 (*57*). The knockdown of the gene encoding FANCD2 or FANCA induced telomere loss and T-SCE reduction in ALT cells. In DDR, the monoubiquitination of FANCD2 and the formation of FANCD2 nuclear foci required ATR kinase. Interestingly, the localization of FANCD2 at APB was also dependent on ATR. These suggest that FANCD2 may be regulated by ATR-dependent monoubiquitination for its proper localization and function on telomere in ALT cells.

Other factors related to ALT pathway

Tumour suppressor p53 is a crucial regulator of genome stability. Human p53 is localized at APB in ALT cells; however, it is not necessary for APB formation (*46*). In an ALT cell line established by the transformation with the T antigen of SV40, p53 was activated by the knockdown of the T antigen gene. APBs of those cells were enlarged (*58*), suggesting that p53 is a positive regulator of APB formation. This effect is dependent on the function of p21 and heterochromatin protein HP1, both of which are also APB components.

Several novel APB proteins were identified using a unique purification method to concentrate chromatin proteins associated with telomere DNA (*59*). The inhibition of COUP-TF2, a novel APB protein and an orphan nuclear receptor, caused a defect in APB formation and the shortening of telomere DNA. The molecular function of those novel APB proteins in the ALT pathway remains to be elucidated.

The phenotypes of ALT cells were suppressed in hybrid cell lines of ALT and telomerase-positive cells (*60*). This suggests that ALT cells may have some recessive mutations in gene(s) required for the inhibition of the ALT pathway. The genes proposed in that study have not been identified; nevertheless, they are expected to provide some hints for the ALT regulation mechanism. The coexistence of ALT and the lengthening by telomerase was apparently observed (*17*), indicating that telomerase activity alone is not sufficient to suppress the ALT phenotypes. This also suggests that some telomerase-positive cancer cells may activate the ALT pathway at the same time.

Similarities in telomerase-independent lengthening mechanisms

Cells that develop telomerase-independent lengthening mechanisms have been providing valuable hints for the ALT pathway. In the case of budding yeast *Saccharomyces cerevisiae* and its related species, survivors were recovered from cells with a disrupted TR gene and classified into two groups: type I cells have amplified subtelomeric DNA and depend on Rad51, and type II cells have elongated telomere repeats and require the function of MRX (MRN homologue) (*61*, *62*). As type II yeast cells and ALT cells have elongated telomere repeats, it is thought that these pathways depend on a conserved mechanism. It is significant that both human RAD51 and MRN are localized at APBs, suggesting that both of them are related to the ALT pathway. It is unknown whether the ALT pathway is made up of subpathways, as in types I and II cells of budding yeast.

As in human ALT cells, budding yeast type II cells harbour t-circles. When a model circular telomere DNA was introduced into type II cells, this DNA was copied to chromosome ends to form a tandem array (*63–65*). These results can be explained by RCR, in which the model circular DNA is utilized as a template for amplification. RCR may also play a crucial role in the replication of the linear mitochondrial DNA of the yeast *Candida parapsilosis*. Replication intermediates of the ends of mitochondrial DNA include a strand-specific ss DNA structure (*33*) that is commonly observed in RCR. These findings imply that RCR may be a conserved and general mechanism for the telomerase-independent pathway in eukaryotes.

The mouse knockout mutant of any genes of interest is one of the best model systems for human biology. Survivor cells from *Terc* $(TR)^{-/-}$ apparently maintained their telomere DNA in a telomerase-independent manner. In a cell line established from $Terc^{-/-}$ embryonic stem (ES) cells, the tandem arrays of telomeric and non-telomeric sequences were observed at the chromosome ends (*66*). Interestingly, most of the chromosome ends contained the same arrays. This suggests that these cells can amplify the arrays to maintain the chromosome ends. An array of this unique sequence was possibly built at one chromosome end at first, and then it expanded to the other chromosome ends. In an example of a human telomerase-negative cell line, the array of telomeric and ectopic DNA sequences (derived from SV40 DNA) was attached to most of the chromosome ends (*50*, *51*). These cells harboured circular extrachromosomal DNA that includes the similar sequence to the array at the chromosome ends, suggesting that the circular DNAs may be analogous to t-circles in ALT cells. It is interesting to examine whether the circular episome is required for the lengthening of the chromosome ends in these cells. Another survivor cell line from $Terc^{-/-}$ mouse embryonic fibroblasts showed heterogeneous telomere DNA lengths and the formation of APBs (*67*). This would serve as an invaluable

model system for the human ALT pathway and cancer development.

The ES cells from knockout mice mutated in DNA methyltransferase genes showed frequent T-SCE and APB formation, even though the cell lines were positive for telomerase activity (*68*). DNA hypomethylation at the subtelomere region was observed in those cells. This suggests that the epigenetic state of the cells may affect the frequency of T-SCE and APB formation. In human ALT cells, on the other hand, the relationship between the methylation status at the subtelomere region and T-SCE frequency was less clear (*69*).

Conclusions

Several lines of evidence strongly suggest that the ALT pathway is dependent on HR. First, a DNA fragment in the telomere repeats of chromosome ends can be copied into the ends of different chromosomes in ALT cells. Second, several kinds of proteins essential for HR are localized at APB. Third, a defect in some HR proteins induces erosion of telomere DNA in ALT cells. Lastly, the unique structures of telomere DNA found in ALT cells, such as ss telomere DNA and circular ECTR, may represent the intermediates and/or substrates of the elongation mechanisms based on HR. Importantly, the similar telomere DNA structures are also observed in cells that utilize HR-mediated telomere maintenance in other organisms. For the further elucidation of the ALT pathway, it is essential to establish a model experiment system in order to acquire direct evidence of the mechanism of HR-dependent telomere elongation.

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Conflict of interest

None declared.

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