Focus Review

A ‘higher order’ of telomere regulation: telomere heterochromatin and telomeric RNAs

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Protection of chromosome ends from DNA repair and degradation activities is mediated by specialized protein complexes bound to telomere repeats. Recently, it has become apparent that epigenetic regulation of the telomeric chromatin template critically impacts on telomere function and telomere-length homeostasis from yeast to man. Across all species, telomeric repeats as well as the adjacent subtelomeric regions carry features of repressive chromatin. Disruption of this silent chromatin environment results in loss of telomere-length control and increased telomere recombination. In turn, progressive telomere loss reduces chromatin compaction at telomeric and subtelomeric domains. The recent discoveries of telomere chromatin regulation during early mammalian development, as well as during nuclear reprogramming, further highlights a central role of telomere chromatin changes in ontogenesis. In addition, telomeres were recently shown to generate long, non-coding RNAs that remain associated to telomeric chromatin and will provide new insights into the regulation of telomere length and telomere chromatin. In this review, we will discuss the epigenetic regulation of telomeres across species, with special emphasis on mammalian telomeres. We will also discuss the links between epigenetic alterations at mammalian telomeres and telomere-associated diseases.

Keywords: epigenetics; silencing; telomeres; telomeric chromatin

Introduction

Telomeres are nucleoprotein structures that protect the ends of linear chromosomes from degradation and from being detected as double-strand DNA breaks (Chan and Blackburn, 2004; Palm and de Lange, 2008). A tri-partite organization of telomeres is a canonical feature of chromosome termini in eukaryotes. Telomeres consist of (i) a capping structure, which protects the end of chromosomes from degradation and from eliciting a DNA damage response (DDR), and also controls the extension of telomeric repeats; (ii) a stretch of double-stranded repetitive and transcribed DNA elements; and (iii) repetitive telomere-associated sequences (TAS) also referred to as subtelomeres (Riethman et al, 2005; Blasco, 2007; Anderson et al, 2008). Whereas yeast, vertebrate, and plant telomeres consist of short-tandem repeats, Drosophila melanogaster chromosomes terminate in arrays of telomere-specific non-long terminal-repeat (LTR) retrotransposons (Pardue and DeBaryshe, 2003; Chan and Blackburn, 2004; Zellinger and Riha, 2007). Telomere function depends on a minimal length of telomeric repeats and the functionality of the associated protein complexes. In addition, higher-order DNA conformations, such as the T-loop, are thought to contribute to telomere function (Griffith et al, 1999). In most species, telomeres are maintained by telomerase, a reverse transcriptase that adds telomeric repeats de novo after every cell division, thereby counteracting incomplete DNA replication of telomeres due to the so-called end-replication problem (Collins and Mitchell, 2002; Chan and Blackburn, 2004). Drosophila melanogaster compensates the lack of telomerase by transposing telomere-specific LTR retrotransposons to chromosome ends (Pardue and DeBaryshe, 2008). Alternative pathways involving telomere recombination (ALT, alternative lengthening of telomeres) have been also described in mammals (Collins and Mitchell, 2002; Pardue and DeBaryshe, 2003; Muntoni and Reddel, 2005).

In adult mammalian tissues and adult stem cells, telomerase activity is not sufficient to maintain telomeres during cell division and tissue renewal (Collins and Mitchell, 2002; Flores et al, 2005; Sarin et al, 2005). Progressive telomere shortening leads to telomere dysfunction and elicitation of a DDR, which result in cell cycle arrest/senescence or apoptosis (Harley et al, 1990; d’Adda di Fagagna et al, 2003). In vivo, critically short telomeres result in stem cell dysfunction, premature loss of tissue regeneration, and reduced life span, as shown in the context of telomerase-deficient mice (Blasso et al, 1997; Herrera et al, 1999; Rudolph et al, 1999; Gonzalez-Suarez et al, 2000; Collins and Mitchell, 2002; Blasco, 2005; Garcia-Cao et al, 2006). In contrast, over-expression of telomerase is sufficient to immortalize most human cell types in vitro and leads to a significant extension of the median life span of Tert transgenic mice with increased cancer resistance (Bodnar et al, 1998; Gonzalez-Suarez et al, 2001; Artandi et al, 2002; Canela et al, 2004; Tomas-Loba et al, 2008).

Pioneer studies in yeast indicated the involvement of chromatin modifications in the control of telomere function and telomere length. In particular, reporter genes introduced...
in proximity to telomeres were found to be silenced, suggesting a repressive chromatin environment at yeast telomeres, which was later also reported for D. melanogaster and mammals (Palladino et al., 1993; Cooper et al., 1997; Baur et al., 2001; Koering et al., 2002; Biessmann et al., 2005; Mason et al., 2008). Whereas telomeric repeats are devoid of histones in yeast, the accumulation of repressive histone modifications at mammalian telomeric and subtelomeric repeats, as well as the hypermethylylation of subtelomeric DNA, has been recently shown to have a central function in mammalian telomere-length homeostasis (Blasco, 2007).

Recent discoveries of transcripts derived from yeast and vertebrate telomeres, as well as rasiRNAs derived from Drosophila melanogaster telomeric retrotransposons, suggests the involvement of non-coding RNAs in telomere structure and telomere regulation across species (Savitsky et al., 2006; Azzalin et al., 2007; Schoeftner and Blasco, 2008). Mammalian and yeast telomeric RNAs have been proposed to control telomere structure as well as telomere elongation by telomerase (Azzalin et al., 2007; Luke et al., 2008; Schoeftner and Blasco, 2008).

In this review, we provide an overview on the epigenetic regulation of yeast, D. melanogaster, and vertebrate telomeres, with a special emphasis on the regulation of mammalian telomeric chromatin during development and in the context of telomere-associated diseases.

**The telomere-binding proteins**

From yeast to man, telomeres are bound by specialized protein complexes that regulate telomere length and telomere capping. In *Saccharomyces cerevisiae*, Cdc13 binds to the G-strand overhang and controls telomere elongation by telomerase, whereas Rap1 (repressor–activator protein 1) recruits the silent information regulator proteins Sir2, Sir3, Sir4 and the telomere-length regulators Rif1 and Rif2 to telomeres, forming the so-called ‘telosome’ (Wright et al., 1992; Tham and Zakian, 2002). Rap1–Rif1 complexes act as a counting mechanism to negatively regulate telomere length (Kyrion et al., 1992; Krauskopf and Blackburn, 1996; Marcand et al., 1999; Levy and Blackburn, 2004). Homologues of *S. cerevisiae* Rap1 and Rif1 have also been described in *Schizosaccharomyces pombe*. In *S. pombe*, Rap1 and Rif1 are recruited to double-stranded telomeric repeats through association with the telomere repeat-binding protein Taiz1, thus regulating telomere length and telomeric silencing (Kanoh and Ishikawa, 2001). The *S. pombe* G-strand overhang is protected by Pot1. Pot1 associates with Tpz1, Ccq1 and Poz1 and contacts the Taiz1–Rap1 complex located at double-stranded telomeric repeats (Miyoshi et al., 2008).

Telomere-binding proteins in *S. pombe* telomeres are highly related to components of the mammalian shelterin complex. In functional analogy to Taiz1, the mammalian shelterin components TRF1 and TRF2 bind to double-stranded telomeric repeats and recruit TPP1 (orthologue of *S. pombe* Tpz1), Rap1, TIN2, and the poly(ADP)-riboylasylases Tank1 and Tank2 to telomeres (Palm and de Lange, 2008). The single-stranded 3′ overhang is bound by Pot1, which contacts with TRF1 and TRF2 at double-stranded telomere regions through TPP1.

*D. melanogaster* lacks telomerase activity and maintains arrays of telomere-specific LTR retrotransposons by retro-

transposition or gene conversion (Biessmann and Mason, 2003). In contrast to yeast and vertebrate telomeres, chromosome capping in *D. melanogaster* is mediated by an alternative mechanism, which is dependent on the ‘terminin’ protein complex containing the heterochromatin protein 1 (HP1), HOAP (HP1/ORC-associated protein), and the mod-iglani (moi) gene product (Cenci et al., 2005). The dependence of chromosome capping on HP1, a major component of heterochromatin, shows the strong bias of *Drosophila melanogaster* telomere regulation towards the use of general chromatin regulators.

**Epigenetic regulation of yeast telomeres**

*S. cerevisiae* telomeres consist of 350 ± 75 bp of C1–2A/TG1–3 histone-free DNA repeats that terminate in a single 3′ overhang (Wright et al., 1992). Adjacent subtelomeric Y′ and X repeats are assembled into nucleosomes and extend several kilobases towards centromeres (Louis, 1995). The silencing of reporter genes introduced into *S. cerevisiae* subtelomeric regions, a phenomenon also referred to as ‘telomere position effect’ (TPE), provided early evidence for a repressive chromatin environment at telomeres (Gottschling et al., 1990; Tham and Zakian, 2002). As discussed above, histone-free telomeric repeats are bound by Rap1, which recruits the silent information regulator Sir4. Sir4 further attracts Sir2 and Sir3 to telomeres. The NAD-dependent deacetylase activity of Sir2 is essential for telomere repression and the spreading of silencing, whereas Sir3 and Sir4 act as structural components. Sir2 de-acetylates the tails of histones H3 and H4 with preference for acetylated lysine 16 on histone H4 (H4K16Ac), thereby creating a high-affinity-binding site for Sir3 and Sir4 (Hecht et al., 1995; Tanny et al., 1999; Imai et al., 2000; Carmen et al., 2002). Mutations in residues K16–K20 of histone H4, as well as loss of Sir2, result in loss of telomeric repression (Johnson et al., 1990; Aparicio et al., 1991; Tanny et al., 1999). Binding of Sir3 and Sir4 is further enhanced by the production of 2′-O-acetyl-ADP-ribose (O-AADPR), a side product of the NAD + hydrolysis by Sir2 (Liou et al., 2005; Martino et al., 2009). Thus, a positive-feedback loop based on cycles of histone H3 and H4 de-acetylation, Sir protein recruitment and O-AADPR-mediated stabilization allows the Sir complex to spread along subtelomeric nucleosomes and silence promoters kilobases away from Rap1-determined silencing nucleation. Silencing is further enhanced by the formation of a telomeric fold-back structure and the association of telomeres with the Sir-rich nuclear periphery (Mailet et al., 1996; Strahl-Bolsinger et al., 1997; de Bruin et al., 2000). Spreading of telomeric silencing is antagonized by Sas2, a specific MYST-type family acetylase of the SAS complex that competes with Sir2 in controlling the acetylation status of H4K16 (Osada et al., 2001; Kimura et al., 2002; Suka et al., 2002; Shia et al., 2005). H4K16 acetylation by Sas2 is important for the subsequent incorporation of H2A.Z that forms a chromatin boundary preventing the propagation of silencing (Meneghini et al., 2003; Shia et al., 2006). Hyperacetylated H4K16 also drives Sir3 displacement and allows binding of the histone methyltransferase Dot1 that methylates the histone H3 lysine 79 residue, further antagonizing the spreading of Sir complexes (Park et al., 2002; van Leeuwen and Gottschling, 2002; van Leeuwen et al., 2002; Ng et al., 2002a; Altf et al., 2007; Fingerman et al., 2007).
addition, the ubiquitination of lysine 123 of H2B by the ubiquitin-ligating enzyme Rad6 is required for efficient H3K79 methylation and the methylation of histone H3K4 by Set1, another marker of telomeric chromatin (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002b; Sun and Allis, 2002; Shahbazian et al., 2005). Together, this indicates the existence of a network of trans-histone pathways to tune repression at telomeres and subtelomeres.

The role of these epigenetic modifications in the regulation of yeast telomere length is well documented. Several mutations that disrupt telomeric silencing also decrease the length of telomeres (Palladino et al., 1993; Greenwell et al., 1995; Porter et al., 1996; Nislow et al., 1997). In addition, the Rap1 counting pathway seems to be indirectly regulated by the Sir proteins (Marcand et al., 1997). Furthermore, anchoring of telomeres to the nuclear periphery seems to regulate telomere length in cells that are compromised for the Rap1 counting pathway (Gartenberg et al., 2004; Berthiau et al., 2006; Hediger et al., 2006). Notably, deletion of Rif2 can also lead to recombination-dependent telomere elongation (Teng et al., 2000), suggesting a link between telomeric chromatin and recombination. Recently, S. cerevisiae and vertebrate telomeres were shown to be transcribed by RNA Polymerase II, giving rise to single-stranded telomeric repeat-containing RNAs (TERRA/TelRNAs). Yeast TERRA was reported to form RNA/DNA hybrids negatively regulating telomerase-dependent telomere elongation; however, the possible role of TelRNA/TERRA in defining telomeric silencing has not yet been addressed (Azzalin et al., 2007; Luke et al., 2008). Studying the involvement of TERRA in the regulation of yeast telomeric chromatin will reveal novel pathways of telomere control.

**Epigenetic regulation of* S. pombe* telomeres**

Telomeres in fission yeast* S. pombe* share features with* S. cerevisiae* and mammalian telomeres. Similar to budding yeast, *S. pombe* telomeric repeats are devoid of nucleosomes; however, telomere-binding proteins and the telomeric chromatin structure are highly related to that of mammals. Mutations in telomere-binding proteins and telomere heterochromatin regulators, such as Tal1, Rap1, Swi6, and Clr1-4, are known to affect telomeric silencing (Thon and Klar, 1992; Allshire et al., 1995; Cooper et al., 1997; Nimmo et al., 1998; Chikashige and Hiraoi, 2001; Kanoh and Ishikawa, 2001; Sugiyama et al., 2007). In addition, disruption of telomeric heterochromatin results in increased subtelomeric recombination, which, similar to mammals, can impact on telomere-length homeostasis (Kanoh et al., 2003; Bisht et al., 2008). Fission yeast telomeric heterochromatin is enriched for Swi6, the orthologue of* D. melanogaster* HP1. HP1 recruitment to telomeres is dependent on H3K9 methylation by the SET domain-containing histone methyltransferase Ctr4 (orthologue of mammalian Su(var)39h HMTases) that methylates the histone H3 lysine 9 residues at telomeres (Bannister et al., 2001; Nakayama et al., 2001). The chromatin structure of* S. pombe* telomeres is similar to that found at centromeric regions and the mating-type locus where H3K9 methylation by Ctr4 is dependent on the generation of small RNAs derived from heterochromatic regions by Dcr1 (the homologue of mammalian Dicer 1) (Ekwall et al., 1995, 1996; Nakayama et al., 2000, 2001; Reinhart and Bartel, 2002; Motamedi et al., 2004; Noma et al., 2004; Verdel et al., 2004; Kato et al., 2005). However, only the combined ablation of the telomeric repeat-binding protein Tal1 and proteins involved in NAI-mediated heterochromatin formation releases Swi6 from telomeres, suggesting that telomeric heterochromatin is recruited by Tal1 and components of the NAI machinery (Kanoh et al., 2005). Recently, the multi-enzyme complex SHREC, which mediates heterochromatin transcriptional gene silencing in* S. pombe*, was shown to be recruited to telomeres by redundant pathways involving Tal1 and Ccq1, as well as the RNAi machinery (Sugiyama et al., 2007). SHREC contains the histone deacetylase Clr3 and the chromatin remodelling factor Mit1 and both activities are required to silence reporter genes at subtelomeres (Sugiyama et al., 2007). Interestingly, in addition to recruiting SHREC, Ccq1, which is functionally linked to the telomeric single-stranded-binding protein Pot1, also recruits telomerase and prevents telomeric recombination (Miyoshi et al., 2008; Tomita and Cooper, 2008). Finally, absence of SpSet1p, a histone H3 lysine 4 methyltransferase associated with transcriptional activation, also results in impaired telomeric silencing and telomere elongation (Kanoh et al., 2003). In summary, the regulation of telomeric heterochromatin in* S. pombe* illustrates an interplay between the telomere-binding proteins and general chromatin regulators. Given the high similarity between* S. pombe* and mammalian telomeres, a role for shelterin in telomere chromatin regulation can be anticipated. In this respect, altered nucleosome spacing in cells over-expressing TRF2 provides evidence for such a connection (Benetti et al., 2008b).

**The heterochromatin structure of* Drosophila* telomeres**

In contrast to short telomeric repeats in yeast and mammals, *D. melanogaster* chromosome termini consist of up to 12 kb of tandem arrays of telomere-specific HeT-A, TART and TAHR LTR retrotransposons (Mason and Biessmann, 1995; Mason et al., 2008). These arrays of HeT-A, TART, and TAHR (HTT) retroelements are preferentially maintained by target-primed reverse transcription-based retrotransposition to chromosome ends, or alternatively, by gene conversion. Transposition is dependent on HTT retroelements-encoded reverse transcriptases and occurs to any chromosome end, creating a high heterogeneity in array length (Biessmann et al., 1993; Levis et al., 1993; Walter et al., 1995; Biessmann and Mason, 2003; Abad et al., 2004; Pardue et al., 2005). Telomere capping is mediated by the ‘terminin’ complex comprising HP1, the telomere-specific HOAP (HP1/ORC-associated protein), and the modigliani (moi) gene product (Silva et al., 2004; Bi et al., 2005; Clapponi et al., 2006; Okemus et al., 2006; Raffa et al., 2009). Interestingly, HP1, encoded by Su(var)205, is recruited to chromosome ends independently of the sequence content or presence of H3K9me3 and spreads at lower density into adjacent HTT arrays where HP1 uses its chromodomain to bind H3K9me3 (Fanti et al., 1998; Andreyeva et al., 2005; Frydrychova et al., 2008). Su(var)205 mutants display telomere fusions, increased HeT-A transcript levels, and increased retroelement addition leading to telomere elongation (Savitsky et al., 2002). Thus, *Drosophila* telomere length is controlled by an interaction of H3K9me3 and HP1 in silencing HTT arrays, whereas
chromosome capping by HP1 controls the addition of retro-elements to chromosome ends (Perrini et al., 2004). In addition to siRNAs and miRNAs, a third RNA silencing system based on the Piwi subfamily of Argonaut proteins has evolved that prevents the spreading of selfish DNA elements such as telomeric retro-transposons in the germline (Hartig et al., 2007). In the first step of the repeat-associated short-interfering (rasi)RNA pathway, rasiRNAs are generated from damaged inactive copies of transposable elements. These antisense rasiRNAs then target transcripts of functional transposons in a process that depends on the action of the Piwi proteins (Saito et al., 2006; Brennecke et al., 2007; Gunawardane et al., 2007). Complementary relationships of sense and antisense RNA populations indicate the existence of a positive-feedback loop, also described as the ‘ping-pong model’ that ensures efficient elimination of transcripts derived from active transposons (Brennecke et al., 2007). Consistent with this model, transcript levels from functional telomere-specific retrotransposons are significantly increased in germline mutants for components of the rasiRNA pathway and the RNA helicase gene spr-E (Savitsky et al., 2006; Klenov et al., 2007; Shpiz et al., 2007). Furthermore, decreased rasiRNA production is accompanied by reduced H3K9me3 and HP1 levels at HTT arrays and by an abundant retrotransposition of HeT-A elements (Savitsky et al., 2006; Klenov et al., 2007). In line with this, Piwi is reported to localize to chromatin in a complex with HP1a, providing further evidence for a role of the rasiRNA pathway in telomere regulation (Brower-Toland et al., 2007; Klenov et al., 2007).

Telomere-associated sequences (TAS) located adjacent to HTT arrays sequences have been reported to have a role in silencing (Mason et al., 2008). TAS are enriched for the H3K27me3 mark and bound by Polycomb proteins, which in turn impact on TPE (Boivin et al., 2003; Mason et al., 2004; Andreveya et al., 2005; Shanower et al., 2005; Doheny et al., 2008). Interestingly, TAS are also subjected to regulation by the rasiRNA pathway. However, in contrast to HTT repeats where mutations of the rasiRNA pathway result in loss of telomeric heterochromatin, reduced TAS-originated rasiRNAs are associated with a loss of euchromatic marks (Yin and Lin, 2007). This discrepancy in chromatin regulation indicates that repetitive elements in HTT arrays and TAS sequences underlie distinct mechanisms of epigenetic regulation. A functional conservation of the rasiRNA pathway in telomere regulation in the mammalian germline is not known to date.

**Vertebrate telomeric heterochromatin**

Similar to *D. melanogaster* and *S. pombe*, vertebrate telomeres are enriched for the H3K9me3 mark, imposed by the Suv39h HMTases, the mammalian homologues of *S. pombe* Ctr4 (Peters et al., 2001, 2003; Garcia-Cao et al., 2004). H3K9me3 provides a high-affinity-binding site for HP1 and promotes the imposition of the H4K20me3 mark by the Suv4-20h1 and Suv4-20h2 HMTases (Bannister et al., 2001; Lachner et al., 2001; Nakayama et al., 2001; Schotta et al., 2004, 2008; Benetti et al., 2007b) (Figure 1). In addition to these heterochromatic histone marks, telomeric repeats also contain di-methylated H3K79, which is mediated by the Dot1L HMTase (San-Segundo and Roeder, 2000; Shanower et al., 2005). Dot1L activity is also required for efficient imposition of the H4K20me3 mark at telomeres, suggesting that both Suv39h HMTases and Dot1L are acting upstream of the Suv4-20h HMTases (Jones et al., 2008) (Figure 1) Interestingly, although telomeres display normal H3K9me3 levels, the abundance of H3K9me2 is markedly reduced at telomeric repeats in cells lacking Dot1L. This suggests that additional H3K9-specific HMTases, such as G9a of ESET, could be involved mediating H3K9me2 at telomeres (Jones et al., 2008). In addition to repressive histone marks, telomeric H3 and H4 histones are under-acetylated (Benetti et al., 2007a). In this regard, lack of the histone deacetylatase SIRT6 results in elevated H3K9-acetylation levels at human telomeres and can lead to telomere dysfunction (Michishita et al., 2008).

**DNA methylation at subtelomeric repeats**

DNA methylation is known to regulate mammalian development and to specify silent chromatin regions in both eu- and heterochromatin (Chen and Li, 2006). In contrast to *S. cerevisiae* and *D. melanogaster*, which lack or display low levels of DNA methylation, mammalian subtelomeric regions are heavily methylated (Tommerup et al., 1994; van Overveld et al., 2003; Steinert et al., 2004; Gonzalez et al., 2006) (Figure 1). Importantly, TTAGGG repeats remain unmethylated because of the lack of methylate-able cytosine. It has been proposed that DNA methylation at subtelomeric repeats acts as an additional mechanism in mammals that enforces TPE (van Overveld et al., 2003; Pedram et al., 2006). DNA methylation patterns in mammalian cells are established by three main DNA methyltransferases (DNMTs). *De novo* methylation patterns are established by DNMT3a and DNMT3b and maintained by DNMT1, which copies parental-strand methylation onto the *de novo* synthesized daughter strand after DNA replication (Okano et al., 1998). DNA methylation is enriched at repetitive elements such as the pericentric regions and is regarded to prevent frequent recombination events (Bender, 1998; Maloisel and Rossignol, 1998; Dominguez-Bendala and McWhir, 2004; Gonzalez et al., 2006; Jaco et al, 2008). Consistent with this, deficiency of DNMT1 or DNMT3ab causes a dramatic elongation of telomeres, which is driven by increased homologous recombination events between telomeric sister chromatids (Gonzalo et al., 2006). The mechanism of DNMT recruitment to subtelomeres remains however unclear. Whereas DNA methylation at pericentric repeats is reduced in the absence of Suv39h HMTases and an interaction between HP1 and Suv39h1 had been reported, loss of Suv39h HMTases does not affect subtelomeric DNA methylation (Fuku et al., 2003; Lehnertz et al., 2003; Benetti et al., 2007b). This suggests the existence of an alternative pathway of DNMT recruitment to subtelomeres.
Rb family proteins regulate telomeric and subtelomeric chromatin status

A major tumour suppressor pathway in mammals is centred on the family of retinoblastoma (RB) proteins, consisting of RB1, RBL1 and RBL2 (Weinberg, 1995; Lipinski and Jacks, 1999; Classon and Harlow, 2002). RB proteins are transcriptional repressors that control cell cycle genes through interaction with E2F family of transcription factors, as well as by direct recruitment of chromatin regulators to promoters (Harbour and Dean, 2000a, b). In addition to their role at specific promoters, RB family proteins also influence global H4K20me3 and DNA methylation levels, impacting on the epigenetic regulation of telomeres and centromeres (Gonzalo et al, 2005). In particular, RB proteins promote the recruitment of Suv4-20h HMTase and HP1 to telomeres, thereby negatively regulating telomere length and telomere recombination (Gonzalo and Blasco, 2005). In addition, mouse Rbl2 acts as a transcriptional repressor of DNMTs, thereby influencing telomere length and telomere recombination (Kimura et al, 2003; Gonzalo and Blasco, 2005; McCabe et al, 2005; Benetti et al, 2008a) (Figure 1). In particular, the lack of a functional miR290 cluster targeting Rbl2 in embryonic stem (ES) cells deficient for Dicer results in elevated levels of Rbl2 (Sinkkonen et al, 2008; Benetti et al, 2008a). In turn, increased Rbl2 levels repress DNMT expression and result in loss of global as well as subtelomeric DNA methylation, which drives increased telomeric recombination and aberrant telomere elongation (Benetti et al, 2008a). Indeed, Dicer1-null ES cells phenocopy telomere defects of DNMT-deficient cells, suggesting that Rbl2 and the miR290 cluster are major determinants controlling DNA methylation in ES cells (Gonzalo et al, 2006; Benetti et al, 2008a) (Figure 1). Remarkably, Dicer deficiency does not result in a loss of heterochromatic histone marks at telomeres, excluding a direct involvement of Dicer-dependent small RNAs in the assembly of telomeric heterochromatin (Benetti et al, 2008a). The antagonistic role of Rbl2 on DNA methylation is at first glance in contradiction to the reduced DNA methylation levels observed in primary mouse embryonic

![Figure 1 Assembly of mammalian telomeric and subtelomeric heterochromatin.](https://example.com/figure1.png)
fibroblasts (MEFs) lacking Rb, Rbl1 and Rbl2 proteins; however, this discrepancy can be explained by the fact that Rbl2 is not expressed in MEFs (Gonzalo and Blasco, 2005). In summary, loss of RB proteins results in improved telomere maintenance due to a more relaxed telomeric chromatin structure. Given the central role of RB proteins as tumour suppressors, it will be very interesting to investigate the contribution of improved telomere maintenance to proliferative capacity of tumour cells lacking RB proteins.

**Telomere repeat-associated transcripts (TERRA/TelRNAs)**

On account of their compact heterochromatic structure, telomeres were not regarded to be permissive for transcription. However, other heterochromatic domains in the genome, such as mouse major satellite or human heterochromatic satellite III repeats, were already shown to be efficiently transcribed by RNA polymerase II, giving rise to non-coding RNAs (Lehnertz et al., 2003; Jolly et al., 2004; Rizzi et al., 2004). Recently, two independent reports showed that the telomeric C-rich strand is frequently transcribed by RNA polymerase II, giving rise to UUAGGG-repeat containing non-coding RNAs (TERRA or TelRNA) (Azzalin et al., 2007; Schoeftner and Blasco, 2008). Although formal evidence is still missing, the detection of subtelomeric sequences in TelRNA/TERRA molecules strongly suggests the existence of transcriptional control elements at subtelomeres (Azzalin et al., 2007). Up to date, transcripts containing telomeric repeats have been described in *Mus musculus*, *Homo sapiens*, *S. cerevisiae* and *Danio rerio* (Azzalin et al., 2007; Luke et al., 2008; Schoeftner and Blasco, 2008). The fact that retrotransposition events at HTT arrays of *D. melanogaster* also depend on transcription suggests that transcription is a universal process occurring at the ends of linear, eukaryotic chromosomes. Importantly, telomeric RNAs can be detected at telomeres by RNA-FISH techniques, suggesting that TERRA/TelRNAs can associate with telomeric chromatin in cis, a feature reported earlier for the non-coding XIST RNA that controls mammalian dosage compensation (Azzalin et al., 2007; Payer and Lee, 2008; Schoeftner and Blasco, 2008). Interestingly, in a panel of female mouse cell lines, TERRA/TelRNA form accumulations (Tacs) in the immediate vicinity of the territory of inactive X chromosome (Xi), suggesting an involvement of TERRA/TelRNA in the biology of X inactivation (Schoeftner and Blasco, 2008). TERRA/TelRNA molecules range between ca 100 bp and >9 kb in length and were reported to form intermolecular G-quadruplex structure with single-stranded telomeric DNA, but can also fold into a compact repeated structure containing G-quartets (Azzalin et al., 2007; Schoeftner and Blasco, 2008; Xu et al., 2008; Martadinata and Phan, 2009; Randall and Griffith, 2009). Several lines of evidence exist implicating TelRNA/TERRA in the negative control of telomere length (Schoeftner and Blasco, 2008). Increased TelRNA/TERRA levels by interfering with TelRNA/TERRA decay, such as the impairment of non-sense-mediated RNA decay in human cells or by deletion of the 5'–3'exonuclease Rat1p in *S. cerevisiae*, are associated with a loss of telomere reserve (Azzalin et al., 2007; Luke et al., 2008). Current models propose a role for TelRNA/TERRA in controlling telomerase activity. In yeast, the formation of a DNA/RNA hybrid between TelRNA/TERRA and telomeres is thought to inhibit elongation by telomerase, whereas in mammals, TelRNA/TERRA was shown to efficiently inhibit telomerase activity in vitro, presumably by base pairing with the template region of the RNA component of telomerase (TERC) (Luke et al., 2008; Schoeftner and Blasco, 2008) (Figure 2). These working models are supported by expression data showing low TelRNA/TERRA levels during mouse embryogenesis and in cancer cells—two biological conditions that are characterized by rapid cell proliferation and dependence on high telomerase activity (Schoeftner and Blasco, 2008). On the other hand, accumulation of TelRNA/TERRA in adult tissues could be coupled to telomerase inhibition and ageing (Schoeftner and Blasco, 2008). Importantly, in immortal cell lines, as well as during nuclear reprogramming, TelRNA/TERRA levels correlate with the average telomere reserve (Schoeftner and Blasco, 2008; Marion et al., 2009). Together with the fact that TelRNA/TERRA can be localized to telomeric DNA repeats this suggests that TelRNA/TERRA could locally control telomerase activity in cis, a mechanism that could explain the preferential elongation of the shortest telomere in yeast and mammals on the molecular level (Marcand et al., 1999; Hemann et al., 2001; Samper et al., 2001; Teixeira et al., 2004; Schoeftner and Blasco, 2008). In addition, this mechanism would also preclude excessive telomere elongation by telomerase (i.e. telomere elongation during nuclear reprogramming, Marion et al., 2009), a condition that was found to be associated with impaired female fertility and fecundity in *D. melanogaster* (Walter et al., 2007). However, until formal evidence for a direct role of TERRA in telomerase inhibition has been presented, a speculative role of telomerase recruitment by TelRNA/TERRA should be considered (Figure 2).

Interestingly, long non-coding RNAs transcribed by RNA Pol II have been shown earlier to be involved in the epigenetic regulation of the genome (Bernstein and Allis, 2005). In particular, XIST and roX RNAs are chromatin-associated non-coding RNAs that regulate mammalian and *Drosophila melanogaster* dosage compensation, respectively (Deng and Meller, 2006; Payer and Lee, 2008). In addition, other non-coding RNAs such as the *Air* or *Kcnq1or1* RNAs are involved in genomic imprinting (Paule et al., 2007; Pandey et al., 2008). Functional evidence is still missing, but it is expected that non-coding TelRNA/TERRA may also influence the chromatin status at subtelomeres and telomeres. Although small *Dicer*-dependent double-stranded small RNAs are not involved in the generation of telomeric heterochromatin (Benetti et al., 2008a), a possible contribution of small single-stranded TelRNA/TERRA molecules, processed from a larger RNA precursor, has to be considered. In this respect, it will be particularly interesting to explore a possible connection between TeRNA/TERRA and the mammalian Piwi proteins, which generate small single-stranded RNAs from transcripts derived from repetitive elements (Aravin et al., 2007; Carmell et al., 2007; Kuramochi-Miyagawa et al., 2008).

**Epigenetic regulation of telomere length and telomere recombination**

Heterochromatic marks at telomeres have been proposed to act as negative regulators of telomere elongation (Blasco,
Telomeric chromatin during differentiation and reprogramming

Telomere length is a major regulator of telomeric chromatin status in a given cell type and is assumed to change over the lifetime of organisms due to progressive loss of telomere reserve (Benetti et al., 2007a). In mouse embryos, telomere length is reset to a maximum length until the blastocyst stage in a telomerase-independent manner (Liu et al., 2007). In particular, increased recombination events at telomeres of mouse zygotes and two-cell embryos suggest that ALT is the driving force for the resetting of telomere length at early cleavage embryos (Schaetzlein et al., 2004; Liu et al., 2007). These data suggest that (sub-)telomeres are organized into a relatively open chromatin structure that favours telomeric recombination until the blastocyst stage. Resetting of telomere length at early cleavage embryos is necessary for the proper development of the organism. This dynamic regulation of telomere length is essential for the survival and adaptation of organisms. Telomere length can be recapitulated by nuclear cloning using terminally differentiated cells. Animals derived from differ-
entiated cells with short telomeres were shown to display normal telomere length even after several cycles of nuclear transfer (Lanza et al., 2000; Wakayama et al., 2000). More recently, nuclear reprogramming has been achieved in vitro. Retroviral transduction of pluripotency factors into primary MEF, gives rise to induced pluripotent stem cells (iPSC), which are functional equivalents of mouse ES cells (Takahashi and Yamanaka, 2006; Maherali et al., 2007; Takahashi et al., 2007; Nakagawa et al., 2008; Stadtfeld et al., 2008; Wernig et al., 2008). This reprogramming event is accompanied by a dramatic telomerase-dependent telomere elongation that continues post-reprogramming until reaching the length of ES cell telomeres (Marion et al., 2009). During this process, high densities of H3K9m3 and H4K20me3 at telomeres of primary MEF are converted into a more open—ES cell-like—chromatin structure at iPSC telomeres (Marion et al., 2009). In parallel with telomere elongation, TERRA levels are efficiently upregulated in iPSC compared with MEF, a phenomenon that may serve to negatively regulate telomerase activity once iPSC reach the ES cell-like telomere length (Marion et al., 2009) (Figure 3). The reprogramming of telomeres during iPSC generation provides formal evidence that telomeric chromatin structure is defined by cell-type-specific epigenetic programmes that can be reversed by reprogramming. In line with the need for sufficient telomere reserve for stem cell functionality, reprogramming efficacy of telomerase-deficient MEF is dramatically reduced due to the appearance increased chromosome end-to-end fusions (Allsopp et al., 2003; Flores et al., 2005, 2008; Marion et al., 2009). Together, this indicates a complex regulation of telomeric heterochromatin during development and cellular differentiation, which is expected to impact on human disease.

### Implications of telomere chromatin regulation for human disease

Telomere maintenance is essential for tumour cells to escape cell arrest/senescence and apoptosis. Tumour formation often occurs in the context of altered DNA methylation, loss of H4K20me3, and altered expression of Suv4-20h and Suv39h HMTases (Fraga et al., 2005; Gonzalo and Blasco, 2005; Pogribny et al., 2006; Ting et al., 2006; Tryndyak et al., 2006). Furthermore, loss of H3K9me2 and H3K9me3 in Suv39h HMTase double null-mice results in an increased incidence of B cell lymphomas (Peters et al., 2001). Along this line, it has been recently shown that the methylation status of subtelomeric DNA repeats negatively correlates with telomere length and telomere recombination in a large panel of human cancer cell lines (Vera et al., 2008). This suggests that telomeres suffer epigenetic alterations during tumourigenesis, which in turn are important drivers of telomere length changes in cancer cells. These epigenetic alterations are also expected to impact on the telomeric chromatin structure, improving telomere maintenance by ALT or providing improved access for telomerase to the G-strand overhang (Blasco, 2007). It is not known, however, whether increasing telomere compaction can affect the proliferative potential of cancer cells and impact on telomere homeostasis during organismal ageing.

**Figure 3** Reprogramming of telomeres upon induction of pluripotency in differentiated cells. Telomeres in primary MEFs are shorter than in ES cells and are organized into a highly compact chromatin structure with low TelRNA/TERRA expression. Induction of pluripotency by retroviral transduction of Oct4, Sox2, Klf4, (c-myc), results in nuclear reprogramming and the generation of pluripotent iPSC cells, which are functionally equivalent to ES cells. Reprogramming results in a dramatic upregulation of telomerase activity concomitant with a reduction of H3K9me3, H4K20me3, HP1, and DNA methylation at telomeres and subtelomeres as well as an increase in TelRNA/TERRA expression. Telomerase efficiently elongates telomeres until the natural limit of telomere length of pluripotent mouse ES cells has been reached.
Some severe premature ageing syndromes are caused by mutations in telomerase components giving rise to human syndromes such as Aplastic anaemia (TERC, TERT) (Yamaguchi et al., 2005), Dyskeratosis congenita (DKC1, TERC) and idiopathic pulmonary fibrosis (Tsakiri et al., 2007), or by mutations in various DNA repair genes such as Ataxia telangiectasia (ATM), Werner (WRN) and Bloom syndromes (BLM), Fanconi anaemia (Fanc genes), and Nijmegen breakage syndrome (NBN) (reviewed in Blasco, 2005). These patients display a substantially increased risk of developing disease states characterized by a premature loss of tissue renewal; however, the possible contribution of epigenetic defects at telomeres is still unclear (Mason et al., 2005). Similarly, accelerated telomere shortening can also occur due to environmental influences. In this regard, human population studies recently linked environmental influences (smoking, obesity, or stress) to an accelerated rate of telomere shortening (Cawthon, 2005). Similarly, accelerated telomere shortening will foster our understanding of general telomere derivatives from telomeres and the epigenetic control of telomeric transcripts. The detailed investigation of function of RNAs derived from telomeres and the epigenetic control of telomeric transcripts. The detailed investigation of function of RNAs derived from telomeres and the epigenetic control of telomeric transcripts.

The recent discovery of TelRNA/TERRA transcription is linked to telomere shortening in humans and yeast. The fact that TelRNA/TERRA can antagonize telomere maintenance by telomerase, and the presence of decreased TERRA levels in human cancer samples, could point towards a relevant role of TelRNA/TERRA in limiting telomerase-dependent telomere elongation in cancer cells (Schoeftner and Blasco, 2008). This pinpoints TelRNA/TERRA as a candidate for cancer therapies based on the inhibition of telomerase (Harley, 2008). Another interesting line of evidence for a role of TelRNA/TERRA in disease comes from patients suffering from autosomal-recessive ICF (immunodeficiency, centromeric region instability, facial anomalies) syndrome. These patients display subtelomeric DNA methylation defects and abnormally short and or undetectable telomeres on some chromosome arms. Increased TelRNA/TERRA transcription in these patients points towards a role of telomeric transcripts in ICF (Yehezkel et al., 2008).

We are just beginning to understand the complex regulation of telomeric chromatin and the regulation of telomeric transcripts. The detailed investigation of function of RNAs derived from telomeres and the epigenetic control of telomeres will foster our understanding of general telomere regulation. This line of research is also expected to provide important insight into the roles of telomeres during development, ageing, and a panel of important telomere associated human diseases.

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