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# Epigenetics of Human Telomeres

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Human telomeric heterochromatin is unusual in that it does not show the enrichment of canonical repressive histone marks H3K9me3 or H4K20me3 seen in constitutive heterochromatin. Instead, human telomeres exhibit both facultative heterochromatin and euchromatin marks, consistent with their epigenetically regulated transcription into TERRA noncoding RNA. Additionally, telomeric DNA is out of phase with the DNA helical repeat and has no nucleosome positioning signal. Yet, human telomeric DNA forms a columnar structure of tightly stacked nucleosomes, alternating with open states, and regulated by histone tails and shelterin protein binding. We discuss the proposed mechanisms regulating human telomeric chromatin and the consequences that telomeric chromatin properties have on various cellular processes, such as telomere transcription, the regulation of shelterin binding, and the activation of the alternative lengthening of telomeres mechanism. Together, we summarize current evidence on the combination of hetero- and euchromatic properties of human telomeres that may help explain their crucial protective functions and plasticity to regulate telomere maintenance pathways and damage signaling.

In 1942, Conrad Waddington first defined epigenetics as the study of "the mechanisms by which the genotype produces the phenotype in the context of development" (Waddington 1942; Cavalli and Heard 2019). Since then, epigenetics has been repeatedly redefined to account not only for transgenerational inheritance but also for mitotic inheritance of chromatin state (Cavalli and Heard 2019). Bird (2007) defines epigenetics as the study of "structural adaptations of chromosomal regions so as to register, signal or perpetuate altered activity states" (Cavalli and Heard 2019), and this definition probably better describes the epigenetic regulation of telomeres. The main carriers of epigenetic information include posttranslational modifications of histone tails and readers of these marks, such as heterochromatin protein 1 (HP1), Polycomb (PRC1 and PRC2) and Trithorax (COMPASS) complexes, noncoding RNAs, and DNA methylation (Cavalli and Heard 2019).

Histone-modifying complexes constantly remodel nucleosomes, the basic units of chromatin consisting of DNA wrapped around octamers of histone proteins made of H2A, H2B, H3, and H4 (Cavalli and Heard 2019). Posttranslational modifications of histones, occurring primarily on lysine residues located on their amino-terminal tails, influence local chromatin structure and mark regions of either euchromatin or facultative or constitutive heterochroma-

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tin (Grewal 2023). Histone marks, such as H3K9me3, and DNA methylation are also highly interrelated and rely on each other for the formation of constitutive heterochromatin (Rose and Klose 2014). One of the main functions of constitutive heterochromatin at highly repeated DNA sequences is to suppress recombination and, thereby, promote genomic stability through chromatin compaction. Constitutive heterochromatin regions, such as pericentromeres, are characterized by a high density of H3K9me3 and H4K20me3 histone marks, and compaction is classically achieved through the oligomerization of H3K9me3-bound HP1α and β isoforms (HP1α/β) (Canzio et al. 2011). According to this definition, mouse telomeres and subtelomeres are classified as constitutive heterochromatin domains (García-Cao et al. 2004; Blasco 2007). However, in striking contrast to their mouse counterparts, although present, H3K9me3 and H4K20me3 marks do not show enrichment at human telomeres, as they do in pericentromeric regions of the human genome (Rosenfeld et al. 2009; O'Sullivan et al. 2010; Arnoult et al. 2012; Cubiles et al. 2018; Udroiu and Sgura 2020). Whether human telomeres can be classified as canonical constitutive heterochromatin regions thus stands as an open question.

Pioneering studies have revealed that telomeric DNA forms nucleosomes with an estimated repeat length of ~157 bp in rat liver nuclei, a value much lower than the 197 bp average repeat of bulk rat liver chromatin (Makarov et al. 1993). This unusually short nucleosome repeat length appears to be a general feature that also applies to human telomeric chromatin (Makarov et al. 1993; Tommerup et al. 1994; Fajkus and Trifonov 2001; Mechelli et al. 2004; Episkopou et al. 2014). Together with the fact that telomeric DNA does not carry any nucleosome positioning signal, this led Fajkus and Trifonov (2001) to propose a columnar packing model of telomeric nucleosomes, with continuous winding of the DNA around the stacked histone cores. This model was recently confirmed by cryogenic electron microscopy (cryo-EM) (Soman et al. 2022a), suggesting that telomeric chromatin compaction occurs through mechanisms distinct from the canonical mechanisms of constitutive heterochromatin formation.

Here, we will review current knowledge on the chromatin characteristics of human telomeres. We will then address the mechanisms regulating human telomeric chromatin, such as the binding of shelterin proteins, the role of histone tails, the noncoding RNA TERRA, and the interaction of telomeres with the nuclear lamina. Finally, we will delve deeper into the—sometimes interdependent—impacts of telomeric chromatin on various aspects of telomere biology, focusing on shelterin protein binding, telomere transcription, the so-called telomere position effect (TPE), the alternative lengthening of telomeres (ALT) mechanism, telomere movement, and the synthesis of telomeric DNA.

## HUMAN TELOMERES DISPLAY CHARACTERISTICS OF MULTIPLE CHROMATIN TYPES

Classically, heterochromatin is classified as either constitutive or facultative. Mouse telomeres, with enrichment in H3K9me3 and H4K20me3 marks, display the classical features of loci showing constitutive heterochromatin (García-Cao et al. 2004; Blasco 2007). Although H3K9me3 and H4K20me3 marks are present at human telomeres, they are not enriched as they are in constitutive heterochromatin regions such as pericentromeres (Rosenfeld et al. 2009; O'Sullivan et al. 2010; Arnoult et al. 2012; Cubiles et al. 2018; Udroiu and Sgura 2020). The reason why mouse and human telomeres show differences in their enrichment in constitutive heterochromatin marks could be related to the distinct length of their telomeres, as telomeres of laboratory mice are 5–10 times longer than human telomeres. Supporting this hypothesis, on the one hand, the density of H3K9me3 and H4K20me3 marks was decreased at the shortened telomeres of telomerase-deficient mice (Benetti et al. 2007) and, on the other hand, the experimental lengthening of human telomeres, via ectopic overexpression of telomerase, increased H3K9me3 telomeric density (Arnoult et al. 2012). The mechanisms underlying the increased density of H3K9me3 at elongated telomeres are not yet clear, but may

involve various histone methyltransferases and, possibly, the chromatin remodeler ATRX. In support of this, long human telomeres exhibit an increased density of G-quadruplex (G4) structures (Yang et al. 2021), which were proposed to promote ATRX-regulated deposition of H3K9me3 (Teng et al. 2021). It has also been proposed that the epigenetic characteristics of telomeres are not spatially homogenous and that the proximal and distal parts of the telomeres might exhibit distinct chromatin properties (Udroiu and Sgura 2020). In this case, it is possible that the observed differences between human and mouse telomeres result from a shift toward the chromatin characteristics of the more distal telomeric repeats in cells with very long telomeres.

Facultative heterochromatin, originally attributed to developmentally regulated heterochromatinization, identifies genomic regions that can adopt open or compact conformations, being transcriptionally silent with the potential to transition to a euchromatic state allowing transcription (Trojer and Reinberg 2007). Known human genomic regions of facultative heterochromatin include the inactive X chromosome and autosomal imprinted genomic loci that typically display a panel of histone marks, including hypoacetylation, H3K27me3, H3K9me2, and H4K20me1 (Trojer and Reinberg 2007). Strikingly, human telomeres display strong enrichment of H4K20me1 (Cubiles et al. 2018) compared to pericentromeres and the presence of H3K27me3 and H3K9me2 marks, as well as low abundance of the H3K9ac mark (Arnoult et al. 2012; Porro et al. 2014; Cubiles et al. 2018). Facultative heterochromatin is further characterized by the presence of HP1γ isoform, which was also detected at human telomeres (Déjardin and Kingston 2009; Canudas et al. 2011; Arnoult et al. 2012), thus highlighting the fact that human telomeres exhibit some properties of facultative heterochromatin.

In addition to facultative heterochromatin marks, the H3K4me3 mark, associated with active transcription, was detected at human telomeric chromatin (Rosenfeld et al. 2009; Cubiles et al. 2018). This histone modification, when co-occurring with H3K27me3, marks the transcriptional start site region of CpG-rich bivalent promoters that set the genes in a poised state during the initial stages of organismal development (Voigt et al. 2013). Since a large fraction of human chromosome ends is characterized by CpG-rich subtelomeric promoters, located directly upstream of the *TTAGGG* repeats (Nergadze et al. 2009) and regulating the expression of the telomeric repeat-containing RNA, TERRA (Fig. 1A; Azzalin et al. 2007), telomeres may resemble bivalent genes. Interestingly, non-*CG* DNA methylation, previously linked to genomic imprinting and cellular reprogramming (Lister et al. 2013), was also detected at telomeres of human embryonic stem cells (Lister et al. 2011).

An overview of the telomeric chromatin marks mentioned above is given in Figure 1B. In summary, although we have not given an exhaustive list of histone marks detected at human telomeres, it appears that the picture of human telomeric chromatin is still rather unclear, and likely distinct from mouse telomeric chromatin.

## COLUMNAR PACKING OF HUMAN TELOMERIC NUCLEOSOMES AND THE ALTERNATIVE OPEN STATE

Despite the lack of H3K9me3 enrichment at human telomeres and the fact that, with its 6 bp *TTAGGG* repeats, telomeric DNA is out of phase with the 10.4 bp DNA helical repeat and lacks any nucleosome positioning signal, human telomeric nucleosomes are organized into regular chromatin structures. The crystal structure of a telomeric nucleosome core particle (NCP) reconstituted with recombinant human histone octamers revealed that its overall structure is similar to previously published NCP structures (Soman et al. 2020). In solution, however, the telomeric NCP exhibited increased dynamics and decreased stability compared to canonical nucleosomes (Soman et al. 2020). Going further, the Nordenskiöld group then characterized 3-kb-long telomeric chromatin fibers using negative stain electron microscopy and single-molecule magnetic tweezers and obtained the cryo-EM structure of the condensed telomeric tetranucleosome and its dinucleosome unit (Soman et al.

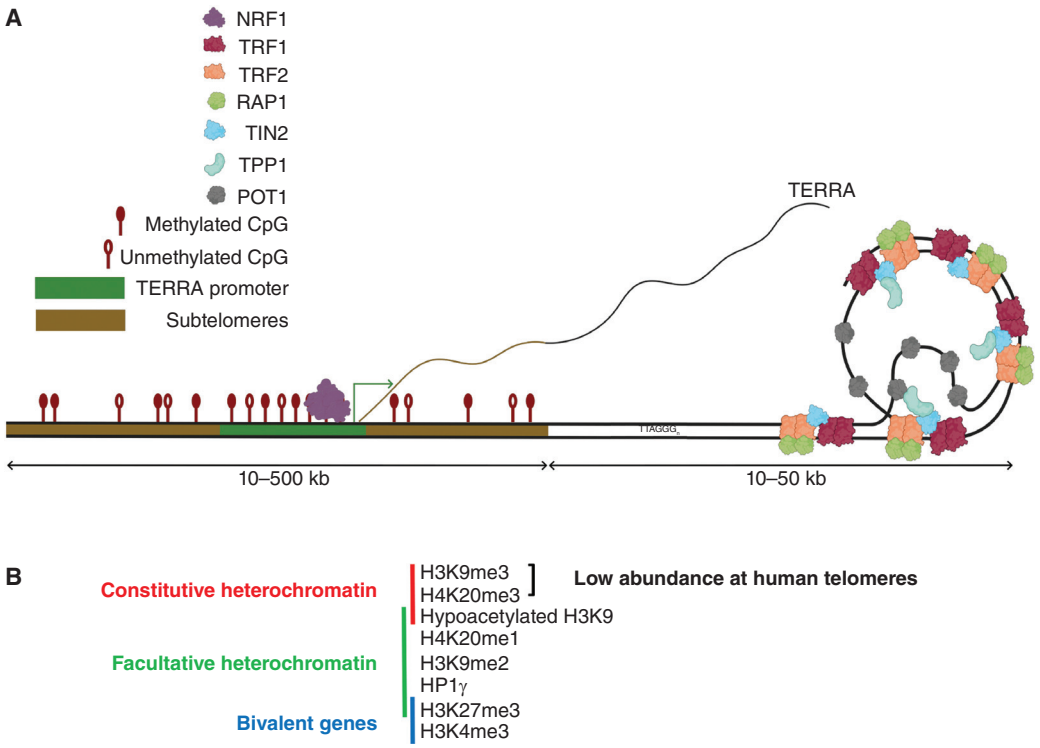


**Figure 1.** General organization of human chromosome ends. (*A*) Human subtelomeres, located directly upstream of telomeric repeats, are highly heterochromatic and exhibit CpG methylation. Subtelomeric promoters have been identified as drivers of the expression of TERRA noncoding RNA. A small subset of activators/repressors of TERRA transcription is shown. The ends of chromosomes fold into a T-loop structure stabilized by a complex of six telomere-specific proteins called shelterin (de Lange 2005). (TSS) Transcription start site, (DNMTs) DNA methyltransferases. (*B*) Selected chromatin marks detected at human telomeres (see text for details).

2022a). Consistent with the initial hypothesis of Fajkus and Trifonov (2001), the structure they obtained displayed close stacking of nucleosomes with a columnar arrangement, which was found to be primarily stabilized by the carboxy-terminal tail of H2A and the amino-terminal tails of other histones (Fig. 2A,B; Soman et al. 2022a). This work revealed that human telomere compaction occurs through mechanisms similar to archaeal histones (Mattiroli et al. 2017).

Further elucidation of the telomeric nucleosome structure revealed that an alternative open state coexists with the tightly stacked columnar telomeric fibers (Fig. 2A; Soman et al. 2022a). The alternative open state has been proposed to occur in the absence of heterochromatin decompaction and to result from unstacking and flipping out of one nucleosome to expose the H2A-H2B acidic patches (Soman et al. 2022a). This biphasic model of telomeric nucleosome structure provides an explanation for the access of factors required for telomere maintenance and the necessary DNA damage response (DDR) at telomeres, which is known to happen in the absence of chromatin decompaction (Timashev et al. 2017; Vancevska et al. 2017; Soman et al. 2022a). Several epigenetic modifications previously observed at telomeres, such as acetylation or methylation of lysine residues on the amino-terminal tails of H3 or H4, were predicted to be located at the interface between two stacked nucleosomes and to get exposed in the open state, indicating that these modifications may have an important structural role (Soman et al. 2022a).

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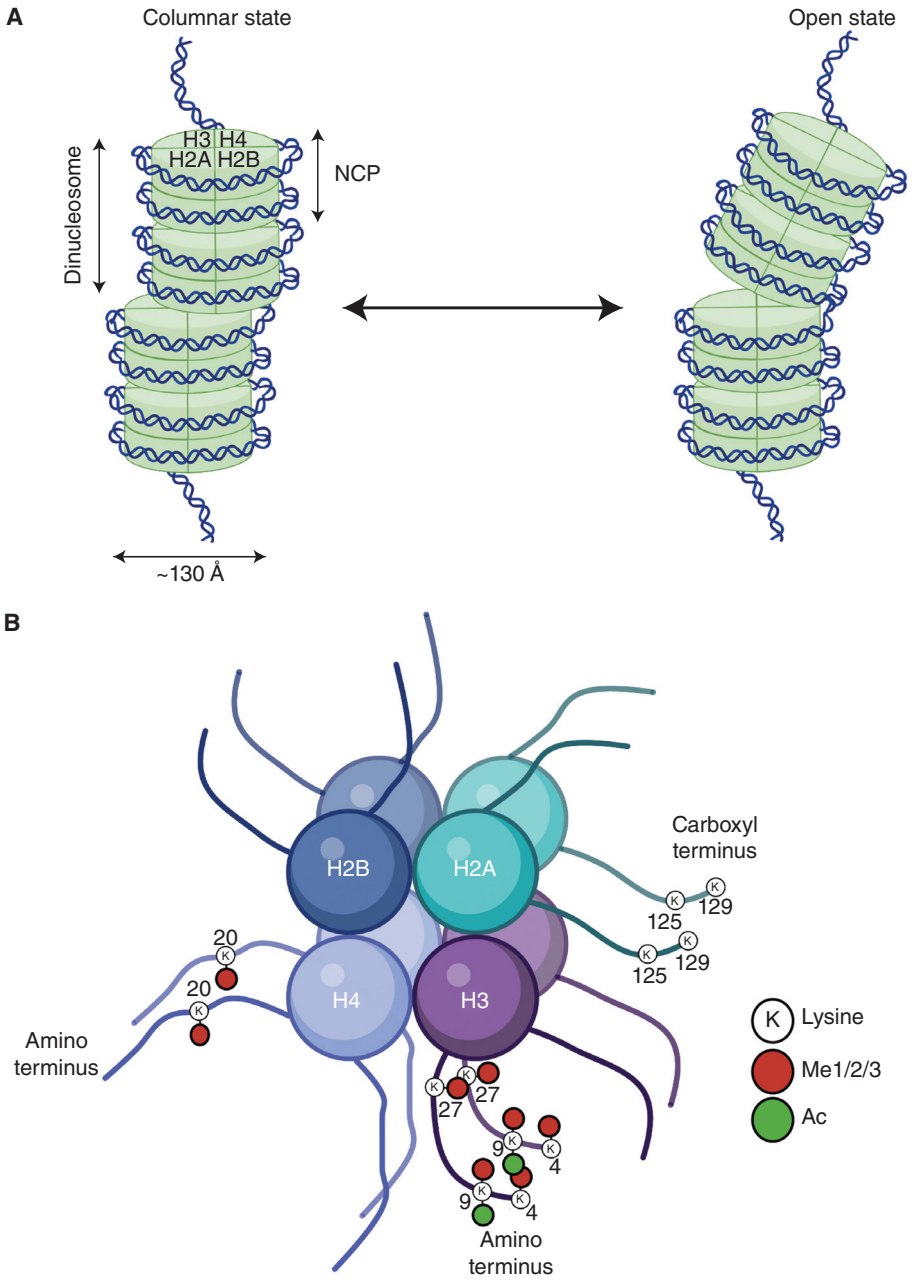


**Figure 2.** Columnar structure and alternative open state of human telomeric chromatin. (*A*) (*Left*) Structure of the human telomeric tetranucleosome. At telomeres, nucleosome core particles (NCPs) display close stacking with a columnar arrangement and comprise ~132 bp of DNA wrapped around the histone octamer, composed of two copies each of H2A, H2B, H3, and H4. (*Right*) The telomeric chromatin structure also exists in an alternative open state where one nucleosome is flipped out. (*B*) Schematic representation of the histone octamer. The carboxy-terminal tails of H2A, which comprise two positively charged lysine residues, K125 and K129, likely play an important role in mediating and stabilizing the columnar structure (Soman et al. 2022a). The posttranslational modifications on H3 and H4 amino-terminal tails that are mentioned in this review are highlighted. (Figure based on data from Soman et al. 2022a,b.)

Taken together, although obtained in the absence of shelterin proteins, this first cryo-EM structure of human telomeric chromatin supports the hypothesis of a distinct mode of chromatin compaction at telomeres compared to canonical constitutive heterochromatic loci and suggests that telomeres have a unique columnar/open conformation that likely ensures both protection and the highly specialized and dynamic functions of telomeres.

## REGULATION OF HUMAN TELOMERIC CHROMATIN

### By the Shelterin Complex

The interaction of telomeric DNA with the telomere-binding proteins TRF1 and TRF2 has long been recognized as an important regulator of chromatin compaction (Cacchione et al. 1997; Rossetti et al. 1998; de Lange 2005; Pisano et al. 2007, 2008; Ichikawa et al. 2014). However, although they share two important domains, the TRF homology (TRFH) dimerization domain and the Myb DNA-binding domain (Broccoli et al. 1997; Fairall et al. 2001), TRF1 and TRF2 have distinct effects on telomeric chromatin. The carboxy-terminal Myb DNA-binding domain of TRF2, by reducing the negative surface charge density of nucleosomal array fibers, mediates telomeric chromatin compaction (Baker et al. 2009), while its amino-terminal domain negatively regulates compaction (Poulet et al. 2012). Cumulatively, TRF2 functions to compact telomeric chromatin. Conversely, the acidic domain of TRF1 inhibits its ability to condense DNA (Poulet et al. 2012). The affinity of the TRF1-Myb domain for telomeric DNA is also higher than that of the TRF2-Myb domain (Hanaoka et al. 2005).

Recently, cryo-EM experiments confirmed distinct roles of TRF1 and TRF2 in telomeric chromatin compaction. Consistent with the compactor role of TRF2, the protein, whether full-length or lacking its amino-terminal part ($TRF2^{\Delta N}$), was found to induce and stabilize columnar fibers (Wong et al. 2024). The binding of TRF2/$TRF2^{\Delta N}$ to supergrooves on the surface of the columnar form of telomeric chromatin occurred without eviction of histone proteins and increased fiber width, while reducing internucleosomal distance (Fig. 3A; Wong et al. 2024).

The impact of TRF1 binding on telomeric nucleosomes was reevaluated on a cryo-EM structure determined to 2.5 Å resolution using a reconstituted human telomeric NCP with a 145 bp telomeric DNA (Hu et al. 2023). The structural analysis suggested that TRF1 binds at the junction between the nucleosome and linker DNA (Fig. 3A; Hu et al. 2023), inducing a register shift of the nucleosomal DNA by 1 bp. This confirmed the previous findings that TRF1 recognizes telomeric repeats in a nucleosomal context and alters nucleosomal structure (Galati et al. 2006; Pisano et al. 2010), binding preferentially to the end of nucleosome (Galati et al. 2006) or to the linker DNA (Galati et al. 2015). The model suggests that the binding mode of TRF1 likely disfavors the binding of H1 to the linker DNA (Hu et al. 2023), and explains the underrepresentation of histone H1 at telomeres (Déjardin and Kingston 2009). The model, however, is not compatible with the columnar structure of human telomeres, in which the linker DNA is not accessible to protein binding (Soman et al. 2022a). To allow TRF1 binding, Hu et al. (2023) therefore proposed that the columnar structure would need to be disrupted. This hypothesis is consistent with the observation that TRF1 alters telomeric nucleosome spacing in vitro (Galati et al. 2015). The cryo-EM data further suggested that the interaction between the phosphorylated carboxy-terminal part of the TRF1-Myb domain and the amino-terminal tail of histone H3 plays a crucial role in stabilizing the complex (Hu et al. 2023). The phosphorylatable serine 435 residue of TRF1, which is involved in the interaction with histone H3, is highly conserved in mammals but absent in TRF2. This suggests that TRF2 binding may occur without the disruption of the columnar structure, nearby outer nucleosomal DNA gyres (Fig. 3A; Hu et al. 2023). Despite its inability to condense telomeric chromatin, it is, however, possible that, through its interaction with TIN2, which links TRF1 and TRF2, TRF1 nevertheless facilitates the compact state of telomeres by promoting intramolecular bonds at telomeres (Fig. 3B; Soman et al. 2022b).

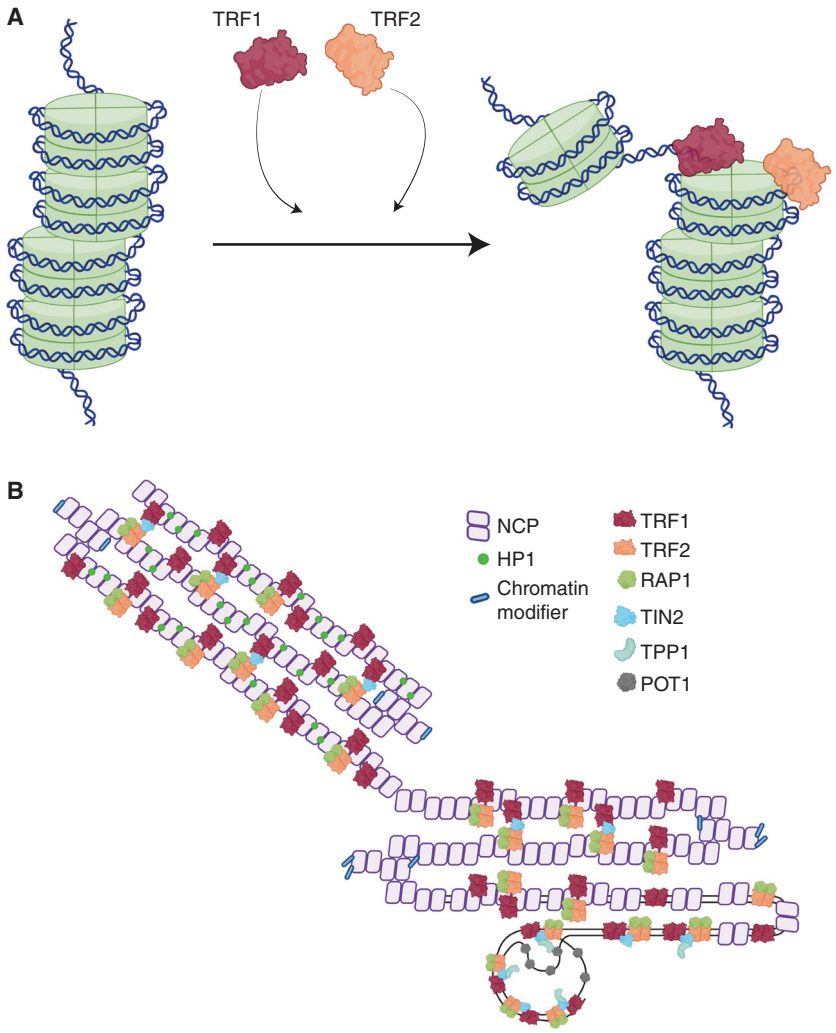


**Figure 3.** A model for the impact of the TRF1 and TRF2 shelterin proteins on human telomeric chromatin. (*A*) The binding of TRF2 on the surface of the telomeric columnar structure occurs without eviction of histones, resulting in no disruption of the packed state of telomeric chromatin. TRF1 binding, likely at the junction between the nucleosome and the linker DNA, instead, leads to a shift in the register of the nucleosomal DNA. The columnar structure of human telomeres needs to be disrupted to allow TRF1 binding. (Panel based on data from Hu et al. 2023.) (*B*) To account for the observed diameter of telomeric fibers of up to 35 nm, Soman et al. (2022b) proposed that TRF1, via its interaction with the shelterin protein TIN2, which can bridge TRF1 and TRF2, could contribute to higher-order formation of telomeric chromatin by promoting intramolecular bonds. (Panel based on data from Soman et al. 2022b.)

Finally, another study revealed that the role of TRF2 in establishing telomeric loops and protecting telomeres from ATM checkpoint activation depends on its ability to modify the topology of DNA by wrapping 90 bp of telomeric DNA around its TRFH domain (Benarroch-Popivker et al. 2016), suggesting a histone-like function for TRF2, possibly at the very ends of telomeres. Future investigations will be required to fully understand how TRF1, TRF2, and the other shelterin proteins regulate telomeric nucleosomal organization.

### By Amino- and Carboxy-Terminal Histone Tails

The carboxy-terminal tails of histone H2A have been proposed to stabilize the columnar structure of human telomeres (Soman et al. 2022a). Histone H2A, however, is not always present in nucleosomes because, upon activation of the DDR, it is replaced by the histone variant H2AX, which lacks the positively charged residues Lys125 and Lys129 of H2A and is phosphorylated on Ser139 by various DDR kinases (Fig. 4A). DDR activation could thus induce an overall shift from a positively charged H2A C-tail to a negatively charged γ-H2AX C-tail, which is expected to destabilize the telomeric chromatin structure (Fig. 4B; Soman et al. 2022a,b). From this point of view, DDR kinases, such as ATM or ATR, could thus alter the structure of telomeres.

In addition to the carboxy-terminal tail of H2A, the cryo-EM structure of the human telomeric NCP revealed that several posttranslational modifications on the amino-terminal histone tails, predicted to be located at the interface between two stacked nucleosomes, would likely modulate the open state and telomeric chromatin dynamics (Soman et al. 2022a). Several histone-modifying enzymes could therefore affect the structure of human telomeric chromatin, such as MLL1, the methyltransferase from the COMPASS complex responsible for H3K4me3

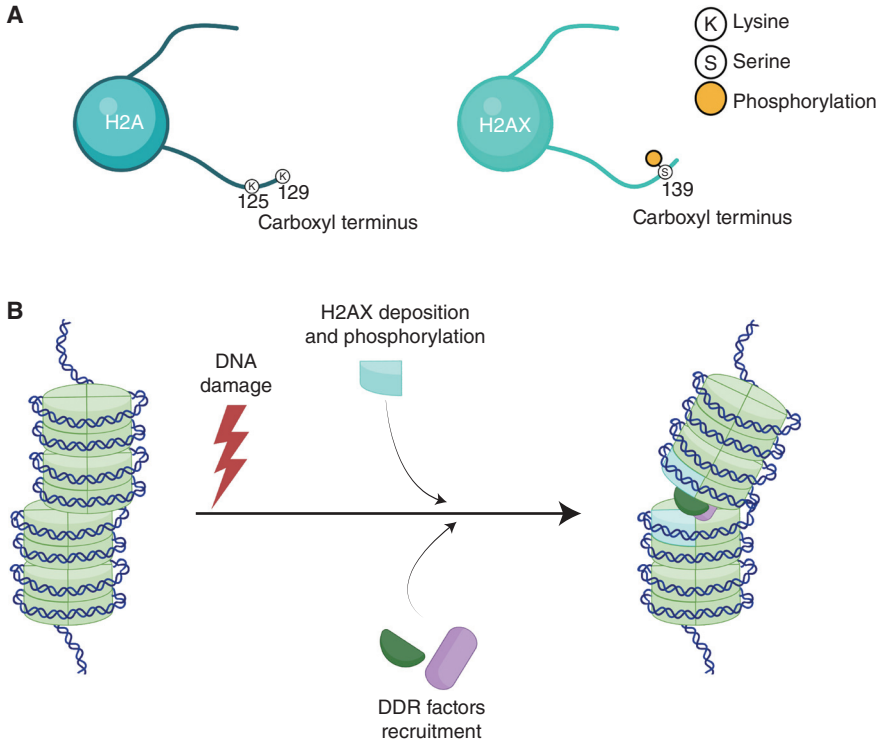


Figure 4. A model for the impact of DNA damage response (DDR) activation on the open state of human telomeric chromatin. (*A*) The carboxy-terminal tails of H2A and the histone variant H2AX are distinct and Lys125 and Lys129 residues are missing from the H2AX carboxy-terminal tail. (*B*) Activation of the DDR at telomeres can modulate the open state without massive decompaction of chromatin structure. Upon DNA damage, histone H2A is replaced by the H2AX variant and the latter is phosphorylated by DDR kinases, such as ATM and ATR, on Ser139 residue. This could favor the open state and allow the recruitment of additional DDR factors, which would ultimately lead to the repair of the damage. (Panel *B* based on data from Soman et al. 2022b.)

(Caslini et al. 2009). Structural analysis demonstrated that the open state would be compatible with the recruitment of the SIRT6 $NAD^+$-dependent deacetylase of H3K9 to telomeres (Michishita et al. 2008; Soman et al. 2022a), suggesting that hypoacetylation of H3K9, by altering the charge of the amino-terminal tail of histone H3, may also regulate the human telomeric chromatin structure.

As discussed later, it is important to emphasize that some posttranslational modifications of amino-terminal histone tails may also indirectly impact telomeric chromatin properties through their impact on the expression of the TERRA telomeric long noncoding RNA.

### By TERRA Telomeric Noncoding RNA

The discovery that Xist noncoding RNA, through its recruitment to chromatin, silences an entire chromosome to equalize X chromosome expression in males and females propelled chromatin-associated RNAs to the rank of chromatin regulators (Johnson and Straight 2017). At telomeres, the RNA TERRA is also involved in chromatin regulation (Bettin et al. 2019). The first evidence was provided by the demonstration that TERRA, through its interaction with TRF2, facilitates the establishment of H3K9me3 and H3K9me2 marks and the recruitment of HP1 at human telomeres (Deng et al. 2009). Subsequently, correlative approaches unveiled that, in cells with highly elongated telomeres, telomeric establishment of H3K9me3 and recruitment of HP1α follow the same timing as TERRA expression throughout the cell cycle (Arnoult et al. 2012). An additional role of TERRA in shaping telomeric chromatin was provided by the identification of chromatin remodeling factors, such as ORC1 (origin of replication complex 1), NoRC (nuclear remodeling complex), and MORF4L2 (a component of the NuA histone acetyl transferase complex), among other proteins found to interact with the telomeric RNA (Deng et al. 2009; Postepska-Igielska et al. 2013; Scheibe et al. 2013; Chu et al. 2017a). TERRA has also been suggested to play a role in establishing heterochromatin at human subtelomeres, by influencing not only H3K9me3 density but also subtelomeric DNA methylation (Deng et al. 2010). This, as we will discuss below, has the potential to modulate the transcriptional activity of the human subtelomeric promoters and places TERRA at the heart of a negative feedback mechanism controlling its own transcription.

Taken together, there is strong evidence that the telomeric RNA transcript TERRA modulates the deposition of both telomeric and subtelomeric heterochromatin marks. Whether TERRA is directly involved in the formation of the columnar structure of human telomeres and/or its alternative open state is however unknown.

### Through Interaction with the Nuclear Lamina

The perinuclear zone has been associated with the induction of heterochromatin, thanks to the demonstration that artificial tethering of genomic loci to the nuclear periphery favors gene silencing (Finlan et al. 2008; Reddy et al. 2008). Human telomeres were first reported to attach to the nuclear matrix more than 30 years ago (de Lange 1992), but it is not yet clear whether perinuclear localization of telomeres is involved in their heterochromatinization.

H3K9me2 appears to specifically mark the heterochromatin localized at the nuclear periphery (Poleshko et al. 2017), whereas euchromatin and H3K9me3-enriched heterochromatin are distributed in the nuclear interior (van Steensel and Belmont 2017; Poleshko et al. 2019). Consistent with the presence of the H3K9me2 mark at human telomeres, almost half the telomeres dynamically relocate to the nuclear periphery during nuclear assembly after mitosis (Crabbe et al. 2012). Several mechanisms of telomere anchoring to the nuclear lamina were proposed: (1) through interactions between the shelterin protein RAP1 and the inner nuclear membrane SUN1 (Crabbe et al. 2012), (2) through interactions between TRF2 or TRF1 and Lamin A or B1 (Burla et al. 2016; Pennarun et al. 2023), (3) through interactions between telomeres and Lamina-associated peptide 2α (LAP2α) (Dechat et al. 2004), or (4) through interactions between the subtelomeric insulator protein CTCF and A-type lamins (Ottaviani et al. 2009). Expression of

progerin, a mutated form of Lamin A found in patients suffering from the Hutchinson–Gilford progeria syndrome (Arancio et al. 2014), reduces the association of telomeres with LAP2α and the global levels of H3K27me3 (Chojnowski et al. 2015). Whether the telomeric abundance of H3K27me3 was reduced, however, has not been evaluated and additional studies will be needed to clarify the role of the nuclear lamina in human telomere heterochromatinization.

## IMPACT OF TELOMERIC CHROMATIN ON TELOMERE BIOLOGY AND CELLULAR HOMEOSTASIS

### Shelterin Binding and Chromosome-End Protection

Resolution of cryo-EM structures revealed intimate contacts between shelterin proteins and histone tails, particularly between the TRF1-Myb domain and the amino-terminal tail of histone H3 (Hu et al. 2023). These observations suggest a reciprocal impact of the telomeric nucleosome on shelterin binding, consistent with the previous report that removal of histone amino-terminal tails significantly reduces the affinities of TRF1 for telomeric NCP (Galati et al. 2015). Posttranslational modifications of histone tails were proposed to affect the binding of shelterin proteins to telomeres and to promote the transition from a protected to a deprotected telomeric state (Galati et al. 2015). The same study further proposed that phosphorylation or acetylation of histone tails might regulate the binding affinity of TRF1 and TRF2 differentially, given their distinct amino-terminal domains, rich in acidic residues for TRF1 or in arginine residues for TRF2 (Galati et al. 2015). It will be of future interest to determine the impact of various posttranslational modifications of histone tails on the ability of shelterin proteins to bind telomeric chromatin.

Given the numerous functions played by the shelterin complex and, which are reviewed elsewhere, the interplay between the telomeric nucleosome and shelterin proteins may thus have a profound impact on telomere functions (Fig. 5).

### Telomere Transcription

Transcription of telomeres into TERRA is initiated from the heterochromatic subtelomeric regions of chromosomes (Fig. 1A; Nergadze et al. 2009). The discovery of transcriptionally permissive histone marks at telomeres, as well as the demonstration that H3K9me3, H3K9me2, and HP1γ are, in fact, associated with transcription elongation (Vakoc et al. 2005), were consistent with transcriptional activity at telomeres. There is clear evidence, however, that human telomere transcription is regulated by epigenetic modifications.

The first evidence in favor of an epigenetic regulation of telomere transcription came from the observation that, in cells derived from patients suffering from the immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome, caused by a mutation in the *DNMT3b* gene coding for DNA methyltransferase 3b, TERRA levels are strongly up-regulated (Yehezkel et al. 2008). Consistent with an impact of DNA methylation on telomere transcription, impaired activity of DNMT1 and DNMT3b in cancer cells increased TERRA expression (Nergadze et al. 2009), and TERRA levels were found to inversely correlate with subtelomeric DNA methylation in human cell lines (Ng et al. 2009; Arnoult et al. 2012; Diman et al. 2016). Both telomerase expression (Ng et al. 2009) and telomere length (Buxton et al. 2014) were also positively associated with methylation levels in subtelomeric DNA, although the underlying molecular mechanisms have not been elucidated. It was also proposed that the recruitment of DNMT3b at subtelomeres may imply specific telomeric histone marks as well as TERRA itself, in a negative feedback mechanism (Deng et al. 2010). The observation that the DNA methylation-sensitive transcription factor NRF1 regulates human telomere transcription, through its recruitment to bona fide binding sites located in the CpG-rich regions of human subtelomeres, also established a link between telomere transcriptional activity and DNA methylation (Fig. 1A; Diman et al. 2016; Le Berre et al. 2019).

In addition to subtelomeric DNA methylation, the repression of telomere transcription

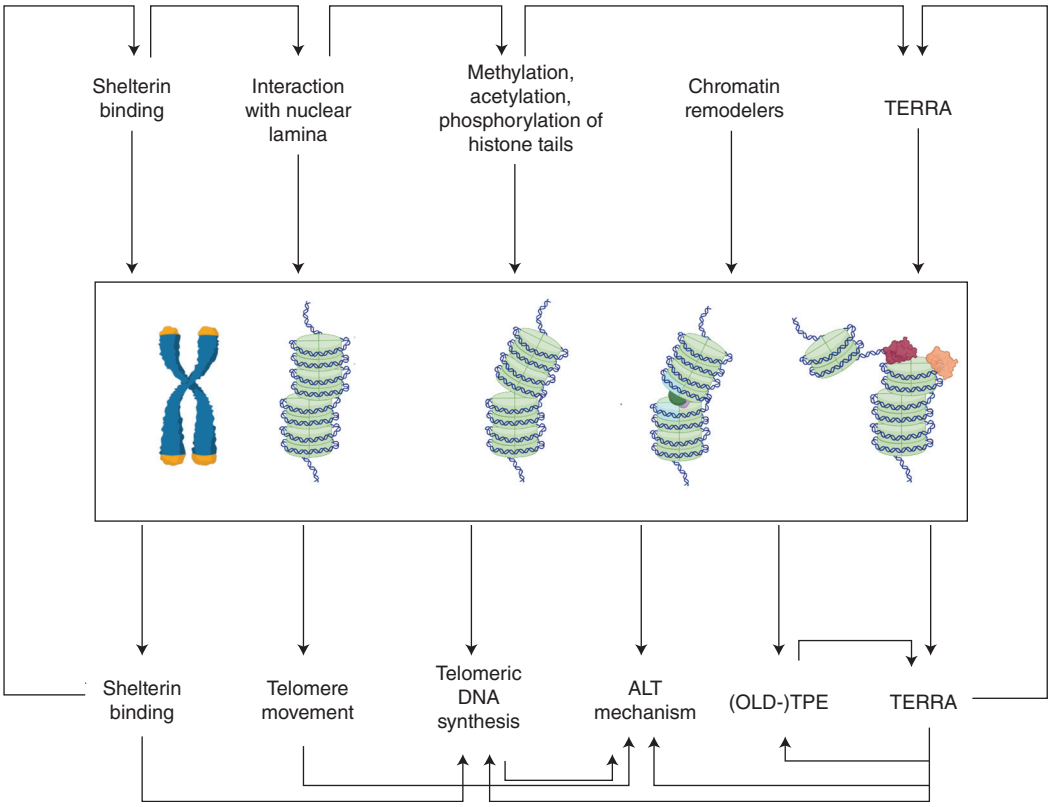


**Figure 5.** Upstream and downstream from human telomeric chromatin. See text for details.

may also result from increased telomeric density of H3K9me3 in human cells with overly elongated telomeres (Arnoult et al. 2012). The causative role for the H3K9me3 repressive mark in the down-regulation of telomere transcription was suggested by the observation that depletion of the SUV39H1 H3K9 methyltransferase abolished the observed telomere length-dependent repression of TERRA (Arnoult et al. 2012). SUV39H1 depletion, however, failed to increase TERRA expression in cells with nonelongated telomeres, suggesting that H3K9me3 may not regulate TERRA expression in human cells with physiological telomere length (Arnoult et al. 2012). In light of these data, the reported enrichment of the constitutive heterochromatin mark H3K9me3 at long mouse telomeres could possibly explain why mouse telomeres are silent. In fact, in mouse, TERRA molecules mainly come from intrachromosomal *TTAGGG*-rich loci and not from telomeres (Chu et al. 2017b; Diman and Decottignies 2018; Viceconte et al. 2021). While DNA methylation and repressive histone marks negatively impact telomere transcription, recruitment of the MLL1 H3K4 methyltransferase to telomeres was found to positively correlate with TERRA transcription (Caslini et al. 2009).

From the above, it appears that TERRA is subject to strict epigenetic regulation in a process that likely involves various negative feedback mechanisms (Fig. 5), and that telomere transcription may be in a poised state, allowing rapid activation upon dedicated environmental stimuli or developmental cues (Voigt et al. 2013).

It is important to mention that, in light of the multiple roles that TERRA plays at telomeres (reviewed elsewhere), it is not always easy to distinguish a direct impact of telomeric chroma-

tin on telomere functions and an indirect impact via regulation of TERRA expression.

## Telomere Position Effect

The spreading of the repressive chromatin environment of long telomeres was previously associated with a local transcriptional silencing mechanism dubbed the TPE. TPE was initially discovered in *Drosophila melanogaster*, where the insertion of genetic elements within telomeres was associated with lower expression levels mediated by an HP1- and H3K9me3-dependent mechanism (Hazelrigg et al. 1984; Perrini et al. 2004). Later, TPE was discovered in budding (Gottschling et al. 1990; Fourel et al. 1999; Pryde and Louis 1999) and fission yeast (Nimmo et al. 1994). While TPE has been well described in flies and yeast, its existence in mammalian cells has long remained controversial, likely due to the paucity of protein-encoding genes near human telomeres. Using an integrated telomeric transgene, Tennen et al. (2011) provided the first evidence for the existence of human TPE and showed that enhanced telomere silencing in response to telomere elongation required the SIRT6 H3K9 deacetylase. The discovery that human telomeres are transcribed (Azzalin et al. 2007) was next seized as an opportunity to assess the existence of TPE at endogenous human chromosomal loci. Isogenic cell lines with distinct telomere lengths revealed that long telomeres negatively regulate TERRA expression in a process involving the histone methyltransferase SUV39H1 and HP1α, providing the first demonstration that human telomere transcription is subject to TPE (Arnoult et al. 2012).

In addition to the phenomenon of TPE described above, evidences were later provided for the existence of a TPE mechanism which, in *trans*, induces gene silencing over long distances (TPE-OLD). The work by the laboratory of Shay and Wright demonstrated that, in the presence of long telomeres, chromosome looping can bring the telomere close to genes that are located up to 10 Mb away (Robin et al. 2014). Both the h*TERT* telomerase gene and the *PPP2R2C* tumor suppressor gene were found to be regulated by TPE-OLD (Kim et al. 2016; Jäger et al. 2022). Interestingly, consistent with the demonstration that telomere shortening alleviates TPE-OLD (Robin et al. 2014), replicative senescence up-regulates the expression of *hTERT* and *PPP2R2C* genes (Kim et al. 2016; Jäger et al. 2022), providing new insights into how replicative aging changes the cell's susceptibility to cancer initiation. These observations go hand in hand with the demonstration of an age-dependent reprogramming of telomeric chromatin (O'Sullivan et al. 2010). The mechanisms underlying TPE-OLD are not entirely elucidated but likely rely on interactions between TRF2 and, possibly, TERRA, with interstitial *TTAGGG* repeats (Kim et al. 2016). The association of telomeres with the repressive environment of the nuclear lamina may further participate in TPE (Burla et al. 2016).

Thus, telomeric chromatin can regulate gene expression both locally—by affecting TERRA expression—and over long distances in a telomere length-dependent manner (Fig. 5).

## Activation of Alternative Lengthening of Telomeres

To acquire an indefinite replication potential, cells activate either telomerase or an alternative mechanism of telomere lengthening, dubbed ALT, which is based on homologous recombination (HR) events at telomeres (for review, see O'Sullivan and Greenberg 2025). How ALT is activated is still not fully understood, but it is clear that epigenetic alterations at telomeres alleviate the natural protection of telomeres against recombination to allow the access of HR factors. Increasing evidence has emerged over the past decade regarding the role of telomeric chromatin alterations on ALT activation (O'Sullivan and Almouzni 2014). One of the strongest evidences that chromatin dysfunction promotes ALT came from the observation that mutations in the *ATRX* or *DAXX* genes, which encode the subunits of a chromatin remodeling complex catalyzing H3.3 variant incorporation at telomeres (Goldberg et al. 2010; Wong et al. 2010; Clynes et al. 2015), correlate with ALT activation in pancreatic neuroendocrine tumors (Heaphy et al. 2011). Agreeing with the crucial role of H3.3 alterations in ALT activation, other

ALT tumors were subsequently found to harbor mutations in the *H3F3A* gene encoding H3.3 (Schwartzentruber et al. 2012; Wu et al. 2012). While several groups reported that the inactivation of the ATRX/DAXX/H3.3 pathway on its own is not enough to activate ALT (Lovejoy et al. 2012; O'Sullivan et al. 2014), the ectopic expression of ATRX is able to suppress ALT activity (Clynes et al. 2015; Napier et al. 2015).

ATRX depletion in human fibroblasts was associated with progressive telomeric chromatin decompaction and reduced telomeric density of H3 and H3.3 (Li et al. 2019). These observations were consistent with the reduced H3 and H4 histone density and the increased access to micrococcal nuclease (MNase) at telomeres of spontaneously immortalized $ALT^{+}ATRX^{-}$ cell lines (Episkopou et al. 2014). In support of a role for telomeric chromatin alterations in ALT induction, the codepletion of ASF1a/b H3.1/H3.3-H4 histone dimer chaperones was sufficient to activate ALT in vitro (O'Sullivan et al. 2014), even though the relevance for ALT activation in vivo remains to be established. Codepletion of ASF1a/b similarly increased the susceptibility of telomeric chromatin to MNase digestion (O'Sullivan et al. 2014). Together, these studies support a role for lack of compaction of telomeric chromatin in activation of the ALT mechanism (Fig. 5). It will be interesting to assess the contribution of various ALT telomere features, such as increased abundance of G4 structures (Yang et al. 2021), the presence of telomeric repeat variants, and binding of various nuclear receptors at ALT telomeres (Marzec et al. 2015) to this change in chromatin structure, and to test whether the columnar packing model of human telomeric nucleosomes is disrupted at ALT telomeres.

Although chromatin at ALT telomeres appears to be more relaxed, conflicting data have been reported regarding the abundance of repressive histone marks (Udroiu and Sgura 2020). In the immortalized human fibroblast lines used in Episkopou et al. (2014), the overall H3K9me3 density was reduced at ALT telomeres, but H3K9me3/H3 ratios were similar in ALT and telomerase-positive cells. These observations suggested that the methyltransferase activity on telomeric H3K9 was not reduced in ALT cells, in agreement with independent observations obtained in ALT and non-ALT cell lines (Conomos et al. 2014). Conversely, based on ChIP-seq data, ALT activity in childhood neuroblastoma was associated with an increased abundance of H3K9me3 marks at telomeric repeats (Hartlieb et al. 2021). In the latter study, however, telomeric repeat reads appeared to be mostly located outside of the terminal sequences of chromosomes and the amount of *TTAGGG* repeats obtained in non-ALT samples was very low (Hartlieb et al. 2021). A careful interpretation of the ChIP-seq data may therefore be needed to conclude on the abundance of these histone marks at the level of telomeres. In a separate study, the depletion of SETDB1 alone reduced some markers of ALT activity in human cancer cells (Gauchier et al. 2019). Whether this was the direct result of changes in H3K9me3 or H3K9me2 density at telomeres was however not evaluated. Interestingly, SETDB1 was proposed to primarily act on euchromatic regions of the genome (Schultz et al. 2002), further strengthening the idea that ALT telomeres do not show the canonical features of telomeric heterochromatin.

Whether ALT telomeres show increased or reduced abundance of repressive histone marks is therefore still unclear. A possible increased abundance of repressive histone marks at ALT telomeres, however, would still be in line with a reduction of telomere compaction which, as noted above, likely occurs through a mechanism independent of the canonical repressive histone marks of constitutive heterochromatin.

Although the impact of histone tail modifications on ALT telomeric chromatin compaction is still unclear, histone mark modifications are nevertheless likely to modulate ALT activity. The impact of histone marks on TERRA production emerged as an obvious candidate to promote ALT activity (reviewed elsewhere). Work from Pickett and Reddel's laboratories also demonstrated that the increased recruitment of the NuRD-ZNF827 complex at ALT telomeres not only causes histone hypoacetylation but also leads to (1) an increase in telomere–telomere interactions, (2) the recruitment of HR enzymes and the inhibition of shelterin

protein binding, and (3) the enhancement of HR activity at ALT telomeres (Conomos et al. 2014; Pickett and Reddel 2015). Whether NuRD-ZNF827-dependent ALT telomere hypoacetylation impacts telomeric chromatin compaction has not been tested but would be important in supporting the hypothesis of a distinct impact of histone marks on ALT activity and telomeric chromatin compaction.

Therefore, similar to the lack of consensus on the epigenetic features of mammalian telomeres, the picture of epigenetic modifications at ALT telomeres is also unclear. In the future, it will be interesting to evaluate how histone modifications separately modulate ALT activity and chromatin compaction to gain a better overall picture of the impact of telomeric chromatin on ALT activity.

### Telomere Movement

Telomere movement is involved in various cellular processes such as telomere positioning in the nucleus (Pandita et al. 2007), end-to-end fusions (Dimitrova et al. 2008), and ALT activity (Jegou et al. 2009; Lamm et al. 2021) and is likely facilitated by telomeric chromatin decondensation (Fig. 5; Gao et al. 2018). Along these lines, the role of SIRT6 in facilitating the directional movement of damaged telomeres was related to its ability to recruit the ATP-dependent chromatin remodeling enzyme SNF2H (Gao et al. 2018), an enzyme involved in nucleosome repositioning (de La Serna et al. 2006). Consistent with the proposed lack of compaction of ALT telomeres and the link to telomere movement, a fraction of telomeres in U2OS ALT cells exhibit extended mobility, which has been proposed to promote ALT activity by facilitating telomere–telomere interactions and their association with PML bodies (Fig. 5; Jegou et al. 2009). The Smc5/6 complex, previously detected at ALT telomeres (Potts and Yu 2007), is required for the directed movement of heterochromatic double-strand breaks (Caridi et al. 2018), suggesting a similar role in the movement of ALT telomeres. Interestingly, telomere mobility is increased in Lamin A–depleted cells (de Vos et al. 2010; Bronshtein et al. 2015), reinforcing the idea that the interaction of telomeres with Lamin A could regulate telomeric chromatin.

### Telomeric DNA Synthesis

The low levels of telomeric H3K9 acetylation are necessary for the efficient replication of human telomeres and the prevention of structural abnormalities (Michishita et al. 2008). By creating a specialized chromatin state that allows the stable association of telomere-processing factors in S phase, such as WRN, the H3K9 histone deacetylase SIRT6 thereby protects telomeres from dysfunction (Fig. 5; Michishita et al. 2008). Further evidence supporting the role of histone hypoacetylation in telomeric DNA synthesis was provided by the demonstration that recruitment of the NuRD-ZNF827 complex to ALT telomeres, linked to the recruitment of histone deacetylases, enhanced the break-induced replication activity at telomeres, as suggested by the increased abundance of C-circles products (Conomos et al. 2014).

Telomerase also synthesizes telomeric DNA and its activity at telomeres may be modulated by telomeric chromatin, as the artificial tethering of HP1α, via its fusion to the TRF1 shelterin protein, attenuated the telomere extension mediated by telomerase (Chow et al. 2018). However, given that TERRA modulates human telomerase access to telomeres (Bettin et al. 2024), it remains to be verified whether the observed negative impact on telomerase-dependent extension was directly mediated by HP1α or whether it was a consequence of a possible deregulation of TERRA (Fig. 5). Similarly, TERRA transcripts have been implicated in various aspects of human telomere replication, and this, again, complicates the understanding of the direct role of histone modifications on telomeric DNA synthesis (Bettin et al. 2019).

Together, whether by direct or indirect mechanisms, telomeric chromatin thus appears to modulate various telomeric DNA synthesis pathways.

## CONCLUSION

In summary, we have emphasized that current knowledge calls for a reassessment of the tradi-

tional view of constitutive heterochromatin in human telomeric chromatin. We propose that the properties of human telomeric chromatin align more closely with those of facultative heterochromatin. Additionally, we have outlined the existence of tightly regulated and interconnected regulatory mechanisms that, together, likely account for the numerous important functions played by the telomeric chromatin structure (Fig. 5). The recent development of cryo-EM approaches will need to be implemented to study the properties of telomeric chromatin in experimental settings that better resemble physiological conditions by taking into account the diversity of histone variants/marks, telomere-binding proteins, or secondary structures, such as G-quadruplexes, as well as in the presence of TERRA noncoding RNA. Exciting times ahead!

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