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Life and cancer without telomerase: ALT and other strategies for making sure ends (don't) meet

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Abstract

While most cancer cells rely on telomerase expression/re-activation for linear chromosome maintenance and sustained proliferation, a significant population of cancers (10–15%) employs telomerase independent strategies, collectively dubbed Alternative Lengthening of Telomeres (ALT). Most ALT cells relax the usual role of telomeres as inhibitors of local homologous recombination while maintaining the ability of telomeres to prohibit local non-homologous end joining reactions. Here we review current concepts surrounding how ALT telomeres achieve this new balance via alterations in chromatin landscape, DNA damage repair processes and handling of telomeric transcription. We also discuss telomerase independent end maintenance strategies utilized by other organisms, including fruitflies and yeasts, to draw parallels and contrasts and highlight additional modes, beyond ALT, that may be available to telomerase-minus cancers. We conclude by commenting on promises and challenges in development of effective anti-ALT cancer therapies.

Keywords

Telomere; telomerase; genome stability; chromatin; heterochromatin; ALT; HAATI; recombination; DNA damage response

Introduction: Telomeres, telomerase and cancer

Eukaryotic chromosome linearity poses two fundamental problems that impact genome integrity. First, linearity forces cells to grapple with the 'end-replication problem' that arises due to the biochemistry of the semi-conservative DNA replication machinery; this machinery requires a template to copy and a 3' hydroxyl group to which to add nucleotides in a 5' to 3' direction, making it unable to fully duplicate linear chromosome ends. These requirements lead to terminal sequence loss in every cell cycle (Watson, 1972, Olovnikov, 1973, Lingner *et al.*, 1995). Second, cells must differentiate chromosome ends from DNA double strand breaks (DSBs) that occur elsewhere in the genome, as such damage induced DSBs are subject to DNA damage response (DDR) pathways that can include disastrous

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fusion or degradation of chromosome ends. These disasters are averted by the specialized protective nucleoprotein structure termed the telomere (Jain and Cooper, 2010). Telomeric DNA generally comprises simple G-rich repeat arrays culminating in a 3' single-stranded (ss) overhang. This DNA associates with a group of proteins termed 'shelterin', which includes double strand (ds) telomere binding proteins, ss overhang binding proteins, and proteins that bridge these two categories (de Lange, 2005). Shelterin or shelterin-like complexes have been described for diverse eukaryotes including mammals, yeasts and fruitflies (Figure 1). Shelterin integrates myriad activities that collectively protect chromosome ends from non-homologous end joining (NHEJ), extensive nucleolytic attack and homologous recombination (HR). Furthermore, shelterin engages and regulates the reverse transcriptase telomerase, whose associated RNA template is copied to replenish the loss of telomeric repeats at each replication cycle. Electron microscopy and stochastic optical reconstruction microscopy revealed that mammalian telomeres can adopt a protective configuration called the 't-loop' (Griffith *et al.*, 1999, Doksan *et al.*, 2013), in which the 3' ss overhang invades upstream TTAGGG repeats (Griffith *et al.*, 1999), displacing a D-loop, camouflaging the 3' terminus and perhaps sequestering it from the DDR (Figure 2a).

Somatic cells down-regulate the expression of telomerase, resulting in progressive attrition of telomeric sequences with each round of DNA replication. Eventually, this attrition culminates in the inability of chromosome ends to identify themselves as distinct from DSBs; at this crisis point, the resulting DDR halts further cell proliferation. Hence, telomere attrition limits the number of replication cycles that can be undertaken by a cell and comprises an intrinsic source of cellular mortality. However, while telomere loss or dysfunction in a checkpoint-competent cell will elicit mortality, telomere dysfunction in checkpoint-deficient settings can trigger chromosome end fusions and in turn, the genomic instability that promotes tumor development (Sharpless and DePinho, 2004). Hence, proper telomere function can prevent tumorigenesis. Conversely, cancer cells rely on extensive proliferation and must overcome the limitations faced by somatic cells to gain cellular immortality, making telomere function (protection from end-fusion and spiraling genomic instability) a requirement for later stages of tumorigenesis and unrestrained proliferation.

As part of their trajectory to unlimited proliferation, most (85–90%) cancer cells reactivate telomerase. The remaining 10–15% of tumor cells must stabilize their chromosome ends by alternative mechanisms to avert cessation of growth. These telomerase independent strategies are collectively known as Alternative Lengthening of Telomeres (ALT). Hence, while telomerase might appear as an attractive anticancer target for most types of cancers, the existence of ALT means that to be effective in the face of large mutational load and selection, an anti-cancer therapy would ideally be multi-pronged, addressing potential ALT pathways as well. A widely utilized ALT strategy is to maintain telomeric length via HR (Lundblad and Blackburn, 1993, Cesare and Reddel, 2010). This is an intriguing biological phenomenon, as fully functional 'normal' telomeres prohibit local activation of DDRs including HR; therefore, ALT telomeres open themselves to specific DDR pathways while maintaining prohibition on other DDR pathways. The requirements for this strategy have been at least partially defined in a number of ALT cell lines as well as tumors over the past two decades (Bryan *et al.*, 1997, Henson and Reddel, 2010). However, the ALT lines characterized thus far are not entirely uniform, and further ALT strategies are likely to exist.

For instance, studies in low grade pediatric gliomas and medullary thyroid sarcomas have uncovered a subset of tumor types that lack both telomerase and the hallmarks of ALT, suggesting yet additional mechanisms at play (Tabori *et al.*, 2006, Slatter *et al.*, 2010, Wang *et al.*, 2014). Here, we review both the increasingly well-understood canonical ALT pathway, which we refer to for brevity as ‘ALT’, and suggest alternative ALT pathways, which we refer to as ‘non-canonical ALT’. We discuss emerging ideas on the mechanisms by which recombination is achieved in ALT. One facet of the chromosome end that appears to play a prominent role in dictating the ability and type of telomerase-negative survival pathway adopted is the histone modification landscape and resulting local nucleosome dynamics; indeed, while of dubious importance for telomere protection in a telomerase-positive setting, chromatin landscape plays a decisive role in dictating the presence and mode of end-protection in telomerase-negative settings. We also discuss additional and potentially illuminating modes of chromosome end maintenance observed in yeast and fruit flies. These concepts in ALT biology and interspecies comparisons may hint at fruitful avenues for anticancer strategies targeting chromosome end-maintenance.

Features of Canonical ALT

While the sporadic occurrence of ALT has been documented across numerous cancer types including common breast carcinomas (Subhawong *et al.*, 2009), it is particularly prevalent in tumors of mesenchymal or neural epithelial origin, like osteosarcomas, liposarcomas, glioblastoma multiforme (primary malignant brain tumor), and astrocytomas (Shay and Bacchetti, 1997, Hakin-Smith *et al.*, 2003, Costa *et al.*, 2006, Jeyapalan *et al.*, 2008, Villa *et al.*, 2008, Subhawong *et al.*, 2009, Henson and Reddel, 2010). The basis for this preference is not yet known although certain features of mesenchymal stem cells have been suggested as ALT activators. For example, these cell populations show active repression of telomerase RNA and hTERT expression by chromatin remodeling of associated gene promoters. This repression may favor the use of ALT for immortalization (Atkinson *et al.*, 2005). In addition, a genome-wide expression signature in mesenchymal stem cells that might favor repressed TERT expression has been identified (Lafferty-Whyte *et al.*, 2009). However, a recent comprehensive survey of ~6100 primary tumor samples across >90 cancer types has shown a much more widespread occurrence of ALT phenotypes in many carcinomas arising in liver, kidney, lung, bladder and endometrium (Heaphy *et al.*, 2011b).

Despite the absence of active telomerase in ALT cells, their chromosome ends display canonical features like ds TTAGGG repeats with terminal 3'-overhangs, shelterin components (though perhaps with stoichiometries different from those found in non-ALT cells; see below) and other telomere-associated proteins, and the ability to form t-loops (Cesare and Griffith, 2004). In addition, they display characteristics exclusive to ALT. ALT involves extensive copying of telomeric DNA from pre-existing telomeric DNA templates on another chromosome or a sister chromatid, giving rise to telomeric sister chromatid exchanges (T-SCE). T-SCE is evinced by chromosome-orientation fluorescent in situ hybridization (CO-FISH) experiments (Bailey *et al.*, 2004, Williams *et al.*, 2011) and by the observation that an ectopic DNA sequence inserted into one telomere appears at other non-sister telomeres in yeast and human ALT cells (Natarajan and McEachern, 2002); such ectopic sequences can also be duplicated at their original locations (Muntoni *et al.*, 2009,

Neumann *et al.*, 2013). The elevated levels of HR used to maintain telomere repeat tracts result in great telomere length heterogeneity in ALT populations (Murnane *et al.*, 1994, Londono-Vallejo *et al.*, 2004).

ALT cells show an abundance of extra-chromosomal telomere repeat-containing DNA that can take several forms including ds telomeric circles (t-circles), partially ss circles (C-circles or G-circles, depending on the constituent strand), linear ds DNA and very high molecular weight 't-complex' DNA containing branched structures that are likely recombination intermediates (Cesare and Reddel, 2010). These extra-chromosomal recombinogenic telomeric repeat DNAs (ECTR), as well as many protein regulators of ALT, associate with a hallmark nuclear structure termed the ALT-associated promyelocytic leukemia body (APB). APBs are subtypes of canonical PML bodies, which are nuclear structures important for DNA repair, senescence and apoptosis in normal cells. PML bodies rarely associate with telomeres in a telomerase-positive setting. In addition to telomeric DNA and PML, APBs contain the DDR associated helicases BLM and WRN, the ssDNA binding protein RPA required for all transactions that involve DNA unwinding, the DSB sensing complex MRE11/RAD50/ NBS1 (MRN), the DSB repair protein RAD51D, the checkpoint/repair Rad9-Rad1-Hus1 complex, the nuclear receptors COUP-TF2 and TR4, and shelterin components (Nittis *et al.*, 2008). APBs also contain the SMC5-SMC6-MMS21 complex, which drives sumoylation of shelterin components TRF1, TRF2, Rap1 and TIN2; this sumoylation appears to be essential for APB formation as well as ALT telomere maintenance (Potts and Yu, 2007). The concentration of this range of factors within APBs likely increases the occurrence and efficiency of ALT (Yeager *et al.*, 1999). The strong correlation between the presence of APBs, C-, G- and t-circles, long heterogeneous telomeres and ALT has led to the routine use of such features as ALT identifiers.

ALT cells display an increased frequency of telomeric variant repeats like TCAGGG. These variant repeats are presumed to originate from sporadic ongoing mutations in ALT cells that become fixed and spread by hyper-recombination among ALT chromosomal ends (Varley *et al.*, 2002). This increased variant content and its heterogeneous distribution within ALT telomeres has been proposed to alter the protein stoichiometry at the telomere by affecting the balance of shelterin components (Lee *et al.*, 2014). Robust shelterin binding in telomerase-positive cells hinders recruitment of nuclear receptors like COUP-TF2 and TR4 to canonical telomeric repeats. In contrast, the variant repeats found at ALT telomeres represent lower affinity binding sites for the shelterin subunits that bind dsDNA; hence, nuclear receptors are thought to outcompete binding by shelterin components. This altered binding can lead to shelterin under-saturation and phenotypes normally associated with shelterin deficits (Conomos *et al.*, 2012). Such deficits may contribute to replication fork stalling, DDRs and enhanced chromatin accessibility at ALT telomeres, considered below.

Global players act locally to regulate chromatin landscape and replication stress at ALT ends

Changes in telomeric nucleosome dynamics have been identified as prerequisites for the accessibility of telomeres to ALT associated HR. Telomeric replication defects also aid in

the recruitment of DNA recombination and repair factors at ALT telomeres. Additionally, a role for transcription-based regulation of ALT telomeric chromatin has been in the spotlight recently. We consider these interdependent chromatin alterations in detail below to illustrate multiple mechanisms at play in ALT cells (summarized in Figure 2b).

Chromatin landscape of ALT telomeres

Although telomeric repeats are amongst the lowest affinity nucleosome binding sequences mammalian telomeres are packaged into nucleosomes (Makarov *et al.*, 1993, Tommerup *et al.*, 1994, Nikitina and Woodcock, 2004). The said nucleosomes are more closely spaced than bulk nucleosomes. Silencing of nearby transcription (Gottschling *et al.*, 1990, Palladino *et al.*, 1993, Nimmo *et al.*, 1994, Cooper *et al.*, 1997, Bourc'his *et al.*, 2001) and enrichment of repressive histone modifications (Kano *et al.*, 2005, Blasco, 2007, Grewal and Jia, 2007) are conserved features of telomeric chromatin. These modifications include heterochromatic histone H3-K9 trimethylation, histone H4-K20 trimethylation, heterochromatin protein 1 (HP1) binding and histone hypoacetylation (Dejardin and Kingston, 2009). The diminution of this repressive domain appears critical to the ability of cells to engage in ALT. Studies in mouse cells show that loss of telomeric heterochromatin results in phenotypes reminiscent of ALT including the appearance of T-SCEs and APBs (Gonzalo *et al.*, 2006, Benetti *et al.*, 2007, Benetti *et al.*, 2008). Indeed, a genome-wide principle of the DDR is that loss of heterochromatic marks that result in 'open' chromatin facilitate HR-based DDR while 'closed' chromatin facilitates NHEJ (Miller and Jackson, 2012). Micrococcal nuclease digestion patterns indicate lower telomeric nucleosome density in ALT cells compared to isogenic telomerase-positive counterparts (Episkopou *et al.*, 2014). Notably, the inhibition of histone deacetylases (HDACs) with trichostatin A (TSA) abolishes heterochromatin and in doing so, enhances T-SCE levels (Jung *et al.*, 2013), reinforcing the notion that a tight nucleosome landscape prevents ALT.

Amongst the most consistent genetic alterations characterizing ALT cells are those that inactivate one or both of the ATP-dependent chromatin remodelers, ATRX (α -thalassemia/mental retardation X-linked) and DAXX (death associated protein-6). Mutation or deletion of ATRX (and to lesser extent DAXX) is found both in ALT cell lines and tumors, including pancreatic neuroendocrine tumors, pediatric glioblastomas, neuroblastomas and medulloblastomas (Heaphy *et al.*, 2011a, Lovejoy *et al.*, 2012, Schwartzenuber *et al.*, 2012). Definitive evidence for a role of ATRX in repressing ALT was provided by the observation that ATRX knockdown in SV40-transformed fibroblasts increases the frequency of ALT while transient expression of ATRX in ALT cells reduces APB formation and c-circle counts (Napier *et al.*, 2015). ATRX not only associates with HP1 proteins and promotes their association with telomeres, but also binds to G-rich sequences (including telomeres) with the propensity to form G-quadruplexes, highly stable non-B-form DNA structures involving runs of G-residues. This G-quadruplex binding ability, along with the known translocase activity of ATRX, led to the hypothesis that ATRX converts G-quadruplex structures to ds DNA (Law *et al.*, 2010, Whitehouse and Owen-Hughes, 2010). The association of ATRX with DAXX also promotes assembly of telomeric regions into H3.3-containing nucleosomes (see below), which may further promote the transition from G-quadruplex to ds DNA. Hence, loss of ATRX function in ALT cells may allow not only

the destabilization of repressive heterochromatin, but also accumulation of G-quadruplex structures, presenting a barrier to replication and perhaps facilitating nucleosome eviction.

ATRX also acts along with its histone chaperone partner DAXX to assemble chromatin harboring the replication-independent histone variant H3.3 (Tagami *et al.*, 2004). Indeed, while H3.3 is deposited at transcriptionally active chromatin by the HIRA complex, it is also recruited to transcriptionally silenced telomeric and pericentromeric heterochromatin by ATRX/DAXX (Loyola *et al.*, 2006, Goldberg *et al.*, 2010, Lewis *et al.*, 2010). Hence, H3.3 can be associated with either silenced or active chromatin. Recent studies suggest that the modification status of H3.3 dictates its role in these two settings. In particular, H3.3-K9 trimethylation (H3.3-K9-Me3) appears to mark telomeric heterochromatin (Udugama *et al.*, 2015). ChIP studies show that increased replication stress and chromatin disruption lead to enhanced recruitment of H3.3 and increased H3.3-K9-Me3 at mouse telomeres. Moreover, overexpression-induced increases in telomeric H3.3-K9-Me3 reduce the levels of t-SCE induced by a G-quadruplex stabilizing ligand (Udugama *et al.*, 2015). As H3.3-K9 trimethylation is proposed to be important for re-heterochromatinization of telomeric DNA (Clynes *et al.*, 2013, Watson *et al.*, 2013), the absence of ATRX in many ALT cells may confer failure to deposit H3.3 and hence a failure to re-assemble telomeric heterochromatin upon replication stress, in turn furthering local genome instability. Consistently, deletion of the mouse Suvar39h histone H3-K9-methyltransferases yields telomere elongation and increased telomeric recombination (Garcia-Cao *et al.*, 2004).

An additional critical role for ATRX in controlling cohesion between sister telomeres has been highlighted as a mediator of ALT (Ramamoorthy and Smith, 2015). Telomeric cohesion is regulated in a manner distinct from cohesion elsewhere in the genome, as its dissolution requires poly-ADP-ribosylation of TRF1 by Tankyrase I (Dynek and Smith, 2004, Bisht *et al.*, 2013). While this process occurs efficiently in wild type and telomerase-positive cancer cells, sister telomeres in ALT cells remain cohered into mitosis. ATRX binds to the histone variant macroH2A and inhibits its incorporation into chromatin; however, Tankyrase 1 competes with ATRX for macroH2A binding (Ratnakumar *et al.*, 2012). In ALT cells, the ATRX deficit favors Tankyrase 1 binding to macroH2A, reducing the association of Tankyrase 1 with telomeres and prolonging sister telomere cohesion. This persistent telomere cohesion is suspected to promote HR between sister telomeres while preventing HR between non-sisters. Hence, ATRX deficiency influences ALT physiology by abrogating multiple cellular pathways that safeguard genomic stability.

Nucleosome assembly in the wake of DNA replication or repair is regulated by the anti-silencing factor 1 (ASF1) paralogs ASF1a and ASF1b, which transfer H3-H4 dimers to the histone chaperones CAF-1 (chromatin assembly factor 1) and HIRA (histone regulator A) to promote nucleosome reassembly. Depletion of ASF1 leads to failed replication restart at repetitive regions, activating ATR signaling, recombination and repair. Interestingly, co-depletion of ASF1a and ASF1b induces features of ALT including ECTRs, APB formation and telomere length heterogeneity (O'Sullivan *et al.*, 2014). These phenotypes are accompanied by reduced H3 density and enhanced chromatin accessibility over the telomeric region, reinforcing the notion that open telomeric chromatin is an ALT stimulator. Perhaps crucially, ASF1a/b co-depletion also leads to reduced expression of *TERT*, the gene

encoding telomerase; hence, it remains to be determined whether ASF1 inactivation would trigger ALT processes in a telomerase-positive setting. ALT induction upon ASF1 depletion occurs in immortalized cell lines with inherently long telomeres, but not in cell lines with very short or normal telomere length. This observation is consistent with a scenario in which over-elongated telomeres swamp limiting concentrations of TRF1, resulting in severe replication stress and fragile telomeres (Sfeir *et al.*, 2009); stalled replication would in turn stimulate HR and ALT (Hensen *et al.*, 2009). In addition, the observation that ASF1 competes with DAXX for binding to H3-H4 dimers (Elsasser *et al.*, 2012) suggests a histone management function that may become prominent in DAXX-deficient ALT cells. In conclusion, regulation of telomere length, chromatin compaction and heterochromatinization is altered at ALT telomeres.

Replication stress control at ALT telomeres

Along with changes in telomeric chromatin accessibility, a dramatic increase in replication stress at eroded telomeres appears instrumental in ALT etiology, as does DSB induction (Figure 2b). The repetitiveness and G-rich character of telomeres makes them inherently difficult to replicate and indeed, replication fork progression through telomeres requires the presence of specific telomere binding proteins (Miller *et al.*, 2006, Sfeir *et al.*, 2009). Stalled telomeric replication forks can be refractory to replication restart, making them highly recombinogenic both in the presence and absence of telomerase (Rog *et al.*, 2009).

The ds telomere binding proteins TRF1 and TRF2 modulate nucleosome dynamics and telomere function in distinct ways. TRF1 promotes telomeric replication fork passage and negatively regulates telomerase-mediated telomere elongation (van Steensel and de Lange, 1997, Sfeir *et al.*, 2009). In contrast, TRF2 promotes t-loop formation and protects telomeres from local ATM activation and end-fusion (van Steensel *et al.*, 1998, Karlseder *et al.*, 1999). TRF1 and TRF2 bind nucleosomal DNA to different extents *in vitro* and induce different effects on nucleosome mobility. TRF1 binds both nucleosomal and linker DNA and induces nucleosome mobility *in vitro* (Pisano *et al.*, 2010, Galati *et al.*, 2015), while TRF2 binds nucleosomal DNA poorly and increases the spacing between nucleosomes (Galati *et al.*, 2012). TRF2 overexpression in mice leads to reduced histone density at telomeres (Benetti *et al.*, 2008). Moreover, TRF1 and TRF2 binding are modulated by the presence and modification state of histone tails on nucleosomes (Galati *et al.*, 2015). Conversely, TRF2 depletion reduces histone H3K9 methylation (Porro *et al.*, 2014b). Therefore, alterations in the balance of TRF1, TRF2 and proteins that bind variant telomere repeats (like the nuclear receptors TRF2 and TRF4 mentioned above) (Conomos *et al.*, 2012, Lee *et al.*, 2014) will likely affect telomere nucleosome structure and dynamics as well as replication.

Increased local DNA mobility is a crucial regulated feature of damaged DNA ends, allowing them to find templates for homologous repair or even other non-homologous DNA ends ripe for fusion (Dion *et al.*, 2012, Roukos *et al.*, 2013). Telomeres damaged by TRF2 removal in telomerase-positive settings show enhanced mobility, allowing such telomeres to sample volumes as large as 8-fold greater than those sampled by undamaged telomeres (Dimitrova *et al.*, 2008, Chen *et al.*, 2013); this movement depends on ATM and 53BP1, and is required for efficient NHEJ of dysfunctional telomeres. In contrast, the search for homologous

sequences with which ALT telomeres can recombine requires long-range telomere movement. Indeed, direct visualization of damaged ALT telomeres shows rapid, directional movements that lead to pairing with a repair template; such movement is also necessary to achieve telomere clustering within APBs (Cho *et al.*, 2014). This process is Rad51-dependent, 53BP1-independent, and dependent on extensive 5' end resection (which creates binding sites for Rad51 on the resulting 3' overhang), underscoring the different mechanisms underlying telomeric mobility at different cell cycle stages and in telomerase positive *versus* negative settings. Intriguingly, meiosis-specific factors important for meiotic homology search and synapsis are expressed in ALT cells and vital for this movement (Cho *et al.*, 2014).

Factors that promote balanced instability

In addition to shelterin components themselves, several proteins facilitate replication fork progression in difficult-to-replicate telomeric locations. These include helicases like RTEL, SMARCAL, BLM, RecQ, WRN, FANC-J and the SLX4 structure-specific endonuclease complex. Collectively these proteins may resolve or dissolve excess recombination intermediates and in doing so, maintain overall genomic stability in ALT settings.

RecQ helicases, including budding yeast Sgs1 and human WRN and BLM, can disassemble branched DNA structures and unwind G-quadruplexes, and play central roles in general genome stability in the face of replication stress. Consistently, these helicases function in ALT. The break-induced replication pathway used by ALT cells likely requires RecQ-mediated channeling of strand exchange intermediates towards dissolution and away from Holliday junction cleavage reactions (Pickett and Reddel, 2015). The BLM helicase associates with TRF1/TRF2 and APBs and may help unwind complex secondary structures that are byproducts of replication defects. BLM depletion leads to rapid attrition and telomere loss in ALT cells, (Bhattacharyya *et al.*, 2009) and reduces the co-localization of BLM-associated proteins with TRF2 and APBs, suggesting that BLM plays not only an enzymatic role in safeguarding ALT telomeres, but also a recruitment role.

SMARCAL1 (SWI/ SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A-like 1) is an ATP-dependent DNA annealing helicase implicated in chromatin remodeling at stalled forks to promote replication restart (Bansbach *et al.*, 2009, Ciccio *et al.*, 2009, Poole *et al.*, 2015). SMARCAL1 accumulates at ALT telomeres as they experience chronic replication stress, and loss of SMARCAL1 leads to even higher levels of APB formation as well as irreversibly collapsed replication forks (Cox *et al.*, 2016). Hence, SMARCAL1 appears to keep ALT in check, allowing the elevated DDR in ALT cells not to overwhelm the system.

RTEL1 (Regulator of Telomere length 1) an essential helicase that removes t-loops in S-phase, preventing SLX1–4 endonuclease-mediated t-loop excision; RTEL also counteracts telomere fragility as well as G-quadruplex formation genome-wide (Barber *et al.*, 2008, Sfeir *et al.*, 2009, Vannier *et al.*, 2012). TRF2 recruits RTEL1 to telomeres (Sarek *et al.*, 2015). Depletion of RTEL1 in mouse cells results in telomere fragility and phenotypes similar to human ALT. Intriguingly, RTEL1 appears to be dispensable for pluripotent, telomerase-positive mouse embryonic stem cell proliferation but upon differentiation, it

becomes a critical regulator of telomere maintenance (Ding *et al.*, 2004); indeed, loss of RTEL1 in differentiated cells results in telomere dysfunction phenotypes resembling ALT, including elevated c-circle production and telomere length heterogeneity. As proliferating and differentiated cell states differ in replication status, telomerase content (Armstrong *et al.*, 2000) and the repertoire of associated telomeric regulatory factors, these observations likely reflect modulation of RTEL1 function by telomeric chromatin state.

Hence, a picture is building in which ALT cells experience deregulated replication, elevated DSB responses and telomeric chromatin remodeling pathways that enhance accessibility to HR. At the same time, imbalances in shelterin and DDR components at ALT telomeres may exacerbate replication defects and DDR alterations, creating the ‘perfect storm’ in which telomere sister chromatid exchanges generate ALT (Figure 2b).

TERRA – A lncRNA link between chromatin state and telomere maintenance

Despite their heterochromatic state, chromosome ends are transcribed by RNA polymerase II (Azzalin and Lingner, 2015). Mammalian telomeres are generally transcribed from the sub-telomeric region towards the chromosome ends, giving rise to long non-coding transcripts (lncRNA) known as telomeric repeat containing RNA (TERRA) (Azzalin *et al.*, 2007, Schoeftner and Blasco, 2008). The fission yeast telomeric transcriptome comprises TERRA and additional species including C-rich telomeric lncRNAs transcribed from the terminus inwards (Bah *et al.*, 2012, Greenwood and Cooper, 2012). TERRA can interact with a number of telomeric players, including unwound telomeric DNA, telomerase, the RNA and ssDNA binding protein hnRNP A1, the DNA repair factor Ku, the lysine demethylase LSD1 and the heterochromatin proteins HP1 α and HP1 β (Deng *et al.*, 2009, Porro *et al.*, 2014b, Azzalin and Lingner, 2015, Cusanelli and Chartrand, 2015). TERRA levels vary with cell cycle progression, with telomere shortening and deprotection, and with changes in the heterochromatic state of telomeres and subtelomeres. Indeed, evidence is emerging for a causal role of TERRA in these processes.

TERRA is found at APBs and has been implicated as a regulator of ALT. ALT cells show higher levels of TERRA than telomerase-positive cancer cells or primary human fibroblasts (Schoeftner and Blasco, 2008, Lovejoy *et al.*, 2012, Episkopou *et al.*, 2014); this increased TERRA transcription is associated with, and perhaps influenced by, hypomethylation of subtelomeric promoters. Elevated TERRA is also associated with defective telomeric semi-conservative replication (Chawla *et al.*, 2011, Greenwood and Cooper, 2012), a hallmark of ALT. Indeed, TERRA may be not only a consequence, but also a cause, of the ALT mechanism. TERRA transcripts can directly bind to template DNA to form TERRA-containing RNA/DNA hybrids or R-loops. R-loops have been shown to pose a serious threat to genome stability as they can trigger replication fork stalling, recombination and mutations (Aguilera and Garcia-Muse, 2012). Levels of R-loops are restrained by the actions by enzymes like RNase H, which digests the RNA moiety of RNA: DNA hybrids, the Pif1 DNA helicase and the RNA processing complex THO (Boule and Zakian, 2007, Rondon *et al.*, 2010, Aguilera and Garcia-Muse, 2012, Paeschke *et al.*, 2013, Pfeiffer *et al.*, 2013).

Interestingly, experiments performed in budding yeast suggest a role for R-loops in promoting Rad52-dependent HR between telomeres. These elevated recombination rates effectively delay cellular senescence (Wellinger and Zakian, 2012, Balk *et al.*, 2013), much like the averted death conferred by ALT in telomerase-negative human cells. Consistent with a role for TERRA-containing R-loops in eliciting telomeric recombination, R-loops are also found in human ALT cells (Arora *et al.*, 2014). Interestingly, reduction of R-loop levels *via* RNase H overexpression leads to telomere shortening and loss of viability in ALT cells. Conversely, elevating R-loop levels in ALT settings *via* depletion of RNase H leads to excessive levels of ssDNA at telomeres, RPA accumulation, and frequent telomere excision. This telomeric R-loops regulation *via* RNase H1 specifically occurs in ALT cells and not in telomerase-positive cells (Arora *et al.*, 2014).

While the foregoing studies demonstrate a link between modulation of telomeric chromatin and lncRNA transcription in ALT cells, puzzles regarding TERRA and ALT remain. TERRA levels are elevated at ALT telomeres where telomeric chromatin is presumably more open but in contrast, TERRA is also implicated in recruiting the histone methyltransferase Suv39h1, which promotes closed chromatin and favors NHEJ (Porro *et al.*, 2014a, Porro *et al.*, 2014b). Hence, multiple modulating factors likely dictate the outcome of TERRA level. A link has also been suggested between TERRA and ATRX (Goldberg *et al.*, 2010, Flynn *et al.*, 2015), as depletion of ATRX leads to elevated TERRA levels in telomerase-positive or -negative backgrounds. Furthermore, *in vitro* studies suggest a series of hand-offs involving TERRA, the ss binding proteins RPA and hnRNPA1 and telomerase and Pot1 (Flynn *et al.*, 2015). One of the models for ALT induction suggests that loss of ATRX in ALT cells triggers TERRA deregulation and in turn, elevates binding of RPA to telomeres and activates ATR signaling (Flynn *et al.*, 2015). Hence, TERRA may contribute to the causal role of ATRX depletion in ALT survival.

Diverse strategies across evolution illuminate non-canonical ALT pathways

Over the course of eukaryotic evolution, telomerase-based end maintenance has been lost multiple times. A number of organisms including *Bombyx mori* (silkworm), *Allium cepa* (onion), *Giardia lamblia* and *Drosophila melanogaster* (and other *Diptera*) lack canonical telomeres as well as telomerase, and maintain chromosome linearity *via* alternative strategies. Moreover, yeasts like *S.cerevisiae* and *S. pombe* normally maintain chromosomal ends *via* telomerase, but under circumstances of telomerase loss resort not only to ALT-like recombination but also distinct mechanisms of end-maintenance. An understanding of these unusual strategies could inform hypotheses for both canonical and non-canonical ALT mechanisms in mammals.

Informative exception: Fly strategies for linearity

Fly chromosomal extremities lack canonical telomeres and are instead maintained by the retro-transposition of three non-long terminal repeat elements, HeT-A, TART and TAHRE, collectively known as HTT (Capkova Frydrychova *et al.*, 2008). Het-A is the most abundant of these repeat elements; however, as it does not encode a reverse transcriptase, it relies on other elements for transposition. The HTT elements transpose in a sequence-independent

manner, allowing variable combinations of different HTT elements to comprise different chromosome ends. Hence, HTT tract length represents a balance between transposition frequency and sequence loss due to the end replication problem.

Crucially, while HTT elements comprise the most common terminal sequences in flies, their identity as protective structures is not sequence dependent but rather is determined epigenetically. This property is best documented by *de novo* formation of a protective structure at broken chromosome ends lacking any terminal repeat sequence (Titen and Golic, 2010). Indeed, HTT elements themselves are not essential for the protection of chromosomes from end-fusion, though when present, they allow efficient recruitment of end-capping complexes. Population studies have uncovered frequent occurrences of terminally deleted chromosomes in natural populations and terminally deleted chromosomes lacking telomeric retrotransposons are stable for many generations (Gao *et al.*, 2010). An intriguing mystery surrounds how both HTT and these non-HTT sequences assemble end protection complexes which, like shelterin at canonical telomeres, are required to prevent lethal chromosome fusions.

Epigenetic chromatin marks regulate both transcription of HTT arrays and their ability to maintain intact chromosome ends. HP1, a genome-wide component of fly heterochromatin, is enriched at chromosomal ends, recruited by the H3K9Me mark. Mutations in the gene encoding HP1 (*Su(var)205*) not only compromise retrotransposon silencing but also chromosome end-capping as evinced by chromosome end-fusions in *Su(var)205* mutants (Fanti *et al.*, 1998, Perrini *et al.*, 2004) as evinced by chromosome end-fusions in *Su(var)205* mutants. Increased Het-A transcription in a *Su(var)205* mutant background is also associated with elongation of the HTT arrays (Perrini *et al.*, 2004, Silva-Sousa *et al.*, 2012); however, some mutations increase Het-A transcription without elongating the arrays (Torok *et al.*, 2007). Conversely, depletion of the Ku70/Ku80 complex leads to HTT tract elongation without affecting transcription; hence, Ku70/80 appears to regulate accessibility of the chromosome ends to transposition intermediates (Melnikova *et al.*, 2005). Therefore, like the lengths of canonical telomeres in organisms that harbor them, HTT tract length in flies is a useful but complex readout of chromosome end maintenance and protection pathways.

In concert with the absence of canonical telomere repeats, flies lack canonical shelterin components (like POT1, TRF1/TRF2, Rap1); instead they assemble a functionally analogous end-capping complex in a sequence-independent manner (Raffa *et al.*, 2013). This complex, dubbed 'terminin', prevents chromosome end-fusions as the loss of any terminin subunit results in the formation of multi-centric chromosomes with a train-like appearance. The core terminin complex is a set of fast-evolving proteins including HOAP (HP1/ORC-Associated Protein) (Cenci *et al.*, 2003), HipHop (HP1/HOAP interacting protein) (Gao *et al.*, 2010), Moi (modigliani), and Ver (verrocchio) (Raffa *et al.*, 2009, Raffa *et al.*, 2010) (Figure 1d). Biochemical studies demonstrated that chromosome end-associated HP1 interacts directly with HOAP, HipHop and *moi*; HOAP interacts with *moi* and *ver*. Ver harbors an oligonucleotide/oligosaccharide-binding (OB)-fold structural domain similar to that found in the shelterin subunit POT1 and indeed, Ver binds ssDNA *in vitro* as does POT1 (Raffa *et al.*, 2011). As for POT1 in organisms with canonical telomeres, Ver can localize to chromosome ends independently of its OB-fold, presumably via interactions with other

terminin components, but the OB-fold is crucial for preventing end-fusions. It is therefore tempting to speculate that the OB-fold domain of Ver, once recruited to the vicinity by interactions with other terminin subunits, binds 3' overhangs (of HTT sequence) *in vivo* in a manner analogous to Pot1.

Analogous to canonical telomeres, fly chromosome ends associate with non-terminin specific factors including not only HP1 but also the MRN complex, the ATR and ATM kinases, the ATRIP helicase, the E2 ubiquitin conjugating enzyme UbcD1, the putative transcription factor 'without children' (Woc), the variant ubiquitin ligase Pendolino (Peo) (Raffa *et al.*, 2011) and piRNAs. HOAP-depleted chromosomal ends evoke ATM and ATR/ATRIP signaling as well as NHEJ dependent end-fusions, fueling the suggestion that interaction of chromosome ends with terminin results in a conformational change that blocks such DDR activities (Ciapponi and Cenci, 2008, Rong, 2008). In fly germ cells, Woc is found in a terminal deadenylase complex that appears to provide a HTT transcript surveillance mechanism, deadenylating excess transcripts and in turn promoting efficient chromosome end-protection (Morgunova *et al.*, 2015). Depletion of Woc leads to accumulation of poly-adenylated Het-A transcripts, chromosome end-to-end fusions, ring chromosomes and anaphase bridges. As Peo lacks the catalytic cysteine required for Ub transfer and interacts with terminin, telomere fusions spurred by Peo inhibition are thought to be due to altered terminin conformation (Cenci *et al.*, 2015). The piwi-interacting RNA (piRNA) pathway, important for retrotransposon silencing in flies, has also been implicated in fly chromosome end protection. Mutations in the piRNA pathway components Aubergine (an argonaute family member) and Armitage (a RNA helicase) disrupt assembly of terminin and lead to chromosome fusions (Khurana *et al.*, 2010). These mutants also show increased subterminal TART copy number, leading to the hypothesis that piwi-bound piRNAs direct terminin assembly, while increased subterminal TART elements titrate a limiting pool of terminin components (Khurana *et al.*, 2010).

Hence, a picture is emerging in which non-canonical heterochromatin interacts with end-specific factors (Ver, HOAP and HipHop) to provide end protection. In turn, this heterochromatin is maintained in a manner stimulated by, but not completely dependent on, HTT transposition. The presence of terminin and accessory factors in flies points towards a possible evolution from telomerase-dependent to telomerase independent chromosome end maintenance. Loss of telomerase may have led to divergence of terminal DNA sequence, selecting for the evolution of sequence-independent recruitment of end-protecting factors (Raffa *et al.*, 2011). Consistently, while flies lack canonical shelterin (with the exception of Ver), most of the non-terminin proteins controlling *Drosophila* terminus behavior are conserved across eukaryotes (Cenci *et al.*, 2005). This conservation, along with the sequence-independent nature of end protection, suggests the existence of universal epigenetic maintenance mechanisms at chromosome ends under specific circumstances.

Budding and fission yeast – distant cousins, shared and distinct survival strategies

Two highly informative but evolutionarily distant yeasts, *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast), maintain linear chromosomes via telomerase. In both species, telomeric DNA is composed of ~300 bp of G-rich tandem

repeats with a 3' - overhang, and is bound by shelterin-like end-capping complexes (Jain and Cooper, 2010) (Figure 1b,c). The loss of telomerase leads to gradual shortening of telomeric repeats (~5 bp per generation) (Diede and Gottschling, 1999), cell cycle arrest and death. During this period of viability loss, survivors arise in which chromosome ends are maintained *via* alternative means.

Recombination-mediated telomere maintenance was initially discovered in budding yeast (Lundblad and Blackburn, 1993). Following identification of the *EST1* gene, which encodes a crucial conserved telomerase accessory factor (Lundblad and Szostak, 1989), the progressive telomere shortening and decline in viability of telomerase-defective cells was characterized (Beernink *et al.*, 2003, Reichenbach *et al.*, 2003). A subset of cells could escape this senescence via recombination (Lundblad and Blackburn, 1993). Two types of such survivors (types I and II) have been characterized. While the HR protein Rad52 is required for both, type I also require Rad51 whereas type II require Rad50, Rad59 and Sgs1. Type I survivors show amplification of sub-telomeric so-called Y' elements and very short terminal tracts of telomere repeats; extra-chromosomal circles harboring Y' elements are also seen in type I survivor cells. Y' amplification may stem from non-reciprocal translocations or integration of extra-chromosomal Y' circles into short telomeres (Louis and Haber, 1990, Louis and Haber, 1992). In contrast, type II survivors harbor terminal telomere repeat tracts without rearrangement of Y' elements. Telomere length in type II survivors is heterogeneous due to unequal recombination *via* BIR (break induced replication) between telomeric repeats. They also harbor partially ss telomere repeats circles similar to those in human ALT cells (Lundblad and Blackburn, 1993, Larrievée and Wellinger, 2006). Type II survivors generally grow faster than type I, which often convert to type II (Teng and Zakian, 1999). The use of DNA polymerase δ -dependent BIR in type II survival (Lydeard *et al.*, 2007) is analogous to human ALT pathways and underscores the alterations in DDR management that occur in telomerase-negative settings under selection for reasonably stable linear chromosome maintenance.

An intriguing strategy of telomerase-minus survival through formation of terminal palindromes (PAL) has been observed in *S.cerevisiae* that lack HR proteins and the exonuclease ExoI. These survivors maintain linear chromosomes despite suffering large terminal deletions that imply complete loss of telomere sequences (Maringele and Lydall, 2004). The newly acquired chromosome ends comprise large inverted duplications (palindromes) that arise from natural occurring small inverted repeats. It is hypothesized that chromosomal erosion leads to exposure of ssDNA at these inverted repeats, which fold into hairpin structures and prime DNA synthesis. The resulting large palindromic termini can buffer the end replication problem. PAL-like processes may occur during human tumorigenesis in some settings, particularly in cells with reduced exonuclease activity, which may allow the cancer cells to escape checkpoint activation as well as cell cycle arrest (Maringele and Lydall, 2005).

S.pombe can also survive the loss of telomerase using Rad52 dependent HR-based strategies (Nakamura *et al.*, 1998, Subramanian *et al.*, 2008, Rog *et al.*, 2009). Binding of Taz1 to telomeres actively prevents formation of these survivors, at least in part by preventing the accumulation of hyper-recombinogenic stalled replication forks; only in cells lacking both

Taz1 and telomerase do such survivors frequently arise. Rather, the most prevalent mode of telomerase-independent survival in otherwise wild-type fission yeast is the circularization of each individual chromosome, yielding so-called ‘circular strains’. This unusual strategy is begotten by the low chromosome number in fission yeast (three per haploid genome). Telomere loss abolishes protection from end-fusion reactions mediated by the single strand-annealing pathway or, less frequently, by NHEJ (Wang and Baumann, 2008, Almeida and Godinho Ferreira, 2013). In a cell harboring only three chromosomes, the probability of experiencing solely intra-chromosomal fusion events (without inter-chromosomal fusions that would produce lethal dicentric chromosomes) is relatively high. While circular strains are viable, they grow slowly and display defects in chromosome segregation.

Recently, the discovery of HAATI (Heterochromatin amplification dependent and telomerase independent) survivors in *S.pombe* again challenged the idea that telomerase is essential for chromosomal linearity (Jain *et al.*, 2010)(Figure 3). HAATI chromosomes maintain their linearity by replacing telomere repeats with generic heterochromatic repeats. These sequences, usually rDNA repeats, are amplified and moved to all chromosome ends; the terminal heterochromatic rDNA blocks undergo continual expansion and contraction similar to that of HTT arrays in flies. Crucially, these so-called HAATI-rDNA chromosomes depend on the heterochromatin assembly machinery for maintenance of linearity. Along with a nontelomeric (rDNA-containing) terminal 3’ overhang, generated via end replication and/or continual recombination of the rDNA repeats, this heterochromatin engages Pot1 despite the absence of telomere repeats. Thus, as seen in *Drosophila*, fission yeast end protection can be determined epigenetically. Notably, both fly and HAATI-rDNA chromosome ends differ fundamentally from ALT cells in requiring robust heterochromatin machinery to maintain end-protection; in contrast, as discussed above, the ALT pathway characterized thus far appears driven by a decline in terminal heterochromatin. In a rare variant of HAATI termed HAATI-STE, sub-telomeric elements (STE), located proximally to the telomeres of two of the three chromosomes are spread not only to the chromosomal extremities but also multiple internal genomic loci. These rare survivors also utilize Pot1 despite the absence of canonical telomeres, but differ from HAATI-rDNA in terms of heterochromatin requirements. Hence, cells that are initially genetically identical can adopt multiple chromosome end-protection strategies.

HAATI arise infrequently, as propagation of *trt1* cells as single colonies yields ~95% circulars. However, HAATI proliferate more rapidly than circulars and therefore have a competitive advantage in liquid culture. At the same time, the balance of the various telomere proteins clearly affects the choice of survival pathway in telomerase-negative settings, as evinced by the dominance of ALT-like telomere maintenance in conditions of Taz1 inhibition. The as-yet incompletely defined requirements for HAATI-STE further highlight that both stochastic and selective effects dictate survival modes. Specific permutations of attrition of degenerate telomere repeats, natural variations in telomere protein abundance, whether cell proliferation is uninterrupted or punctuated by periods of starvation, all play roles in determining survivor type. The choice of linear chromosome maintenance strategy in cancer cells is a function of these variables as well. Moreover, the existence of HAATI in organisms as evolutionarily distant as flies and fission yeast strongly

suggest that HAATI is a strategy available to human telomerase-minus cancers, whether at a subset of or all chromosome ends.

Control ALT Delete Cancer – challenging paths to therapeutics

There is a growing interest in the use of telomerase-based treatment options as broad spectrum cancer therapies (Ruden and Puri, 2013, Xu and Goldkorn, 2016). While such approaches have great potential for the treatment of telomerase-positive tumors, they would be ineffective for ALT tumors. Telomerase inactivation also faces major theoretical challenges in terms of the lag period between onset of telomerase inhibition and halted proliferation due to the end replication problem, as well as the potential to develop resistance over this time frame. One pathway for resistance is the emergence of telomerase-negative survivors, furthering the need to seek anti-ALT therapies. Indeed, telomerase inhibition in mouse lymphomas resulted in an emergence of ALT populations (Hu *et al.*, 2012). Hence, various clinically relevant target molecules have been tested for anti-ALT activity. The association between ALT and ATR function has led to exploration of several ATR inhibitors as selective anti-ALT strategies (Foote *et al.*, 2013, Josse *et al.*, 2014, Flynn *et al.*, 2015). While successful in terms of abrogating ALT, these inhibitors also face issues like specificity, tissue toxicity and inter-patient response variability.

The virtual ubiquity of ATRX and/or DAXX inactivation in ALT suggests a suite of potential strategies to selectively kill ALT cells. Treatments that reverse or avert ATRX and/or DAXX inactivation could be fruitful though admittedly nontrivial *in vivo*. The complex role played by ATRX in modulating the chromatin state in ALT cells renders them 'ATRX inhibition-sensitized' and suggests avenues for abrogating multiple pathways that contribute to ALT cell robustness. As ATRX is thought to oppose G-quadruplex formation, the introduction of G-quadruplex stabilizing ligands could overwhelm ALT cells with replication stress, massive genomic instability and cell death. A number of DNA helicases including BLM, WRN and FANCI, that assist G-quadruplex unwinding may also serve as potential targets, although the pleiotropic effects of inhibiting such helicases could confound such efforts. Depletion of TOPBP1, a binding partner of BLM, inhibits ALT cell survival (Temime-Smaali *et al.*, 2008), suggesting additional and perhaps combinatorial treatment options. The emerging role of TERRA in ALT etiology is likely to present additional target possibilities for anti-ALT approaches. Indeed, TERRA is at least in some circumstances stimulatory to telomerase activity as well (Cusanelli *et al.*, 2013). Hence, although not a straightforward option, depletion of TERRA in cancer cells could simultaneously affect telomere maintenance in both telomerase dependent and ALT cancer cells.

Notable is the possibility that anti-ALT therapeutic approaches will eventually encounter the emergence of resistance, with selection for mutations that allow a switch back to telomerase positivity. Moreover, the possibility of the emergence of non-canonical mechanisms of chromosome end maintenance cannot be excluded. Thus, combination therapies simultaneously targeting non-canonical ALT pathways may need to be developed. Complexity within the tumor microenvironment and the intra-tumoral heterogeneity (Dawson *et al.*, 2013, Kim *et al.*, 2015) may create conditions ripe for co-existence of cells with telomerase-dependent and -independent strategies as evident in some studies, thus

affecting the response to anti-cancer drugs (Cerone *et al.*, 2001, Perrem *et al.*, 2001). Hence, understanding the environmental and genetic conditions that favor telomerase activation, ALT, or non-canonical ALT pathways like HAATI will be crucial to a comprehensive anticancer approach targeting chromosome end protection. Complicating the matter further, recent characterization of various gliomas and carcinomas including some that frequently show ALT phenotypes, suggests intrinsic molecular-level differences between male and female cancer patients that have otherwise similar tumor characteristics. As more than half of known therapeutic targets or biomarkers showed gender-biased signatures (Yuan *et al.*, 2016), the possibility exists that ALT-targeting strategies could also show such differential behavior.

Notwithstanding the challenges in targeting mortality to telomerase-negative cancers, a complete understanding of the entire repertoire of such cancers and the crosstalk between altered chromatin environment, epigenetic regulation and the DDR therein is likely to reveal viable strategies. A clearer picture of the cause-effect relationships between known regulators of ALT will be crucial, as will determination of whether non-canonical end-protection strategies like those found in flies or fission yeast exist among human cancers. By completing our picture of ALT cell biology, we will be a step closer to solving the long-standing jigsaw puzzle that is cancer.

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(a) *Homo sapiens* **(b) *Schizosaccharomyces pombe*** **(c) *Saccharomyces cerevisiae*** **(d) *Drosophila melanogaster*** **Figure 1.**

Schematic of telomere-specific (shelterin) components in four organisms **a.** Telomeric DNA (TTAGGG)_n in human cells is capped by the six shelterin proteins. TRF1 and TRF2 bind ds telomeric DNA and create a platform for restriction of local DDR activities, promotion of smooth semi-conservative telomere replication, and the association of other shelterin proteins. TIN2 binds TRF1 and TRF2 as well as the TPP1-POT1 dimer. POT1 binds the ss telomeric overhang and regulates telomeric 5' end resection, ATR activation and telomerase. RAP1, a TRF2-binding protein, contributes to the repression of homology-directed repair at

telomeres. The CTC1-STN1-TEN1 (CST) complex binds telomeres as well as other genomic regions and plays roles in replication and in the activation and termination of telomerase activity. **b.** Fission yeast (*Schizosaccharomyces pombe*) telomeres comprise degenerate repeats (TTAC[A]G₂₋₈) bound by a shelterin complex very similar to that of human. The telomeric dsDNA binding protein Taz1 (homolog of TRF1 and TRF2) and ssDNA binding Pot1 are connected by Rap1, Poz1 and Tpp1; Ccq1 promotes telomerase recruitment and connects telomeres with heterochromatin assembly factors. Telomeric protection and access to telomerase is further aided by the Stn1-Ten1 complex. **c.** Budding yeast (*Saccharomyces cerevisiae*) telomeres comprise TG₁₋₃ repeats bound by Rap1, a dsDNA binding protein specific for telomere repeats as well as widespread promoter sequences; Rap1 binds the Rif1 and Rif2 telomere length regulators. Cdc13 binds telomeric ssDNA and interacts with Stn1 and Ten1 to form a complex essential for restraining 5' resection and coordinating replication with telomerase activity. **d.** Chromosomal termini in fruit flies (*Drosophila melanogaster*) comprise HTT repeat arrays bound by terminin, which has some components analogous to those of shelterin. Known components of terminin include HipHop and HOAP, which are connected to the ss overhang binding protein Ver by Moi; terminin relies on local HP1-containing heterochromatin for its assembly. Not shown in the schematic are the many non-telomere specific proteins across different model organisms that associate with telomeres, including those that recruit the heterochromatin assembly machinery (eg, the Sir proteins, which interact with budding yeast Rap1, the SHREC histone deacetylase complex, which interacts with fission yeast Ccq1, and global DDR factors).

(a) Telomerase positive telomeres **(b) ALT emergence in the absence of telomerase** **Figure 2.**

Features of human telomeres in **a.** telomerase-positive and **b.** telomerase-negative scenarios. The G-rich strand (copied by lagging strand replication) is shown in red, the C-rich is in black. Subtelomeric region is in grey shaded boxes. **a.** A t-loop model is shown, in which telomeres are protected from eliciting the DNA damage response (DDR) via the shelterin-induced sequestration of the 3' terminus within subterminal D-loops formed from strand invasion by the terminal ss overhang; shelterin components also have DDR-inhibiting activities that are independent of t-loop formation. This telomeric state actively inhibits cell

cycle arrest, NHEJ and HR. Telomerase replenishes telomeric sequences lost due to the end replication problem and degradation. **b.** In the absence of telomerase, telomeres erode and most will trigger the DDR, leading to senescence/apoptosis. Shelterin is still bound but may be in altered stoichiometry. Altered chromatin organization, due to ATRX/DAXX deficiency, R-loop formation and recruitment of chromatin remodelers leads to a more accessible telomeric chromatin structure. Persistent replication stress due to stalled replication forks and elevated TERRA transcription provoke break-induced replication (indicated by the dotted black line), T-SCEs and ALT, all promoted by chromatin accessibility. These telomeres retain the ability to prohibit NHEJ.

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Figure 3.

Model for HAATI survival in *S. pombe*. In the absence of telomerase, HAATI survivors replace canonical telomeric repeats by either of two non-telomeric repeat sequences, the rDNA (shown) or ‘subtelomeric element’ repeats. Constant expansion and contraction of these sequences buffer unique sequences from the end-replication problem. In contrast to ALT and telomerase-positive cells, robust heterochromatin machinery is essential for end protection in HAATI^{rDNA} cells. The amplified rDNA repeats interact with the SHREC histone deacetylase complex, which interacts with the Pot1-binding protein Ccq1. Nontelomeric 3' ssDNA overhangs assist in concentrating Pot1 at chromosomal termini; in turn, Pot1 prevents fusion HAATI chromosome ends.