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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.

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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 (Blasco *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

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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 hypermethylation 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 *Schyzosaccharomyces pombe*. In *S. pombe*, Rap1 and Rif1 are recruited to double-stranded telomeric repeats through association with the telomere repeat-binding protein Taz1, 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 Taz1–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 Taz1, 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)-ribosylases 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 retrotransposition 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 *modigliani* (*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 $C_{1-2}A/TG_{1-3}$ 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 (Maillet *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; Altaf *et al*, 2007; Fingerman *et al*, 2007). In

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 Taz1, 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 Hiraoka, 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 Clr4 (orthologue of mammalian Suv39h 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 Clr4 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 Taz1 and proteins involved RNAi-mediated heterochromatin formation releases Swi6 from telomeres, suggesting that telomeric heterochromatin is recruited by Taz1 and components of the RNAi machinery (Kanoh *et al*, 2005). Recently, the multi-enzyme complex SHREC, which mediates heterochromatic transcriptional gene silencing in *S. pombe*, was shown to be recruited to telomeres by redundant pathways involving Taz1 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; Ciapponi *et al*, 2006; Oikemus *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 retroelements 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 dependents 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 *spn-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 K3K27me3 mark and bound by Polycomb proteins, which in turn impact on TPE (Boivin *et al*, 2003; Mason *et al*, 2004; Andreyeva *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 H3K9m3 mark, imposed by the Suv39h1 and Suv39h2 HMTases, the mammalian homologues of *S. pombe Clr4* (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-methyalted 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 deacetylase SIRT6 results in elevated H3K9-acetylation levels at human telomeres and can lead to telomere dysfunction (Michishita *et al*, 2008).

Importantly, repressive chromatin marks are also present at subtelomeric repeats. In particular, subtelomeres are enriched for H3K9me3, HP1, H4K20me3, and contain under-acetylated histone H3 and H4 (Benetti *et al*, 2007a, b) (Figure 1). To this end, however, it is not clear whether subtelomeric heterochromatin is a consequence of spreading of a heterochromatic 'island' at telomeres or recruited *in cis* because due to the presence of repetitive elements at subtelomeres.

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; Gonzalo *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 metyltransferases (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; Gonzalo *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 (Fuks *et al*, 2003; Lehnertz *et al*, 2003; Benetti *et al*, 2007b). This suggests the existence of an alternative pathway of DNMT recruitment to subtelomeres.

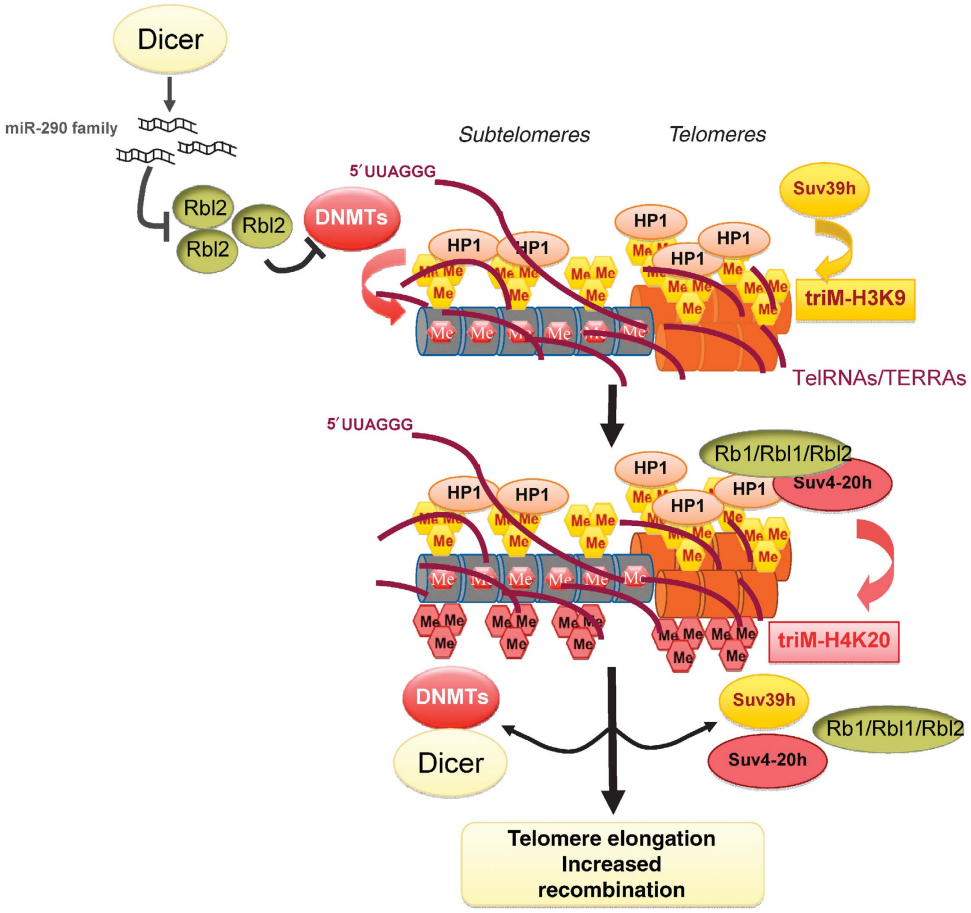

Figure 1 Assembly of mammalian telomeric and subtelomeric heterochromatin. Scheme showing a model for the assembly of telomeric and subtelomeric heterochromatin. Suv39 h1 and h2 HMTases tri-methylate H3K9, which in turn generates a high-affinity site for HP1. HP1 can recruit Suv4-20 h1 and h2 HMTases to telomeres and subtelomeres, thereby tri-methylating H4K20 at these regions. The Rb family proteins (Rb1, Rbl1, and Rbl2) can directly interact with Suv420 HMTases and with HP1, thus influencing the levels of H4K20m3. Dicer is essential for the maturation of miRNAs including the miR290 cluster. miR290 cluster expression in ES cells results in post-transcriptional repression of Rbl2 (p130), a transcriptional repressor of mammalian DNA methyltransferases (DNMTs). Low Rbl2 levels ensure the establishment of global and subtelomeric DNA methylation patterns in ES cells. A lack of mature miRNA290 cluster results in repression of DNMTs by uncontrolled expression of Rbl2. Consequently, a global decrease in DNA methylation unleashes recombination leading to telomere elongation and increased chromatin compaction at telomeric and subtelomeric repeats mediated by Suv39h and Suv4-20h HMTases. Loss of heterochromatin in cells lacking Dicer, DNMTs, Suv39h, or Suv4-20h HMTases results in increased telomeric recombination and telomere elongation.

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 *Dicer1* 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 *Dicer1*-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

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 *Kcnq1ot1* RNAs are involved in genomic imprinting (Pauler *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 *Dicer1*-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 TelRNA/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,

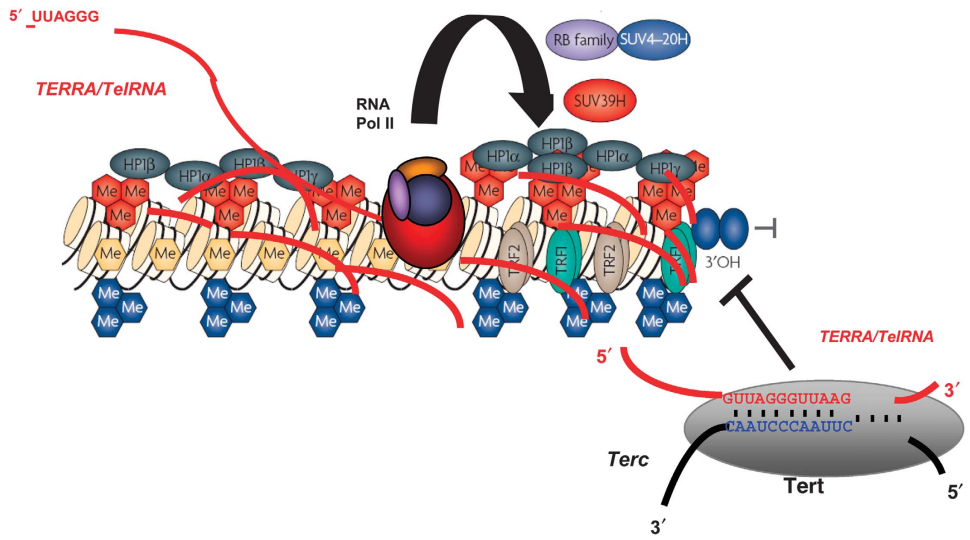

Figure 2 TERRA/TelRNAs associate to telomeric chromatin and may be involved in regulation of telomere length. Model for a role of telomeric RNAs in the regulation of telomere length. TERRA/TelRNA acts as a potent inhibitor of telomerase activity *in vitro*, possibly by formation of RNA:RNA hybrids with the template region of the telomerase RNA component.

2007). This is exemplified by a substantial elongation of telomeres upon the loss of H3K9me3, HP1, and H4K20me3 marks in cells deficient for the Suv39h or Suv4-20h HMTases (Garcia-Cao *et al*, 2004; Benetti *et al*, 2007b) (Figure 1). In both settings, subtelomeric DNA methylation remains unaffected, suggesting that DNMTs can be recruited to subtelomeric regions independently of the Su(var) HMTases. Loss of subtelomeric DNA methylation in DNMT1, DNMT3ab or Dicer deficient ES cells also results in a dramatic telomere elongation, which is accompanied by increased abundance of histone heterochromatic marks at telomeric repeats (Gonzalo *et al*, 2006; Benetti *et al*, 2008a) (Figure 1). In both instances, telomere recombination frequencies are increased, suggesting that repressive marks at telomeric and subtelomeric chromatin are essential to repress recombination events (Figure 1) (Gonzalo *et al*, 2006; Benetti *et al*, 2007a, b). Consistent with this notion, increased numbers of APB bodies (ALT-associated PML bodies) have been detected in all models for impaired telomeric chromatin (Garcia-Cao *et al*, 2004; Gonzalo *et al*, 2006; Benetti *et al*, 2007a, b, 2008a). Of interest, loss of heterochromatic marks at telomeres does not seem to affect TRF1 and TRF2 binding, indicating that shelterin recruitment is uncoupled from telomeric chromatin regulation (Garcia-Cao *et al*, 2004; Gonzalo *et al*, 2006; Benetti *et al*, 2007a, b, 2008a). However, up to date it cannot be excluded that an altered function of shelterin components contributes to ALT.

TPE experiments suggest a model in which increasing telomere-length augments silencing and thus chromatin compaction (Baur *et al*, 2001; Koering *et al*, 2002; Tham and Zakian, 2002). In agreement with this model, progressive telomere shortening in telomerase-deficient MEFs was associated with a continuous loss of H3K9me3, H4K20me3, and HP1 heterochromatic marks at telomeres and subtelomeres, which was accompanied by increased histone H3 and H4 acetylation at these regions (Benetti *et al*, 2007a). Moreover, subtelomeric DNA methylation was significantly reduced upon telomere shortening (Benetti *et al*, 2007a). Similarly, telomere shortening in mice over-expressing negative regulators of telomere length, such as TRF2 transgenic mice, also results in the loss of telomeric and subtelomeric heterochromatic features and altered nucleosome spacing (Benetti *et al*, 2008b). On the other hand, aberrant telomere elongation in the context of DNMT or Dicer1 deficiencies leads to increased density of heterochromatic marks at telomeres (Gonzalo *et al*, 2006; Benetti *et al*, 2007a, 2008a). These findings support a model in which the number of TTAGGG repeats at telomeres directs the epigenetic status of heterochromatin *in cis* and exerts a *trans*-acting effect on chromatin structure at subtelomeric regions. Telomere shortening, as observed during organismal ageing, causes a switch from a repressive to a more open telomeric chromatin status and favour telomere elongation by telomerase or by unleashing telomere recombination (Benetti *et al*, 2007a) (Figure 1). In this regard, recombination-based ALT pathways are activated in telomerase-deficient mice (Blasco *et al*, 1997; Hande *et al*, 1999; Rudolph *et al*, 1999; Herrera *et al*, 2000; Niida *et al*, 2000; Chang *et al*, 2003). More recently, an impact of telomere chromatin on telomerase-dependent telomere elongation has been also shown in the context of nuclear reprogramming (see below).

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 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.

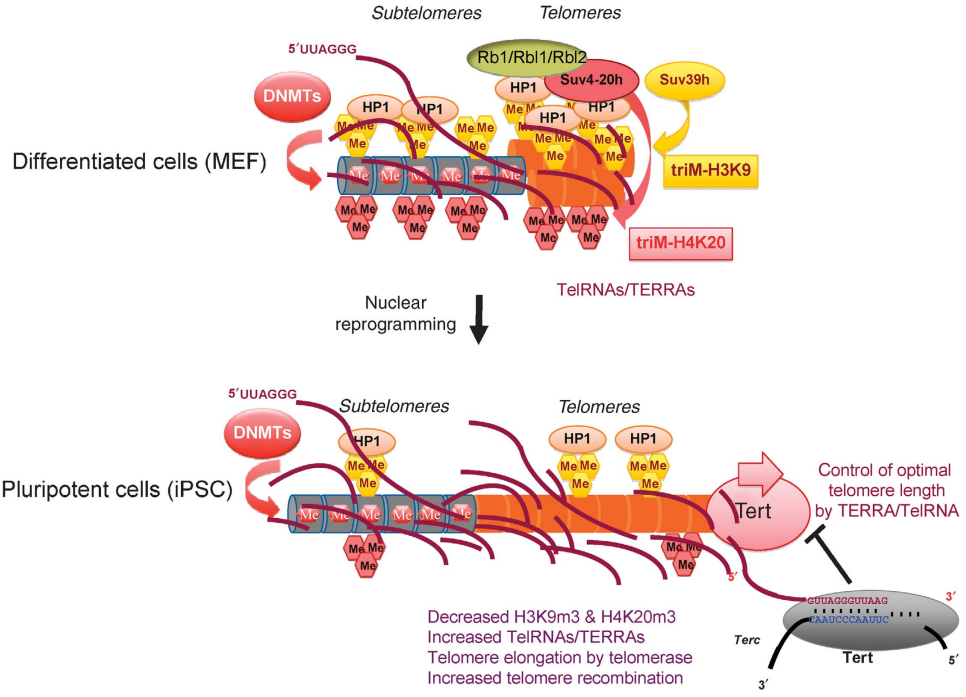

Figure 3 Reprogramming of telomeres upon induction of plutipotency in differentiated cells. Telomeres in primary MEFs are shorter than in ES cells and are organized into a highly compact chromatin structure with low TelRNA/TERRA expression. Induction of pluripotency by retroviral transduction of Oct4, Sox2, Kfl4, (c-myc), results in nuclear reprogramming and the generation of pluripotent iPS 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 idiopatic 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 *et al*, 2003; Epel *et al*, 2004; Valdes *et al*, 2005). Given the important role of epigenetic regulators during organismal ageing, it is tempting to speculate that these factors could also impact on chromatin structure leading to telomere-length abnormalities and disease (Oberdoerffer *et al*, 2008; Dang *et al*, 2009).

The recent discovery of TelRNA/TERRA allows making a new link between disease and telomeres. Increased 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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