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DNA Repair and Telomeres — An Intriguing Relationship

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Additional information is available at the end of the chapter

1. Introduction

Recent advances in DNA repair and telomere biology further establish an intimate interrelationship between these cellular attributes, in the maintenance of genome stability under normal physiological conditions. Consequently, any pathological situation with defect in these signalling pathways may result in genome instability and related diseases. Preservation of genome integrity is depending on effective detection and repair of DNA lesions. Telomeres, the end of linear chromosomes, function to preserve chromosome integrity during each round of DNA replication, thus preventing chromosomal ends from being recognised as DNA damage and drive the cell to 'retire' when reaching specific limits. Therefore, functional telomeres are part of the genome stability maintenance machinery. Telomere dysfunction is directly related to rare diseases like pulmonary fibrosis and dyskeratosis congenita as well as to a growing list of aging related diseases and cancer. Since the pioneering work of Blackburn & Gall in 1978 [1], proving the concept of Muller (1938) that 'the terminal gene must have a special function, that of sealing the end of the chromosome' [2], numerous research publications shed light to aspects of telomere structure / function and its interrelation with DNA repair pathways and genomic stability. Moreover, many comprehensive reviews and book chapters during the recent years describe in details the wealth of information gathered [3-8]. This chapter focuses on the brief description of basics regarding telomere structure – function followed by discussion of selected recent advances, regarding telosome (functional telomere complex) interaction with DNA Damage Response (DDR) and Repair pathways, in order to restore genome information and prevent neoplastic transformation. Unsurprisingly, impairment of DNA repair – telomere function interplay is related to specific aggressive forms of cancer. Moreover, this review will hint at selected points regarding consequences of impair-

ment of telomere integrity accompanied by cellular checkpoints abrogation. Quite interestingly, it seems that the pleiotropic effects governing a cell's decision to senesce vs. undergoing apoptosis when reaching the so-called Hayflick limit [9] (certain number of cell divisions), comprise a fertile environment for cancer formation. Cancer cells have developed numerous strategies towards bypassing these limits on the road to achieving eternal proliferation.

2. Genomic stability and telomeres

Genomic stability is the prerequisite of species survival as ensures that all required information will be passed on to the next generations. In contrast to single-cell – quickly dividing - species, higher order organisms, in order to preserve their genomic information, require more efficient DNA repair mechanisms due to later onset of reproduction. Therefore, a remarkable ability of cells to recognize and repair DNA damage and progress through the cell cycle, in a regulated and orderly manner, has been developed. A vulnerable portion of the genome, especially in eukaryotic organisms whose genome is organised in linear chromosomes, is their edges called telomeres (after the greek words 'τέλος' (télos) and 'μέρος' (méros) meaning 'the ending part'). Telomeres are nucleoprotein complexes that 'cap' the chromosomes' physical ends. In most eukaryotic organisms integral and stable telomeres guarantee the maintenance of genetic information and its accurate transfer to the next generation. In case telomeres are impaired, abnormal ends are recognised by the DNA damage detection machinery as double-strand DNA (dsDNA) breaks, the DDR is activated and the lesion is healed by Non-Homologous-End-Joining (NHEJ) repair activities. The result of this type of repair may be the fusion of chromosomes and the formation of dicentric /polycentric chromosomes leading in turn to further genomic instability. For this reason a number of telomere-binding protein complexes are associated with telomeres to ensure the formation of a proper secondary structure and a capping function. Intriguingly, a number of protein complexes implicated in DNA repair also contribute to telomere stability. The structure of telomeres is intrinsically dynamic, as chromosome ends should relax during genome replication and then re-establish their 'capped' state after replication. Consequently, telomeres may switch between closed (protected) and open (replication-competent) states during the cell cycle. Each state is governed by a number of interactions with specific factors and can lead the cell to either cell division or senescence / apoptosis under normal conditions, or to disorders / cancer in abnormal cases (processes still poorly understood in a large extent) [10-11]. Moreover, during development and in certain cell types in adults, telomere length should be preserved. Thus, multiple physiological processes guarantee functional and structural heterogeneity of telomeres concerning their length and nucleoprotein composition. A functional chromosome end structure is essential for genome stability, as it must prevent chromosome shortening and chromosome end fusion as well as degradation by the DNA repair machinery. Hence, structure and function of telomeres are highly conserved throughout evolution [12]. The cell's inability to properly maintain its telomeres can lead to diseases such as dyskeratosis congenita, pulmonary fibrosis, atheromatosis and cancer. On the other hand, telomere gradual shortening during the cell's life span functions among other things as a protective mechanism against cancer. These characteristics

make telomeres an attractive target for specific anti-cancer therapies. Therefore, analysis of telomere structure-function biology is crucial in order to clarify how telomere length and structure are preserved, together with telomere – DNA repair intercommunication.
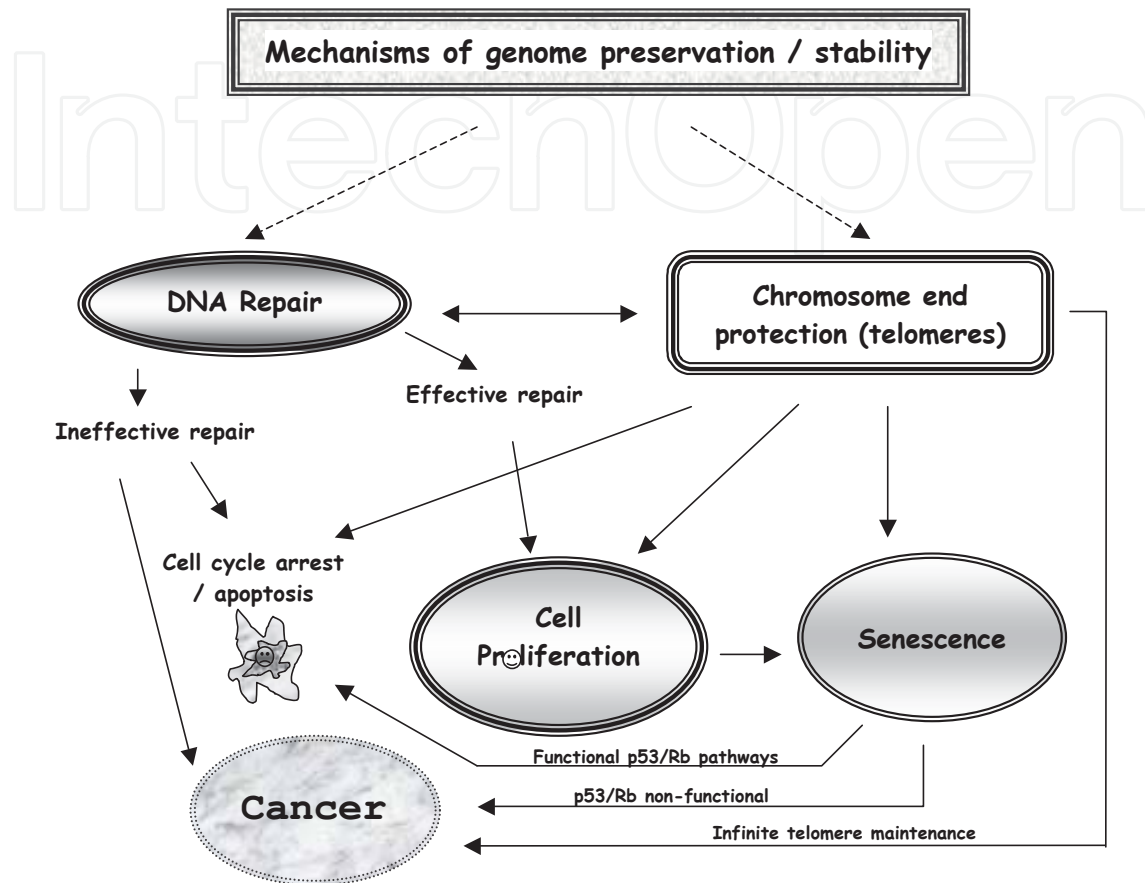

Figure 1. Scheme of Genome preservation mechanisms. Details in the text.

3. Telomere features and replication

Telomeres are long tracts of DNA at the linear chromosome's ends composed of tandem repeats of a Guanine rich sequence motif that vary in length from 2 to 20 kb, according to species. This motif is conserved in lower eukaryotes and in mammalian cells [13]. Exceptionally, the chromosome ends of a few insect species (Drosophila and some dipterans), instead of telomeric motifs, possess tandem arrays of retrotransposons [14]. Telomeric DNA is double stranded with a single – stranded terminus that is on average 130-210nt long in human cells [15]. Under normal conditions, in most somatic cells of an adult organism, telomeres shorten in each cell division (i.e. in humans by about 50–150 nucleotides (nt)). The basic telomere DNA repeat unit in vertebrates is the hexamer TTAGGG, in which the strand running $5´ \to 3´$ outwards the centromere is usually guanine-rich and referred to as G-tail. In order not to leave

exposed a single stranded overhang this G-rich strand protrudes its complementary DNA-strand and by bending on itself it folds back to form a telomere DNA loop (t-loop), while the G-tail 3′end invades into the double strand forming a D-loop inside the t-loop (Figure3) [16]. As a result, the t-loop protects the G-tail from being recognized as a double-stranded break by sequestering the 3′-overhang into a higher order DNA structure. Inability of telomeres to form a t-loop, for example due to a very short length, results in DDR triggering and /or exonuclease degradation, chromosome fusion and further genomic instability. Despite t-loop, the G-tail is also able to form, at least *in vitro*, a secondary DNA structure of intra and intermolecular G-quadruplexes [17-18]. G-quadruplexes are piles of G-quartets, planar assemblies of four Hoogsteen-bonded guanines, with the guanines derived from one or more nucleic acid
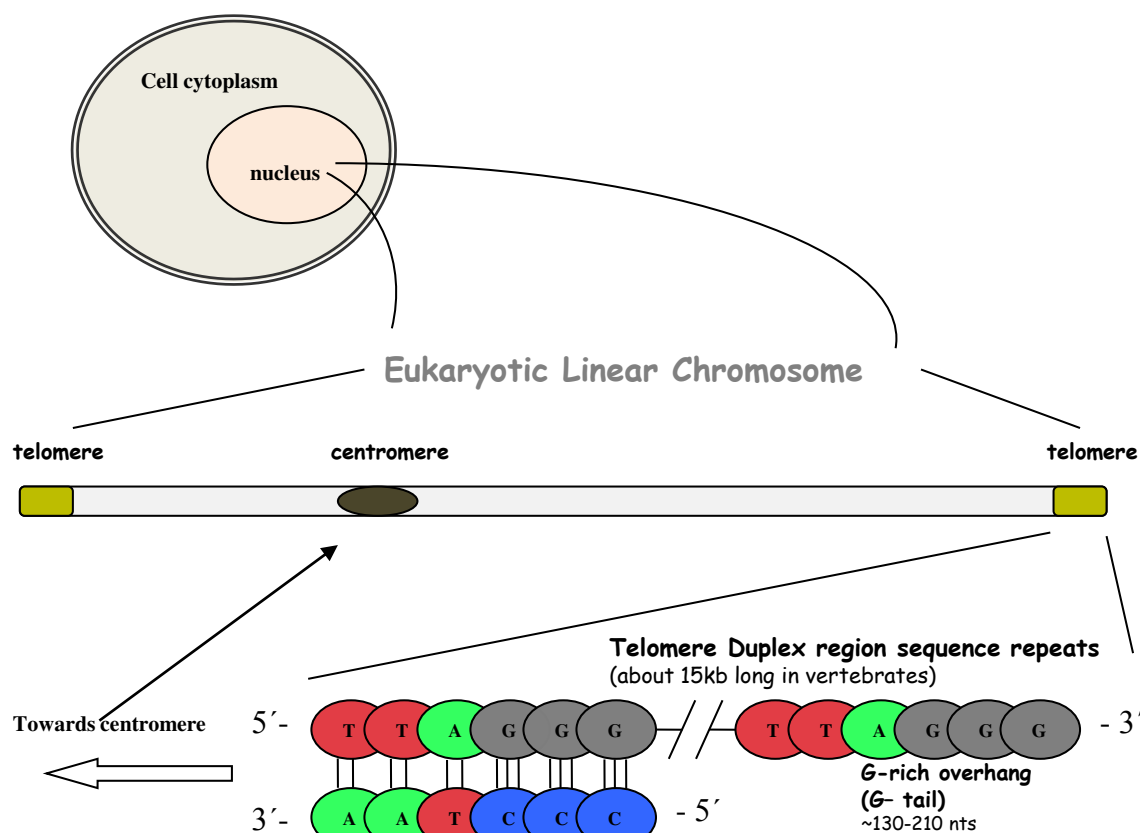

Figure 2. Telomere primary structure scheme. Details in the text.

Such secondary structures of the telomere G-tail, as the t-loop and the quadruplexes, may contribute to telomere stability and chromosome-end protection as they prohibit access of nucleases and DDR detection enzymes. On the other hand, these structures should relax to allow telomere replication. Telomeres, in the absence of any compensation mechanism, become shortened during every cell cycle due to incomplete replication of the lagging strand (referred to as the ''end replication problem''), resulting in cumulative telomere attrition during aging. In addition, loss of telomere DNA also occurs due to post-replicative degradation of the 5′strand that generates long 3′G-rich overhangs [22].

Telomere replication is a multi-step process combining the classical semi-conservative and telomere-specific replication, which necessitates dynamic opening of the telomeric DNA. During genome duplication, replication of the telomere duplex occurs via the conventional replication machinery. As a next step, nucleases cleave the C-strand to generate a G-tail. G-tail then serves as an anchor for a telomere-specific reverse transcriptase (TERT), also termed as telomerase, a nucleoprotein enzyme responsible for telomere end replication. Telomerase function compensates for the inability of DNA polymerases to replicate the 5′ends of eukaryotic chromosomes [1, 23]). This remarkable and unique feature of telomerase is attributed to its specific RNA subunit termed TER. TER sequence is complementary to the G-tail DNA sequence, specifically recognises and binds to two sequential telomeric motifs, with the aid of telomerase and serves as a template [24]. Telomere G-overhang is then elongated by additions of sequence repeats by telomerase leading to the telomere loss counteraction [25, 26, 27]. The complementary C-rich strand is then synthesized by conventional RNA-primed DNA replication [28,29]. Following replication, the telomeres created by the synthesis of the leading strand are either blunt-ended or left carrying a small 50 nt overhang whereas those created by the lagging-strand synthesis have a 3′ overhang with a length determined by the position of the outermost RNA primer [30]. This fact underlines the importance of telomerase activity for genome integrity, especially during development and in certain adult cell types, where telomere shortening during each cell division should at least partially be restored. Many excellent recent reviews extensively cover telomerase structure-function in health and disease [31, 32].

Following telomere synthesis, the created G-tail reforms the t-loop structure and the telomeres are re-bound by shelterin, a specific multi-tasked protein complex (figure 3). Since the role of telomere protection is vital for cell viability, shelterin complex and interacting proteins have evolved to specifically interact with these chromosome end structures and survey proper telomere protection / preservation, depending on the cell's status [3]. The shelterin complex is formed by a core of six proteins including the Sab/Myb-type homeodomain TRF proteins in mammals which bind the duplex form of the telomere repeats, the OB-fold containing protein POT1 in mammals which binds the single-stranded telomere 3′ overhang and by other proteins associated via protein–protein interactions with them [33]. The main roles of shelterins are to repress the DNA repair machinery at telomeres, and regulate telomere length [3, 35, 36] therefore they are evolutionary conserved to a great extent [4].

In addition, telomeres are also associated with a large number of non-telomere specific proteins mainly factors and enzymes involved in DNA double strand signalling and repair. Obviously, intact telomeres are essential for chromosome integrity [37-39]. Therefore, telomere associated proteins protect the ends of eukaryotic chromosomes from being recognized as double strand breaks, and avoid chromosome end degradation by nucleases and non-canonical chromosome-end fusions.

Another intrinsic feature of telomeres is their transcriptional activity, despite their heterochromatin-like structure, giving rise to a long non-coding G-rich RNA (lncRNA) termed TERRA (telomere repeat-containing RNA), which forms an integral component of telomere heterochromatin [4, 5, 40 - 44]. TERRA associates with telomeres and is suggested to be

involved in telomere structure and the state of telomeric chromatin during development and differentiation [41, 45, 46]. TERRA transcription occurs at most or all chromosome ends and is regulated by RNA surveillance factors and in response to changes in telomere length. The accumulation of TERRA at telomeres may also interfere with telomere replication [40, 41, 43].

4. Telomere maintenance / impairment consequences

Telomeres are able to counterbalance incomplete replication of terminal DNA by conventional DNA polymerase and overcome the so-called 'end replication problem' as during each genome replication, due to inability of the DNA polymerase to extend a 5' DNA end, the lagging strand, after removal of the RNA primer, is not copied completely. As a result telomeres gradually shorten with each round of genome replication [47, 48]. Consequently, a mechanism was required to get through this obstacle. Upon each genome duplication, cells would otherwise keep loosing genetic material, eventually resulting in premature cell death, a critical problem for both the species and an individual's survival. This issue is even more prominent especially in multi-cellular organisms with late onset of reproduction. During ontogenesis, eukaryotic organisms solved this problem by preventing telomere attrition in dividing cells, through recruitment of the specialized and unique reverse transcriptase that replicates telomeric DNA sequences (telomerase), thereby maintaining them at a 'constant' length, as a limited telomere length is a prerequisite for cell replication [49]. Telomerase is routinely active only during embryogenesis and development, while in adults is expressed only to rapidly dividing cells (i.e. proliferative skin and gastrointestinal cells, activated lymphocytes, specific bone marrow stem cells and dividing male germ cell lineages [50].

In most adult cells telomerase is not expressed. Consequently, after a number of cell divisions, telomeres reach a critical length and chromosomes become uncapped. This leads, depending on the cellular context in which the uncapping occurs, either to a permanent cell cycle arrest (termed cellular senescence) or to apoptosis (programmed cell death) [51,52]. Extreme telomere shortening leads to chromosome instability, end-to-end fusions, and checkpoint-mediated cell cycle arrest and/or apoptosis [for review see 52 - 53]. All these processes are related in mammals not only to aging, but also to several age associated diseases such as cancer, coronary artery disease, and heart failure [54-57]. Cells programmed to enter senescence may escape this procedure due to checkpoint dysfunction and instead continue infinite proliferation, leading to oncogenesis. In such cases genomic stability has to be re-established and telomere length has to be restored by a Telomere Maintenance Mechanism (TMM). In most of tumor cells telomere maintenance is achieved by re-expression of telomerase. Interestingly, tumors have been described where telomerase could not be detected. Further studies revealed that in addition to the role of telomerase in maintaining telomere length, homologous recombination (HR) constitute an alternative method (ALT ''alternative lengthening of telomeres'') to maintain telomere DNA in telomerase- deficient cells. ALT TMM, in contrast to telomerase dependent TMM, results in telomeres with high heterogeneity in length and at least in the well-studied model of S. cerevisiae, consists of two pathways. While the bulk of cancer and

immortalized cells utilize telomerase re-expression to maintain telomere length, about 10-15% of tumors described operate using the ALT mechanism [58-60].

5. Telomere structure — Function relationship

As aforementioned, in the absence of telomerase, telomeres become non-functional, shorten with successive cell divisions, and chromosome termini can fuse as a consequence of de-protection. Telomere fusions are the result of non-homologous-end-joining (NHEJ) which is one of the prevailing mechanisms of a double strand break (DSB) healing. The outcome of such events could be the creation of chromosomes bearing more than one centromeres, which will likely be pooled to opposite poles during mitosis, resulting in chromosome breakage and further genomic instability through repeated fusion – breakage events. In vertebrates, the role of chromosome end protection in order to be distinguished from chromosome breaks is attributed to a specific complex of proteins collectively referred to as shelterin. Shelterin complex is basically composed by six proteins. Two members of the shelterin complex, TRF1 and TRF2 (from Telomere Repeat-binding Factor 1 and 2) bind directly to double stranded telomeric sequence, while POT1 binds ssDNA. TRF2 interacts with and recruits RAP1, while TIN2 mediates TPP1 – POT1 binding to the TIRF1 / TIRF2 core complex. POT1 binds to and protects the 3' single-stranded DNA overhang of telomeres (G-tail), while TIN2 likely links the single and double-stranded DNA binding complexes, especially in the area of the telomeric D-loop formation (figure 3) [5]. It seems that this core shelterin complex is mainly located at the telomere end (also referred to as telosome) and serves both in stabilizing t-loop structure, protecting it at the same time from being recognized as DNA damage and repaired by NHEJ. Additionally, shelterin regulates access to restoration processes of telomeric DNA after each genome replication. In general, shelterin complex seems to function as a platform regulating recruitment of a growing list of factors involved in chromatin remodelling, DNA replication, DNA damage repair, recombination and telomerase function, thus regulating telomere access / modification by diverse cellular processes (figure 4), recently reviewed in [61].

Interestingly, it appears that more than one type of core shelterin complex exists and not all of them are necessarily part of the telosome. Complexes containing only TRF1-TIN2-TPP1-POT1 or TRF2-RAP1 have been detected. Recent data measuring the absolute and relative amounts of TRF1 and TRF2 in the cell revealed that TRF2 is about twice as abundant as TRF1 [62] and this is consistent with TRF2 being detected in spatially directed DNA damage induced foci in non-telomeric chromosome regions. TRF2 recruitment to sites of DNA damage is consistent with it playing a critical role in the DNA damage response [63]. The complexity of the telosome created network is practically based on the unique structural features of the shelterin members. TRF1 and 2 bear a SAB/MYB domain by which they both recognise a TTAGGGTTA motif on telomere ds DNA, an acidic rich (D/E) terminal region and a specific docking motif referred to as TRF homology (TRFH) motif [64]. The TRFH domain mediates homo-dimerization of TRF1 or TRF2 [65, 66] but prohibits heterodimerization due to structural constraints [67]. A FxLxP motif and a Y/FxLxP motif are required for TRF1 and TRF2 binding, respectively. These domains are re-
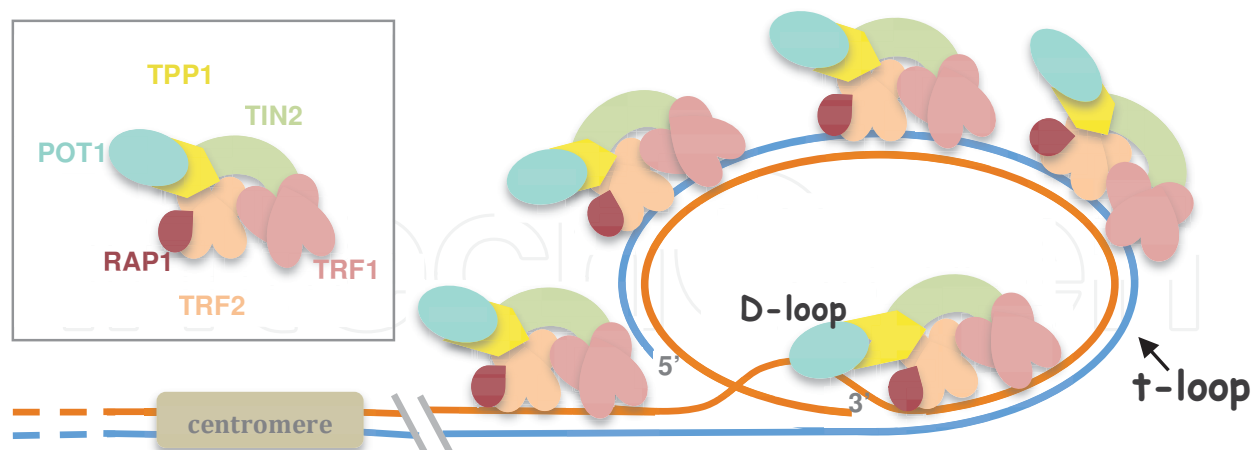
Potential telosome structure

Figure 3. Schematic model of potential telomere capping arrangement by the shelterin complex. Proteins of the shelterin complex participate in telomere protection, replication and length regulation. TRF1 and TRF2 proteins bind specifically to telomeric ds DNA, while POT1 (TPP1) recognizes ssDNA (stabilizing D-loop). TIN2 interconnects ssDNA to dsDNA binding complexes, stabilizing telosome structure. Telomeric DNA consists of repetitive DNA sequence, a duplex region and a ssDNA G-strand overhang (G-strand, orange; C-strand, blue). The shelterin complex binds to both the duplex and ssDNA regions through specific protein–DNA interactions. Formation of the t-loop involves strand invasion of the G-overhang to create a displacement-loop (D-loop). The t-loop is proposed to mask the chromosome end from DNA damage sensors. For simplicity reasons the shelterin complex is depicted as a six-protein complex homogeneously dispersed onto telomere. See text for further details.

ferred to as TRFH Binding Motifs (TBM). The Phe 142 amino acid residue in TRF1-TRFH motif is responsible for TIN2 binding through its TBM region. TIN2-TBM has significantly lower affinity for the corresponding region of TRF2 (Phe 120) due to structural differences in the vicinity of Phe 120 and finally is attached to TRF2 via a unique TRF2 region near the N-terminus of the protein. Nevertheless, Phe 120 residue is crucial for specific interaction with other telomere associated factors like Apollo nuclease, a TRF2 binding partner. Complex formation between shelterin core members and associated factors with TBM like motifs [68] are likely to be also directed by changes in binding affinities due to post-translational modifications. A nice example is the TRF1 parsylation by tankyrase, resulting in significant decrease of the DNA-TRF1 affinity, allowing telomere lengthening and sister telomeres separation by specifically relieving cohesion complex from TRF1 and TIN2 [4, 5, 69-72]. Misbalance of such interactions could be detrimental for genome integrity as shown by elevated levels of TIFS formed in cells overexpressing an isolated TBM as a tandem YRL repeat. Analogous deleterious results were obtained when expressing a TRF2-F120 substitution allele [68].

Recent structural studies of one of the two OB (Oligonucleotide/oligosaccharide-binding) folds of S. pombe Pot1, that comprise the binding site of ssDNA, revealed that non-specific nucleotide recognition of ssDNA is achieved by hitherto unidentified binding modes that thermodynamically compensate for base-substitutions through alternate stacking interactions and new H-bonding networks [73]. Thus, delineating in detail the structure of shelterin members and associated factors is expected to geometrically improve our understanding of the networks

consisted and the way quantity vs. quality changes interfere with structural modifications leading to functional alterations, finely tuning genome stability. Undoubtedly, the wealth of information gathered has already paved the way of using anti-telomerase agents in clinical trials, with robust expected outcome.

Apart from shelterin and interacting partners, another significant complex has recently emerged to be also involved in telomere biology, the CST complex. The CST complex is composed of CTC1, STN1 (OBFC1) and TEN1, and has been attributed the rescue of stalled replication forks during replication stress. The CST complex interconnects telomeres to genome replication and protection independently of the Pot1 pathway [5, 74].

Accumulating evidence by numerous publications quite unexpectedly demonstrated that DNA damage response (DDR) and repair pathways, despite seeming a paradox, share common features with telomere maintenance strategies. DDR early response proteins are recruited to telomeres and proteins believed to function in telomere maintenance have been also evidenced to be involved in DDR. Paradoxically, DDR factors in telomeres, in normal conditions, seem to interfere with telomere restoration and length preservation. This distinct phenomenon is attributed to shelterin co-ordination of DDR factors access and function at telomeres. TRF2 can bind to and suppress ATM, while POT1, when bound to the G-tail through TPP1, inhibits ATR. Suppression of TRF2 activity elicits p53 and ATM activation, leading to telomere dysfunction induced foci (TIFs). TIFs result in end-to-end telomere fusions via the NHEJ pathway and their appearance is correlated with the induction of senescence [75]. The interplay seems to be based on shelterin quantity and telomere length, two parameters directly related to each other, as when telomeres are critically short they are less likely to form a t-loop, a reaction catalysed by TRF2 *in vitro*, and in turn less shelterin is bound on [75]. Consequently, two major telomere maintenance structures are significantly reduced (t-loop and shelterin coating), allowing DDR activation. Yet, quite intriguingly, NHEJ machinery may also exert a protective role at telomeres through the enzymatic activity of Tankyrase related to the promotion of DNA-PKcs stability and prevention of the formation of telomere sister chromatid exchanges (T-SCEs) as a product of inter-telomere recombination [76]

Another intriguing paradigm is the MRN complex (a protein complex of meiotic recombination 11 (MRE11) – RAD50 and NBS1 proteins), where a single NBS1 molecule is associated with two dimers of MRE11 and RAD50 [77]. The MRE11 and RAD50 proteins form a hetero-tetramer that contains two DNA-binding and processing domains that can bridge free DNA ends [8, 77]. The MRN complex localizes to telomeres during the S and G2 phases of the cell cycle through direct interaction of NBS1 with TRF2, presumably contributing to the G-tail formation on the leading telomeric strand and thus to telomere stability [46, 77-81]. In humans, mutation in the NBS1 gene leads to the chromosomal instability disorder, Nijmegen breakage syndrome 1, associated with enhanced sensitivity to ionizing radiation and chromosomal instability and early developing cancer even in NBS1$^{+/-}$ heterozygotes. NBS1 contains a forkhead-associated (FHA), a BRCT (BRCA1 C Terminus) domain, an MRE11-binding domain, and an ATM-interacting domain. Accumulating evidence demonstrates that NBS1 interacts with telomeres and contributes to their stability, at least in human and mouse cells. Indirect immuno-fluorescence experiments revealed that NBS1 co-localizes with TRF2 during the S

phase in cultured HeLa cells [64, 78], possibly by modulating t- loop formation. As TRF2 has also been found on non-telomeric sequences the impact of NBS1 co-localization with TRF2 requires further clarification. Similarly, in mouse embryonic fibroblasts, active recruitment of NBS1 to dysfunctional telomeres has been observed [46, 79, 81]. The MRN complex appears to play a dual role in telomere biology. One is to mediate, at least in part, the ATM response leading to TIF formation after TRF2 deletion [81]. Secondly, by its nuclease activity, it is required for normal telomere formation, as MRN is implicated in the processing of damaged telomeres by influencing the production of the overhang from a blunt end telomere created after telomere replication [46, 79, 81]. Such acceleration of the G-tail formation, following telomere dysfunction / de-protection prevents the fusion of leading blunt-ended strands of de-protected telomeres during S phase. Apollo nuclease may be also recruited and be involved in this process. Direct interaction of NBS1 with telomere repeat-binding factor 1 (TRF1) has been shown for immortalized telomerase negative cells [13] implying that this interaction might be involved in the alternative lengthening of telomeres. Furthermore, in telomerase expressing cells, MRN complex, through downregulation and removal of TRF1 (NBS1-dependent phosphorylation of TRF1 by ATM) may also promote accessibility of telomerase to the 3′ end of telomeres [82, 83]. DNA repair intercommunication with telomere stability is a relationship established quite early in evolution as indicated by the fact that MRE11 and RAD50 together with protein kinases ATM and ATR, are also essential for proper telomere maintenance in plants [4,5].

Recently, another protein phosphatase, PNUTS (phosphatase 1 nuclear-targeting subunit), which interacts with TRF2, inserts another piece in the puzzle of the DDR and telomere relation [68]. In addition, detected by genome-wide searching for TBM containing proteins, the three BRCT domain bearing MCPH1 proximal DDR factor also interacts with TRF2. MCPH1 mutations are associated with developmental defects and increased tumor incidence [84]. MCPH1 depleted cells present decreased levels of BRCA1 and Chk1 and are defective in the G2/M checkpoint [85].

An essential role in telomere integrity is also attributed to BRCA2, a key component of the HR DNA repair pathway. BRCA2 associates with telomeres during the S/G2 cell cycle phases and appears to facilitate RAD51 recombinase loading [86]. Therefore, BRCA2-mediated HR activity is required for telomere length maintenance. These findings may explain, at least in part, the shorter telomeres found in BRCA2 mutated human breast tumors. Therefore, telomere dysfunction may be also implicated in the genomic instability observed in BRCA2-deficient breast and ovarian cancers [86].

In total, a number of DNA repair molecules, which are collectively part of the HR, NHEJ, NER and Fanconi Anemia pathways have been found to be recruited at telomeres, with TRF2 mainly functioning as a protein hub. In normal conditions, ATM/ATR signalling, upon de-protection due to short telomere length and subsequent 'retirement' of the cell (senescence / apoptosis) is part of the normal, tumor-initiation protective mechanism against genome-destabilized cells. In cells bearing normal telomere length there are inhibitory relationships between these different DNA repair systems, preventing each other's activation.
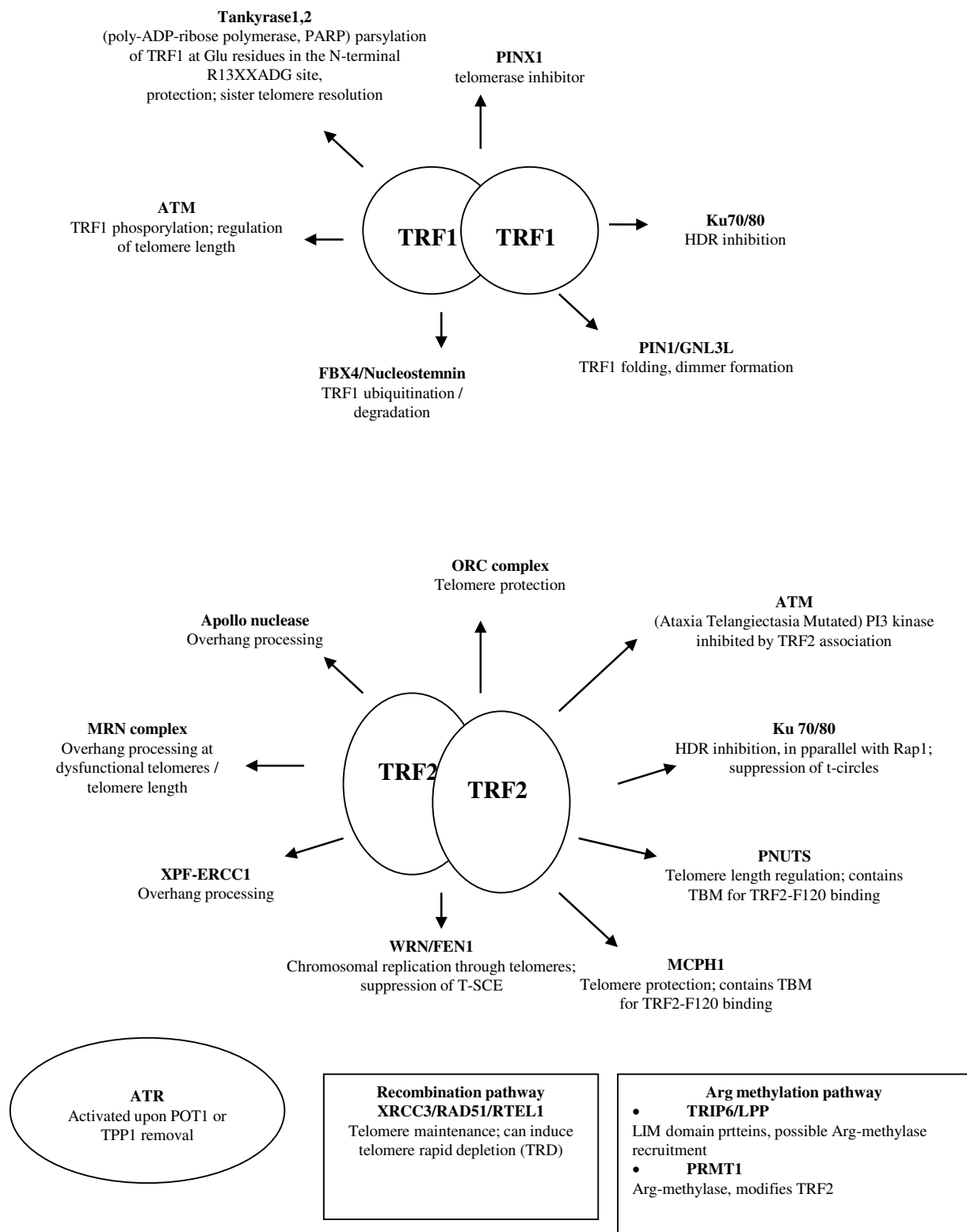
Tankyrase1,2
(poly-ADP-ribose polymerase, PARP) parsylation
of TRF1 at Glu residues in the N-terminal
R13XXADG site,
protection; sister telomere resolution

PINX1
telomerase inhibitor

ATM
TRF1 phosporylation; regulation
of telomere length

TRF1   TRF1

Ku70/80
HDR inhibition

FBX4/Nucleostemnin
TRF1 ubiquitination /
degradation

PIN1/GNL3L
TRF1 folding, dimmer formation

ORC complex
Telomere protection

Apollo nuclease
Overhang processing

ATM
(Ataxia Telangiectasia Mutated) PI3 kinase
inhibited by TRF2 association

MRN complex
Overhang processing at
dysfunctional telomeres /
telomere length

Ku 70/80
HDR inhibition, in pparallel with Rap1;
suppression of t-circles

TRF2   TRF2

PNUTS
Telomere length regulation; contains
TBM for TRF2-F120 binding

XPF-ERCC1
Overhang processing

WRN/FEN1
Chromosomal replication through telomeres;
suppression of T-SCE

MCPH1
Telomere protection; contains TBM
for TRF2-F120 binding

ATR
Activated upon POT1 or
TPP1 removal

Recombination pathway
XRCC3/RAD51/RTEL1
Telomere maintenance; can induce
telomere rapid depletion (TRD)

Arg methylation pathway
• **TRIP6/LPP**
LIM domain prtteins, possible Arg-methylase
recruitment
• **PRMT1**
Arg-methylase, modifies TRF2

Figure 4. Shelterin associated factors also involved in DNA Damage Response. Details in the text

Telomeres are part of heterochromatin structure meaning that specific signals define their location in the nucleus. Although the fact that telomeres are expected to be by definition stable and inert chromosome ends, nevertheless appear to be dynamic nucleoprotein complexes also involved in chromatin remodelling. Recruitment of heterochromatin binding protein HP1 [87, 88], enriched tri-methylation of histone H3 lysine 9 (H3K9) and H4K20 [89], as well as methylation of CpG dinucleotides in subtelomeric DNA repeats [90] support this notion. These heterochromatic marks are replaced by characteristics of open chromatin (increased acetylation on histone tails, etc.) when telomeres become shorter. Such changes imply that a minimum telomere length is required to maintain heterochromatin–like conformation at chromosome ends, a structure that may change following telomere attrition. Moreover, telomeres and the shelterin complex should loosen their tight structure during chromosome replication and re-establish their compact form after completion of DNA duplication. An analogous loosening of telomere structure should be required in cases of telomere restoration by either telomerase or DNA repair mechanisms, although possibly through distinct procedures. In order to achieve this plasticity, chromatin should be remodelled through a number of enzymes, according to a local histone code [91]. A number of histone modifications are implicated where distinct histone tail-protein interactions promote telomere complex structure relaxation or compression [92]. As an example, SIRT6 (a histone H3K9 deacetylase that modulates telomeric chromatin) depletion experiments by RNA interference provided evidence of increased nuclear DNA damage and the formation of telomere dysfunction-induced foci. These experiments suggested that SIRT6 protects endothelial cells from telomere and genomic DNA damage, thus preventing a decrease in replicative capacity and the onset of premature senescence, in this particular case implicated in maintaining endothelial homeostatic functions and delay vascular ageing.

Another important set of factors implicating in telomere biology is the products of ATRX and DAXX genes, which are implicated in chromatin remodelling along with histone H3.3 [93-95]. Mutations or deletions in these genetic loci have been directly correlated with ALT+ status on cell lines or tumors per se [93,95]. According to these findings, screening for ATRX/DAXX mutations/expression may represent the most - up to date - reliable marker for tumors that have chosen the ALT TMM pathway.

Collectively, it is the proper assembly of shelterins in telomeres that is essential for chromosome stability (differentiates chromosome ends from DNA ds breaks and prevents loss of genetic information through either nucleolytic attack (exonuclease-mediated degradation) or aberrant chromosome fusions and undesirable recombination, during a cell's life span. Together with proper structure, functional co-ordination controlling TMM and telomerase activity are strictly regulated throughout the cell cycle by a number of implicated accessory factors, transiently recruited by the shelterin complexes / subcomplexes [75].

Apart from their protective role, proper interaction of shelterins with components of DNA repair machinery as well as telomerase components and telomerase recruitment, allows telomere restoration when appropriate. The importance of the correct structure – function of shelterin components in telomere biology and cancer formation, together with telomere-

associated diseases, are depicted by association of mutation detection in i.e. TIN2 in many of these cases [96].

6. Telomeres and diseases

Telomere function is directly implicated in cellular senescence and therefore is expected to play a fundamental role in aging processes. Indeed, numerous publications the recent years reveal a correlation of telomere maintenance and retardation of aging in both cellular and animal models. Moreover, large epidemiological studies have reported an association between shorter telomere length in peripheral leukocytes and several inflammatory diseases of the elderly including diabetes, atherosclerosis and, recently, periodontitis [97].

To the present, leukocyte telomere length (LTL) serves in many cases as a predictor of age-related diseases and mortality. The potential role of telomere attrition in the onset or evolution of chronic inflammatory diseases, although requiring further investigation, could serve as a monitor of disease progression and effectiveness of treatment schemes. Furthermore, recent work of Entriger et al., provides preliminary evidence in humans, supporting a correlation of maternal psychological stress during pregnancy with the setting of newborn leukocyte telomere length. [98].

Apart from aging and specific syndromes (dyskeratosis congenita, pulmonary fibrosis) directly related to telomere dysfunction, abnormal telomere biology critically interferes with cancer [99-102]. One of the hallmarks of cancer is unlimited cell proliferation therefore tumour cells require a telomere maintenance mechanism (TMM) in order to retain the ability of infinite propagation. This issue will be more extensively discussed in the next paragraphs.

7. Telomere maintenance in non-physiological situations — ALT pathway

In adult vertebrates telomere length is –in most of the cell types - normally reduced during each cell division, while a limited telomere length is a prerequisite for cell replication. Following a certain number of replication cycles, telomere length is gradually shortened and this shortening during cell life span functions among others as a protective mechanism against both organismal ageing and neoplasia development. When telomere length reaches a critical value it triggers DNA Damage Response (DDR) followed by replicative senescence and / or check point-driven cell death, thereby prohibiting cellular aging and the capability of continuous proliferation. On the other hand, critical telomere length causes telomere uncapping and may result in the fusion of chromosomes by the NHEJ mechanism. Random telomere fusions mean either random fusions of various replicated chromosomes or fusion of sister chromatids of the same chromosome. [3,5,103]. In every case the consequences are fatal for genome integrity and normal cell well-being. In case that either senescence or apoptosis will be bypassed by deregulated cell fate control mechanisms, as for example mutated Rb or p53 proteins, then carcinogenesis might occur. Cancer cells depend on extensive cell proliferation

and thus intact telomeres of a minimal length are also required for tumour survival and expansion. Telomere maintenance in cancer is achieved by two major mechanisms. In most of the cases telomere attrition in cancer cells is counteracted by telomerase upregulation [104] but in about 10-15% of tumour telomeres are preserved by telomerase independent mechanisms referred to as the Alternative Lengthening of Telomeres (ALT) pathways which are based on homologous recombination [105,106]. ALT has been detected in many tumour types but is most prevalent in tumours of mesenchymal origin like glioblastomas, osteosarcomas, soft tissue sarcomas, all of which tend to present particularly poor prognosis (table 1). The list includes 20-65% of sarcomas (in approximately half of osteosarcomas and in about one third of soft tissues sarcomas, one fourth of the primary brain tumor, glioblastoma multiforma and 10% of neuroblastomas) and 5-15% carcinomas (approximately half of which is gastric carcinoma and an about 15% adrenocortical & ovarian carcinoma) [94,107-115].

Genetic or epigenetic changes that unleash ALT are not yet deciphered. It seems that human mesenchymal stem cells might have a particular tendency to activate ALT [116]. ALT process has not been detected in normal cells although it might be part of a physiological process with or without modifications, since most, if not all, of the molecules implicated in ALT seem to be present also in normal cells, raising the question what inhibits / prevents ALT under physiological conditions. The Rb family member p130 seems to play a role in ALT inhibition as p130 forms a complex with the RAD50 interacting protein RINT-1, possibly blocking RAD50 from binding to MRE11 towards formation of a functional MRN complex postulated to prevent telomerase independent telomere lengthening in normal cells [117]. A recent report [118] provides evidence possibly explaining how ALT is upregulated in Human Papiloma Virus (HPV) induced cervical cancer. The mechanism involves E7 viral protein, which degrades p130 and by this way ALT TMM is used to prolong telomeres. This observation renders p130 a potential suppressor of ALT pathway, paving the way of using p130 in gene therapy approaches against cervical cancers. ALT is characterized by a number of phenotypic characteristics (figure 5) that have been observed in tumour cells and certain immortalized cell lines. In ALT cells many characteristics of normal telomere biology have been detected as duplex TTAGGG repeats with single stranded G-tails, shelterin complex together with other telomere associated proteins and the ability of t-loop formation. Besides these features, ALT cells present a number of exceptional characteristics with the most prominent being extrachromosomal telomeric sequences detected in many forms. Double stranded telomeric circles (t-circles) [106,119,120] are mainly detected, while partially single stranded circles (either C- or G- circles) are also abundant [121,122]. Moreover, linear ds-DNA [123,124] and very high molecular weight 't-complex' DNA that is likely to contain abnormal, highly branched structures are also been detected [122]. Another quite common but not universal characteristic of ALT cells is the formation of ALT-associated Promyelocytic Leukaemia (PML) nuclear bodies referred to as APBS [125]. APBS are quite interesting macromolecular structures that are considered to represent locations of ALT activity, as they contain telomeric DNA, associated telomere binding, DNA repair and recombination proteins like MRE11 complex, Mus81 and the SMC5/6 sumoylation pathway, [5,6,106,125-136], despite a number of inconsistencies. Moreover APBs might also function in sequestering of extrachromosomal DNA and are also related to cell cycle

Soft tissue sarcomas	• Chondrosarcoma
	• Undifferentiated pleomorphic sarcomas (including malignant fibrous histiocytoma
	• Leiomyorsarcoma
	• Epithelioid sarcoma
	• Liposarcoma
	• Fibrosarcoma (and variants)
	• Angiosarcoma and neurofibroma
Central Nervous System cancer subtypes	• Grade 2 diffuse astrocytoma
	• Grade 3 anaplastic astrocytoma
	• Grade 4 paediatric glioblastoma multiforme (GBM)
	• Oligodendroglioma
	• Anaplastic medulloblastoma
	• Other embryonal tumours
	• Grade 1 pilocytic astrocytoma, nonaplastic medduloblastoma, mengingioma, schwannoma etc
Urinary bladder subsets	• Small cell carcinoma
	• Invasive urothelial carcinoma
Adrenal gland / peripheral nervous system subtypes	• ganglioneuroblastoma
	• neuroblastoma
	• pheochromocytoma
Neuroendocrine neoplasms	• paraganglioma
Kidney subsets	• Chromophobe carcinoma
	• Sarcomatoid carcinoma
	• Clear cell and papillary carcinoma
Lung and pleural subtypes	• Malignant mesothelioma
	• Large cell carcinoma
	• Small cell carcinoma
Skin	• Malignant melanoma
Liver	• Hepatocellular carcinoma
Testis	• Nonseminomatous germ cell tumour
Breast	• Lobular carcinoma
	• Ductal carcinoma
	• Medullary carcinoma
Uterus	• Serous endometrial carcinoma
	• Squamous carcinoma
Ovary	• Clear cell carcinoma
	• Endometrioid carcinoma
Gall bladder	• adenocarcinoma
Oesophagus	• adenocarcinoma

Table 1. ALT + tumour types listed in descending order of prevalence [94].

arrest and senescence. Cesare & Reddel propose a model consistent with more than one type of APBs, depending on the cell cycle stage and the telomere status. According to this hypothesis and in conjunction to the published experimental data there might be two major classes of APBs: large APBs that contain compacted chromatin and accumulated under conditions of cell cycle arrest, including senescence and others that are the sites of ALT activity [106]. As APBs seem to be dynamic structures interacting with PML bodies, chromatin and DNA repair machinery (and also have been detected in many cell cycle stages), it is likely that APBs consist of a core basic domain and interact with the above referred components depending on / sensing

telomere dysfunction status. In the latter case DDR may be elicited and lead cell to senescence. Of course, more experimental approaches are required in order to elucidate APBs' puzzle. Nevertheless, APBs formation, although common, does not appear to be a universal characteristic of the ALT pathway or a prerequisite for ALT activity.

Among ALT features t-circles seem to be involved in both ALT and physiological telomere biology [136]. t-circles could be the by-product of telomere-loop junctions (t-loop) resolution performed by recombination enzymes. This process could result in free t-circles and truncated telomeres [120], although in ALT cells t-circles are detected in significantly higher numbers than normal cells [119,120]. This reaction is dependent on the recombination factors Nijmegen breakage syndrome 1 (NBS1) and X-ray repair cross-complementing 3 (XRCC3) in human cells, while it is suppressed by the basic domain of TRF2 [120,133,137]. t-circles, although found to be more abundant in ALT cells compared to non-ALT cells [119,120], are also detected in telomerase – positive human cell lines with artificially elongated telomeres due to increased expression of telomerase components [138]. Experimental data suggest that human cells have a 'telomere trimming' mechanism that shortens telomeres through telomere-loop junction resolution (t-loop junction resolution). Therefore, abundant t-circles detected in ALT cells may represent the by-product of trimming of overlengthened telomeres and not a direct player in the ALT pathway per se.

On the other hand C-circles (telomeric circles consisting of an essentially complete C-rich strand and an incomplete G-rich strand) [121] seem to be involved in a more direct way with ALT mechanism. A quantitative relationship between the amount of ALT activity and the number of partially ds telomeric C-circles was observed [121], with an estimation of approximately 1,000 C-circles present per ALT cell. C-circles are possibly generated by nucleolytic degradation of the G-rich strand of t-circles, a hypothesis requiring further investigation. G-circles are also detected in ALT cells but reduced by 100-fold. Another result supportive of C-circles being characteristic of ALT cells is their detection in cell lines maintaining telomere length in the absence of telomerase without bearing any other ALT features [121]. Supportive to that is the observation that in immortalized cultured cells onset of ALT activity was temporary correlated with the appearance of C-circles. In accordance, ALT inhibition was accompanied by C-circles disappearance within 24 hours [121]. Taken together, the above reported data together with the fact that C-circles are also detected in blood samples from patients with ALT-positive osteosarcomas, it may be concluded that assaying C-circles may represent one of the most reliable marker of ALT activity. This notion is under validation for use at patient diagnosis level.

Epigenetic changes may also interfere with telomere biology and turn the balance towards the TMM selection. Concomitant with this hypothesis is the increasing evidence that depletion of chromatin remodelling complex ATRX/DAXX has been directly correlated with ALT phenotype, presumably repressing ALT under normal conditions [94,95]. Screening for ATRX/DAXX and the related histone variant H3.3 may therefore represent part of the signature of tumours replenishing their telomeres by homologous recombination pathways. ATRX/DAXX manipulation experiments suggest that their expression deficiency and the concomitant lack of H3.3 deposition into telomeric chromatin, is not sufficient to launch TMM choice in favour of ALT
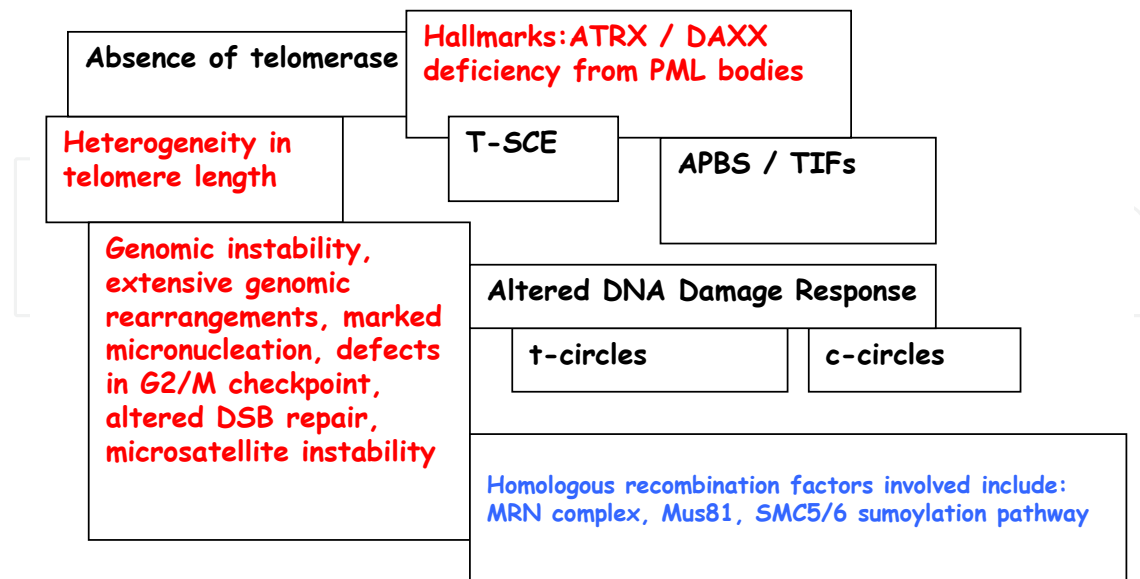
ALT features

Figure 5. Basic ALT features. Telomere length heterogeneity, ARTX/DAXX lack of expression and extensive genomic instability seem to be universal characteristics, whereas APBs and c-circles have not been found in some telomerase negative cases.

pathway, pointing to the need to identify additional co-operating (epi-)genetic changes. ALT cells exhibit a high degree of ongoing genomic instability, including frequent micronuclei, high basal levels of DNA damage foci, and elevated checkpoint signalling in absence of exogenous damage, implying that ATRX/DAXX may interfere with repressing genes involved in telomere recombination, a hypothesis requiring further clarification [95]. Extensive genome instability, accompanied by G2/M checkpoint deficiencies detected in many ALT+ cells may explain how these cells keep on proliferating overcoming DNA damage events. Based on these findings, G2/M checkpoint inhibitors are currently developed and evaluated in clinical trials under the concept of enhancing the efficacy of clastogenic therapies [139].

Taken together, current hypotheses support a model where multiple steps, including loss of ATRX/DAXX function together with defects in the G2/M checkpoint in a high level of spontaneous DNA damage environment, are required for ALT-mediated immortalization. Thus, ALT tumours may present unique vulnerabilities [95] offering the potential for development of selective targeting agents towards personalized treatment schemes. A promising example might be targeting topoisomerase (Topo) IIIα, which associates with BLM helicase, an important player allowing telomere recombination in the absence of telomerase. Repression of Topo IIIα resulted in reduced ALT cells survival, decreased levels of TRF2 and BLM proteins, significant increase in the formation of anaphase bridges, degradation of the G-tail signal and TIF formation while telomerase expressing cells were unaffected [140]. Quite strikingly, Telomestatin, a natural compound functioning as a G-quadruplex ligand, impairs Topo IIIα binding to telomeres. Consequently, the Topo III/BLM/TRF2 complex is depleted from telomeres, APBs are disrupted and uncapped telomeres seem to trigger DDR [141].

In accordance to the multi-step process assumed to be required for activation of ALT TMM, major defects in DNA repair were observed to occur between preneoplasia and breast cancer, as monitored by ATM activation and subsequent significant repression, respectively [142]. Such defects are associated with changes in telomere length between the preneoplastic and the cancer stage.

8. ALT-mediated telomere elongation

Cumulative evidence supports a telomerase-independent, recombination-dependent, telomere length maintenance mechanism (TMM) [110]. Such an ALT process has been found to depend on the function of the homologous recombination gene RAD52 in telomerase-null mutant yeast [143], followed by numerous studies reporting detection of ALT pathways in human cell lines [144-146]. Further evidence established the existence of ALT mechanism as a telomerase maintenance process involving recombination events between non-sister telomeres or extrachromosomal sequences [119-121]. Such TMM activities may also explain the high heterogeneity of telomere length found in ALT cells in contrast to telomerase re-expressing cells. Telomere sister chromatid exchanges (T-SCEs) were also detected in much higher frequencies in ALT cells compared to normal or telomerase-expressing cells [148,149]. A model based on this observation attempted to explain TMM by ALT cells. Normally, SCEs may result from recombinational repair of broken replication forks [151] and therefore the detection of nicks and gaps in telomeric DNA [152] may result in T-SCEs. By this way unequal T-SCE may lead to cells with inherited elongated telomeres, resulting in a prolonged proliferative capacity, while other cells bearing shortened telomeres were characterized by decreased proliferative capacity [153]. Moreover, although there was no increase in SCE frequency detected elsewhere in the genome [148,149], overall recombination activity may be upregulated in ALT cells and not restricted only to telomeres. This could explain the poor outcome of ALT positive cancers as hyper-recombination events might confer to chemoresistance and further genomic instability leading to more aggressive cancer types. Despite data further supporting that ALT mechanism requires DNA recombination processes, the exact mechanism / mechanisms are still under investigation.

A theory consistent with the unequal T-SCE model would be that the same cell would inherit all lengthened telomeres, which would lead to unlimited proliferation of the given cell's descendants, a rather unlikely assumption, despite a few opposing evidences [154]. Such a hypothesis would require a specific telomere length based segregation mechanism, a theory necessitating further exploitation. Such an example is the case of copying of a DNA tag of a single telomere to other chromosome ends only in ALT-positive and not in telomerase-positive cells [147]. Therefore, ALT cells may use the unequal T-SCE model and the homologous recombination (HR) - dependent replication model. It is possible that the two suggested mechanisms are not mutually exclusive.

On the other hand, the HR – dependent telomere replication model, based on the hypothesis that recombination – mediated synthesis of new telomeric DNA occurs using an existing

telomere sequence, is supported by more evidence. In this model telomeres from adjacent chromosomes could serve as templates [147,155], resulting in a net increase in telomeric DNA. In support of the view that ALT TMM functions through homology-directed recombination, an elevated frequency of sequence exchanges between telomeres has been observed in ALT cells [122, 147-149]. Furthermore, ALT cells contain extrachromosomal linear and circular telomeric DNA [119] and often exhibit heterogeneously-sized telomeres. These features, summarized in figure 5, are consistent with hyperactive HR activities, probably by a Break Induced Replication (BIR) – like mechanism [155,156]. In normal cells entering telomere crisis, cellular senescence and apoptosis, in a functional p53 or Rb pathway dependent processes, will occur. Most of the ALT cell lines and tumours lack normal p53 and Rb tumour suppressor functions and they are therefore tolerating persistent DSBs [157-159]. Many DNA repair proteins involved in HR are particularly active in ALT, like Rad52 and MRN complex. Especially, the MRN complex has been found to be necessary for ALT mediated telomere elongation [106,127]. This makes sense as MRN facilitates 5' to 3' resection of the DNA ends to create 3' overhangs for strand invasion, a prerequisite for HR [160]. In ALT cells MRN has been detected in APBs, which in turn recruits BRCA1. As previously mentioned, MRN is also necessary for ATM phoshorylation of TRF1 and its dissociation from telomeres regardless which TMM pathway is active. Therefore, MRN functions in order to facilitate HR events at shortened telomeres [83]. MRN does not seem to be absolutely vital for ALT TMM, as it's depletion did not result in unstable telomeres, implying the existence of related redundant pathways [127,161]. On the other hand, the formation of ALT characteristic c-circles depends on active recombination proteins like XRCC3, NBS1 and Ku70/80 [162], implying that t-circle formation requires NHEJ activity in ALT cells. In the context of HR, BLM RecQ helicase, an ATPase-driven helicase possessing 3'-5' unwinding activity, Holliday junction branch migration and ssDNA annealing function, is particularly active in ALT and may have a crucial involvement in ALT-TMM. Along comes the WRN helicase, possessing exonuclease activity and interacting with DNA-PKcs, RPA, MRN and Rad51 in response to DSBs. WRN has been detected in APBs together with TRF1 and TRF2 in S-phase, presumably resolving T-loops in order to facilitate telomere elongation. Depletion of HR components like Rad51D, MUS81, BLM or FANCA/D2 in ALT+ cells results in extremely shortened telomeres and reduced cell survival [105,106,135,163,164]. These results strongly suggest that HR is a major mechanism of TMM in ALT cells and targeting specific HR components may drive to specific and effective anti-ALT therapies.

As previously mentioned, telomere dysfunction and the resulting genomic instability comprise a fertile environment for carcinogenesis. Most of the cancer types manage to restore telomere length by upregulating telomerase and based on that observation an anti-telomerase oligonucleotide- based therapy (Imetelstat) showed promising results in CLL, MM, breast cancer and NSCLC patients in the context of Phase I clinical trials. Recently, a more advanced vaccine designed to raise immunity against a 16mer peptide from the active sites of human TERT has already entered Phase I & II clinical trials in cases of NSCLC (Non-Small Cell Lung Carcinoma), hepatocellular carcinoma and non-resectable pancreativ carcinoma. Moreover, there is an ongoing randomized Phase III clinical trial in patients with locally advanced or metastatic pancreatic cancer (ClinicalTrials.gov Identifier: NCT00425360) [165-166]. Never-
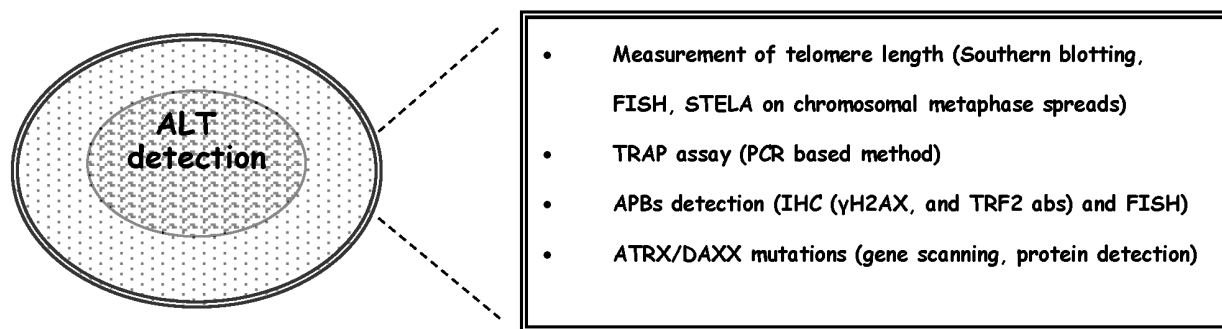
Figure 6. ALT detections approaches based on ALT features and biomarkers.

theless, anti-telomerase therapies are obviously of no value in telomerase non-expressing tumours, not to mention potential toxicity due to off-target effects. Furthermore, anti-telomerase treatment can always drive to selection for resistant cells that may activate an ALT mechanism [167]. These data render ALT an attractive target for anti-tumour therapies based on a personalized treatment approach. Recent reports support this notion, as repression of ALT in ALT-dependent immortal cell lines resulted in selective senescence and cell death [106], while ALT inhibition by siRNA-targeting of ALT components appear to result in more rapid telomere dysfunction [105,106,135,163,164] increasing therapeutic efficacy. Quite fascinatingly, preliminary results from the use of Telomestatin (a macrocyclic compound binding to G4-quadruplexes) exhibited effective elimination of both telomerase-expressing and ALT+ cell lines [141]. Of course there is a scepticism raised here as except of telomeres a significant portion of gene promoters also tend to adopt a G-quadruplex structure [168]. In addition, the puzzle becomes more complicated as transition of TMM pathway from telomerase upregulation to ALT and vice versa has been observed, especially in cases of secondary tumours and cases where both TMM pathways appear to co-exist, although not necessarily in the same tumour cell [169-172].

In conclusion, more extensive analysis of the detailed molecular mechanisms underlying TMM pathways and the structure-function relationship of the components involved is a prerequisite towards individualized treatment schemes with higher efficacy and lower toxicity. Unravelling of the detailed mechanisms incorporated in order to restore a minimum telomere length along with elucidation of the escape pathways that ALT+ cells are thought to use may ultimately lead to design of specific ALT-component directed compounds conferring high selectivity in targeting tumour against normal surrounding tissue cells.

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