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Focus Review

TERRA: telomeric repeat-containing RNA

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Telomeres, the physical ends of eukaryotic chromosomes, consist of tandem arrays of short DNA repeats and a large set of specialized proteins. A recent analysis has identified telomeric repeat-containing RNA (TERRA), a large non-coding RNA in animals and fungi, which forms an integral component of telomeric heterochromatin. TERRA transcription occurs at most or all chromosome ends and it is regulated by RNA surveillance factors and in response to changes in telomere length. TERRA functions that are emerging suggest important roles in the regulation of telomerase and in orchestrating chromatin remodelling throughout development and cellular differentiation. The accumulation of TERRA at telomeres can also interfere with telomere replication, leading to a sudden loss of telomere tracts. Such a phenotype can be observed upon impairment of the RNA surveillance machinery or in cells from ICF (Immunodeficiency, Centromeric region instability, Facial anomalies) patients, in which TERRA is upregulated because of DNA methylation defects in the subtelomeric region. Thus, TERRA may mediate several crucial functions at the telomeres, a region of the genome that had been considered to be transcriptionally silent.

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Introduction

Telomeres are critical at the cellular level for genome stability, whereas at the organismal level, they act as tumour suppressors and may contribute to ageing. The following roles of telomeres are well established: First, telomeres can regulate the lifespan of cells (Harley *et al*, 1990; Allsopp *et al*, 1992; Levy *et al*, 1992; Bodnar *et al*, 1998). Telomere shortening occurs at the distal ends because of a combination of nucleolytic processing of chromosome ends and the end replication problem. This shortening can be counteracted by the cellular reverse-transcriptase telomerase, which uses an internal RNA moiety as a template for the synthesis of telomere repeats (Cech, 2004; Blackburn *et al*, 2006). Telomerase is regulated at individual chromosome ends through telomere-binding proteins to mediate telomere length homoeostasis; however, in humans, telomerase is expressed in most tissues only during the first weeks of embryogenesis (Ulaner and Giudice, 1997). Repression of telomerase in somatic cells is thought to result in a powerful tumour-suppressive function. Short telomeres that accumulate following an excessive number of cell division cycles induce cellular senescence, and this counteracts the growth of pre-malignant lesions. This growth barrier is overcome in most cancers that contain cells that re-express telomerase. Second, telomeres protect natural chromosome ends from unwanted DNA repair activities, which include non-homologous end joining and homologous recombination (Palm and de Lange, 2008). Several telomere-associated proteins are critical for this function. Third, telomeres establish a heterochromatic state at chromosome ends, which is characterized by the presence of trimethylated lysines at position 9 in histone H3 and at position 20 in histone H4, histone hypoacetylation, the accumulation of several isoforms of heterochromatin protein 1 and the hypermethylation of cytosines in CpG dinucleotides that are present in subtelomeric regions (Blasco, 2007; Ottaviani *et al*, 2008). The heterochromatic state of telomeres may contribute to chromosome positioning and movement within the nucleus and also to the regulation of telomerase. Abnormal telomeric chromatin states have been linked to severe stochastic telomere sequence loss phenotypes, suggesting crucial roles of this structure during telomere replication (Michishita *et al*, 2008; Yehezkel *et al*, 2008).

Several telomere-binding proteins that are crucial for the distinct telomere functions outlined above have been identified. These include the six-polypeptide-containing shelterin complex (Palm and de Lange, 2008), which is abundant at telomeres as well as other more rare telomeric proteins. However, relatively little is known mechanistically about which telomere transitions occur between different states for example: when to activate telomerase in a length-dependent and cell-cycle-restricted manner; when to induce cellular senescence when telomeres are very short; and when to lose protection from end fusion upon further erosion. Recent analysis has identified telomeric repeat-containing RNA (TERRA), a large non-coding RNA in animals and fungi, which forms an integral component of telomeric heterochromatin (Azzalin *et al*, 2007; Luke *et al*, 2008; Schoeftner and Blasco, 2008). Earlier reports also indicate the existence of telomere transcription in trypanosomes, birds and Diptera

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(Morcillo *et al*, 1988; Rudenko and Van der Ploeg, 1989; Solovei *et al*, 1994). The finding of ribonucleic acids at telomeres adds a new dimension of how functions at the telomere may be orchestrated and regulated. Here, we review recent progress in the understanding of TERRA biogenesis, turnover and function.

TERRA biogenesis

TERRA is a heterogeneous non-coding RNA that consists of a combination of subtelomeric and telomeric sequences. To date, all individual telomeres tested in mammals can and do produce TERRA transcripts and TERRA is expressed in most tissues (Azzalin *et al*, 2007; Azzalin and Lingner, 2008; Schoeftner and Blasco, 2008). TERRA is transcribed in a centromere to telomere direction, indicating that the transcription start site lies within the subtelomeric sequence. In the yeast, *Saccharomyces cerevisiae*, it has been determined that TERRA transcription occurs at both, telomeres containing or lacking the Y′ element. The Y′ element is a conserved repeat sequence, present at ~50% of all telomeres in *S. cerevisiae*. The 5′-end of TERRA derived from telomeres containing the Y′ element is relatively homogenous, supporting the idea of a defined start site (Luke *et al*, 2008). This suggests that the heterogeneity of TERRA stems from its 3′-end, and indicates either that transcription can terminate in multiple places within the telomeric tract, or that TERRA 3′-ends are differentially processed. TERRA is transcribed largely by RNA polymerase II (RNAPII). This conclusion was reached because of the fact that RNAPII was found associated with telomeric DNA through chromatin immunoprecipitation (ChIP) experiments in human, mouse and yeast cells (Luke *et al*, 2008; Schoeftner and Blasco, 2008). Furthermore, the specific inhibition of RNAPII by α-amanitin reduces the abundance of TERRA in both human and mouse cells (Schoeftner and Blasco, 2008). Moreover, a temperature-sensitive RNAPII allele (*rpb3-2*) prevents the accumulation of TERRA in budding yeast (Luke *et al*, 2008). Although α-amanitin does reduce, to a large extent, the abundance of TERRA in mammalian cells, it is far less efficient in doing so than the general transcriptional inhibitor, actinomycin D. Where actinomycin D treatment resulted in a 50% reduction in TERRA levels within 2.5–3 h (Azzalin *et al*, 2007; Schoeftner and Blasco, 2008), only ~30% of TERRA was degraded after 6 h of α-amanitin treatment, and 20% of initial TERRA was still remaining after 12 h of treatment (Schoeftner and Blasco, 2008). Similar results were seen in mouse cells treated with α-amanitin, in which TERRA levels never decreased below 50% of initial levels even after 12 h of RNAPII inhibition (Schoeftner and Blasco, 2008). These results indicate that although RNAPII has a major function in promoting TERRA transcription, there may be another RNA polymerase (either RNAPI and RNAPIII) that could potentially have a function in telomeric transcription in mammals. The idea of another RNA polymerase being involved in generation of TERRA would be consistent with recent data in which several subunits of RNAPI and RNAPIII (RPABC1, RPAC1, RPA49, RPA2 and RPA1) were found enriched in formaldehyde-crosslinked and partially purified human telomere fractions (Dejardin and Kingston, 2009). Furthermore in trypanosomatida, telomeres can be transcribed both by RNAPII and, in the subset of trypanosomes that show antigenic variation, by the α-amanitin-resistant RNAPI (Rudenko and Van der Ploeg, 1989).

Similar to most products of RNAPII transcription, TERRA is polyadenylated at its 3′-end (Azzalin and Lingner, 2008; Luke *et al*, 2008; Schoeftner and Blasco, 2008). Approximately 7% of human TERRA is polyadenylated (Azzalin and Lingner, 2008), whereas most or all yeast TERRA molecules carry a poly(A) tail (Luke *et al*, 2008). It is unknown whether TERRA polyadenylation involves the 3′-end cleavage of a longer TERRA precursor or whether terminated transcripts are directly polyadenylated. A canonical 5′-AAUAAA-3′ cleavage and a polyadenylation signal is not present at mammalian TERRA 3′-ends, whereas the telomere-derived GU-rich TERRA 3′-ends in yeast bear some resemblance to the canonical U-rich 3′-end-processing signals in this organism. In yeast, the canonical polyadenylation polymerase, Pap1, is responsible for poly(A) addition and it most likely contributes to the stability of the RNA, because TERRA becomes completely destabilized in *pap1-1* temperature-sensitive mutants and polyadenylated TERRA is no longer detectable (Luke *et al*, 2008). How the 5′-end of TERRA is modified has not yet been addressed. If it contains a 7-methylguanosine (m^7G) cap structure as do mRNAs, it likely gets uncapped in *S. cerevisiae* at the time when it becomes a target for Rat1-dependent degradation, which recognizes 5′-uncapped mono-phosphate structures (see below).

Although the specific transcription factors that are responsible for the transcriptional regulation of TERRA have not yet been elucidated, there are indications that some of the telomere-bound proteins that make up the protective cap of telomeres, along with the heterochomatic markers present at telomeres, are involved in controlling TERRA levels. As discussed in the Introduction section, telomeres are often in a heterochromatic state (Blasco, 2007; Ottaviani *et al*, 2008). Trichostatin A, a histone deacetylase inhibitor, results in increased TERRA levels in human cells (Azzalin and Lingner, 2008) as do mutations in the histone methyltransferases, SUV39H1/2 and SUV4-20H1/2, in mouse cells (Schoeftner and Blasco, 2008) and mutations in the DNA methyltransferase 3b (DNMT3B) in human cells (Yehezkel *et al*, 2008). These results indicate that TERRA levels are partially controlled through subtle changes in the local heterochromatin, although indirect effects could not be distinguished in these experiments.

Shelterin is a complex composed of six proteins (TRF1, TRF2, Rap1, TIN2, TPP1 and POT1), which binds to and protects telomeres (Palm and de Lange, 2008). Interestingly, TRF1 can interact with RNAPII as shown through co-immunoprecipitation experiments (Schoeftner and Blasco, 2008). It has been reported that when TRF1 was depleted using small-interfering RNA (siRNA), overall levels of TERRA decreased twofold, which suggests that TRF1 supports TERRA transcription (Schoeftner and Blasco, 2008). It is unlikely, however, that TRF1 acts as a conventional transcriptional activator. First, depletion of TRF1 does not result in a corresponding loss of RNAPII associated with telomeric DNA. Second, TRF1 is restricted to the telomeric tract and likely does not spread into the subtelomere, wherein transcription is presumed to initiate. Finally, the overproduction of TRF1 results in less TERRA production, rather than more, although it should be noted that telomeres shorten in length upon TRF1 overexpression and this may also influence TERRA transcription (Benetti *et al*, 2008; Schoeftner and Blasco, 2008; Munoz *et al*, 2009). Overall, these data suggest that TRF1 promotes RNAPII progression through the telo-

meric tract, but it does not directly influence RNAPII recruitment (see Figure 1, TERRA transcription).

It is important to re-visit all of the known telomere-associated proteins (shelterin and non-shelterin components) and re-assess their functions in terms of TERRA regulation. This also includes several proteins that could be potentially involved in transcription elongation and regulation, which were detected in partially purified telomere fractions (Dejardin and Kingston, 2009).

TERRA at telomeres

TERRA is exclusively found in nuclear RNA fractions from human and mouse cells, and is likely restricted to the nucleus in yeast as well (see below). Furthermore, TERRA can be detected using RNA fluorescence *in situ* hybridization (RNA-FISH) at a subset of telomeres in interphase cells, and it is present on human and mouse chromosome ends in the metaphase of the cell cycle (Azzalin *et al*, 2007; Schoeftner and Blasco, 2008). The fact that TERRA associates with telomeres suggests that TERRA may be an integral part of the telomere and could potentially be important for structural integrity. However, because TERRA is detected by FISH in only a subset of telomeres, it seems more likely that TERRA is not an essential or a permanent constituent of the telomeric chromatin, but rather has a transient structural function during telomere assembly or it may have regulatory roles in a subset of telomeres that may correspond to a specific functional state. A telomere autonomous regulation would be compatible with telomeric-state-dependent functions; for example, in the establishment, but not necessarily the maintenance, of telomeric heterochromatin or in the regulation of telomerase *in cis* in a length-dependent manner. Although TERRA remains associated with telomeres, it has not been shown whether TERRA molecules can move from one telomere to another or whether the RNA remains associated with the telomere that it was transcribed from.

There are two potential means by which TERRA could be tethered to telomeres. One possibility would be through interactions of a telomeric protein with the RNA or, alternatively, through a direct interaction of TERRA with the telomeric DNA. Both modes of association are possible and are indeed not mutually exclusive. In yeast, mainly indirect evidence has hinted that DNA–RNA hybrid formation is occurring to some extent. It was shown that overexpression

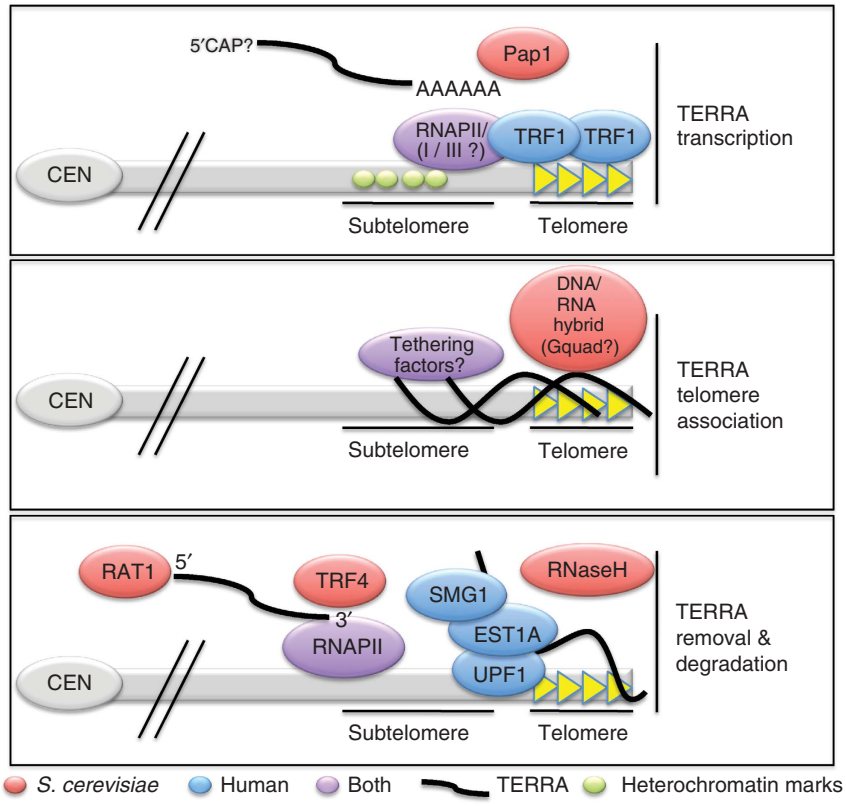

Figure 1 TERRA Biogenesis, telomere association and displacement from telomeres. TERRA Biogenesis (upper panel)—TERRA is an RNAPII-dependent transcript whose transcription initiates within the subtelomeric sequences and proceeds into the telomeric tract. Human TRF1 may promote transcription through the telomere tract through its association with RNAPII. A fraction of TERRA is polyadenylated through the canonical poly(A) polymerase, Pap1. The 5′-end structure that exists on TERRA molecules has not yet been reported. Telomere association (middle panel)—TERRA colocalizes with telomeres as visualized by RNA-FISH experiments on human interphase and metaphase chromosomes. Indirect evidence suggests that at least a portion of TERRA is bound to telomeres through base pairing with telomeric DNA. Undefined RNA–protein interactions or intermolecular G-quadruplex structures (Gquad) may also tether TERRA to telomeric DNA. Telomere removal and degradation (lower panel)—the 5′ to 3′ exonuclease, Rat1, directly degrades TERRA molecules and can itself be found associated with telomeres. Rat1 can degrade its other target RNAs in a co-transcriptional manner (as depicted here), whether TERRA is degraded by Rat1 in a similar manner has yet to be determined. The poly(A) polymerase, Trf4, also contributes to TERRA degradation, although likely as a minor contributor compared with Rat1. The NMD factors, UPF1, SMG1 and EST1A/SMG6, all contribute to TERRA removal from telomeres. Inhibition of any of these factors results in both more and brighter TERRA foci, although overall TERRA levels as assessed by northern blot analysis remain largely unchanged. RNaseH overexpression also reduces cellular TERRA levels when Rat1 function is impaired.

of RNaseH, which degrades the RNA moiety in a DNA–RNA hybrid, could specifically reduce TERRA levels in a genetic background in which TERRA accumulates (Luke *et al*, 2008). Furthermore, it was shown *in vitro* that telomeric DNA and telomeric RNA together form an intermolecular heat-stable G-quadruplex structure (Xu *et al*, 2008). To date, there is no evidence that telomeric proteins are involved in the tethering of TERRA to chromosome ends. POT1, a good candidate that binds to telomeric ssDNA in mammals, does not interact with TERRA *in vitro* (S Redon and JL, unpublished data). Another candidate in mammalian cells is hnRNP A1, an abundant RNA-binding protein that binds specifically to UAGGGA/U repeats *in vitro* (Burd and Dreyfuss, 1994). UUAGGG repeats are very abundant in the portion of mammalian TERRA that is transcribed from telomeric repeats. hnRNP A1 has also been implicated in telomere length control (LaBranche *et al*, 1998) and it binds to single-stranded telomeric repeats *in vitro* (McKay and Cooke, 1992) and to telomeres *in vivo* (Zhang *et al*, 2006). Thus, it might function as a molecular bridge between telomeric DNA and TERRA. Telomere purification also showed other RNA-binding proteins, including FUS, RNA-binding protein 14, cold-induced RNA-binding protein and RNA-binding protein 8A (Dejardin and Kingston, 2009), all of which might be involved in TERRA localization to telomeres (Figure 1, see TERRA telomere association).

TERRA regulators

Several negative regulators of both TERRA stability and TERRA localization have now been documented. The nonsense-mediated RNA decay (NMD) machinery, which is best known for its role in the degradation of mRNAs containing pre-mature termination codons, has also an important function in the displacement of TERRA from human telomeres (Azzalin *et al*, 2007; Chawla and Azzalin, 2008). From the key players in the NMD pathway, UPF1, SMG1 and EST1A/SMG6 were shown to regulate TERRA at telomeres, as their depletion by RNA interference (RNAi) led to a several-fold increase of telomeres with TERRA foci. However, neither total levels of TERRA nor TERRA half-life was affected by the depletion of these factors. NMD proteins may interact directly with TERRA at telomeres because NMD proteins can be detected at telomeres *in vivo* by ChIP (Azzalin *et al*, 2007). Furthermore, the NMD factor, EST1A/SMG6, physically interacts with telomerase (Reichenbach *et al*, 2003; Snow *et al*, 2003; Redon *et al*, 2007). Two possible scenarios on how NMD factors diminish TERRA abundance at telomeres can be envisioned. First, UPF1/SMG1/EST1A may be required only for the displacement of TERRA without influencing the rate of TERRA degradation. Second, UPF1/SMG1/EST1A may be involved in TERRA degradation, but only locally at the telomere, and such small changes in TERRA levels are not discernable on northern blots. In this respect, it is interesting to note that EST1A/SMG6 contains a PIN domain at its C terminus, which shows RNA endonuclease activity during NMD (Huntzinger *et al*, 2008; Eberle *et al*, 2009). Distinguishing between these two possibilities will be technically challenging.

It is not known whether yeast TERRA resides at the telomere as is the case in complex eukaryotes; therefore, regulators of TERRA localization have not yet been addressed in this organism. However, it is intriguing that inactivation of the NMD machinery in *S. cerevisiae* leads to shorter telomeres, although it had been proposed that this effect was due to increased expression levels of the telomere-capping factors, Stn1 and Ten1, which were observed in NMD mutants (Dahlseid *et al*, 2003; Enomoto *et al*, 2004). In yeast, NMD mutants did not affect overall TERRA levels (Luke *et al*, 2008), but similar to human cells, they might only locally affect TERRA, rendering the effect undetectable on northern blots.

It has been determined, however, that the stability of TERRA in yeast is tightly regulated by the 5′- to 3′-exonuclease, Rat1. In wild-type cells, TERRA is almost undetectable using standard northern blotting procedures, but accumulates significantly when Rat1 is inactive. These effects are likely to be direct. Mutation in the *RAT1* gene increases the half-life of TERRA; moreover, Rat1 can be found to be associated with telomeres through ChIP experiments. On the other hand, Xrn1, the cytoplasmic paralog of Rat1, has no effect on TERRA levels, supporting the notion that, similar to its human counterpart, yeast TERRA is nuclear. Trf4, an alternative poly(A) polymerase that is often associated with exosomal-mediated degradation, has no effect on TERRA levels when mutated singly; however; when combined with a Rat1 mutation, the double-mutant shows a dramatic increase in TERRA levels. This suggests that Trf4 has a minor function in TERRA degradation on its own; however, when Rat1 is mutated and TERRA levels are higher, the contribution of Trf4 becomes more noticeable. In this respect, analysis of double mutants (with one mutation being the *rat1-1* mutation) may be instrumental in finding additional regulators of TERRA stability and, furthermore, of TERRA transcriptional activation (Figure 1, see TERRA removal and degradation).

TERRA function

Telomerase

As discussed above, in human cells, TERRA is displaced or degraded at telomeres by NMD factors that physically interact with telomeric chromatin (Azzalin *et al*, 2007). Among these factors, EST1A/SMG6 was also identified through its sequence similarity with the *S. cerevisiae* telomerase-associated protein, Est1 (Reichenbach *et al*, 2003; Snow *et al*, 2003). Moreover, similar to yeast Est1, human EST1A/SMG6 physically interacts with telomerase (Reichenbach *et al*, 2003; Snow *et al*, 2003; Redon *et al*, 2007). The *S. cerevisiae* Est1 protein binds directly to telomerase RNA and to the single-stranded telomeric DNA-binding protein, Cdc13p, to recruit telomerase to chromosome ends in the S phase. Therefore, in *est1*Δ cells, telomerase cannot act and telomeres shorten (Lundblad and Szostak, 1989; Pennock *et al*, 2001; Chan *et al*, 2008). The recruitment and activation of telomerase at chromosome ends is not well understood in complex eukaryotes, including humans, and the role of EST1A/SMG6 in this process is uncertain. The association of EST1A/SMG6 with telomerase is compatible with a role in telomerase regulation, but its effects on TERRA displacement at telomeres suggest that EST1A/SMG6 may regulate telomerase through TERRA. Indeed, the TERRA-mimicking RNA oligonucleotide $(UUAGGG)_3$ inhibits telomerase activity *in vitro* as determined in the TRAP assay (Schoeftner and

Blasco, 2008), and because TERRA is more abundant when telomeres are long (Azzalin *et al*, 2007; Schoeftner and Blasco, 2008), it has been proposed that telomerase may be regulated by TERRA in a telomere-length-dependent manner (Schoeftner and Blasco, 2008). Consistent with a negative regulatory role of TERRA for telomerase is also the finding that in several tumours, lower levels of TERRA were detected than in the corresponding normal tissue (Schoeftner and Blasco, 2008). Furthermore, in tumour-derived and in *in vitro*-immortalized cell lines, the presence of telomerase activity correlates with lower TERRA levels when compared with cell lines that use the recombination-based ALT pathway to maintain telomeric ends (Ng *et al*, 2009). Genetic experiments in *S. cerevisiae* provide more conclusive evidence that TERRA indeed regulates telomerase *in vivo*. In the *rat1-1* mutant background, TERRA is upregulated and telomeres are shorter than in wild-type cells because of an impairment in telomerase-mediated telomere elongation (Luke *et al*, 2008). The overexpression of RNaseH reduced TERRA levels and could overcome the short telomere phenotype, indicating that a DNA/TERRA hybrid was responsible for the effect. Further support for the role of TERRA in inhibiting telomerase *in vivo* stems from an observation that forced telomere transcription (through the use of a strong Gal promoter) leads to telomere shortening of the transcribed telomere *in cis* (Sandell *et al*, 1994). It will be interesting to elucidate the mode of inhibition of the enzymatic activity of telomerase by TERRA and to determine its possible effects on the recruitment of telomerase to telomeres.

Heterochromatin

Large ncRNAs can mediate epigenetic changes by recruiting chromatin-remodelling complexes to specific genomic loci. For example, in the human HOXD locus, the 2.2-kb HOTAIR ncRNA interacts with the polycomb repressive complex 2, resulting in trimethylated histone H3 at lysine 27 (H3K27) (Rinn *et al*, 2007). Similarly, for X chromosome inactivation of one of the two X chromosomes in females, transcription of the 1.6-kb RepA RNA within the Xist locus is involved in recruiting polycomb group proteins for H3K27 trimethylation (Zhao *et al*, 2008). Thus, it would not be surprising if TERRA also functions as a recruiter for chromatin-modifying enzymes at telomeres to establish or stabilize the heterochromatic state.

More clearly established is another parallel between Xist and TERRA, in which both are regulated by SMG proteins. Female mouse embryonic stem (ES) cell lines depleted for RENT1 (the murine ortholog of UPF1) or for UPF2 fail to form Xist RNA domains upon differentiation, and subsequently fail to undergo X-inactivation (Ciaudo *et al*, 2006). Thus, nuclear SMG proteins may contribute to chromatin organization at several loci that involve long ncRNAs. However, methods that allow a direct manipulation of TERRA transcription or stability will be required to elucidate this issue. Furthermore, TERRA has been shown to colocalize with Xist (Schoeftner and Blasco, 2008; Zhang *et al*, 2009) and specifically with the distal telomere of the inactive X chromosome in female mouse embryo fibroblasts (Zhang *et al*, 2009). Curiously, TERRA localization to the inactive X does not depend on Xist expression, which also indicates that TERRA is not dependent on X chromosome inactivation but rather likely plays a telomere-specific heterochromatinization role. In the fission yeast *Schizosaccharomyces pombe*, siRNAs are produced from subtelomeric repetitive elements referred to as dh repeats, whereby both the RNAi RITS (RNA-induced initiation of transcriptional silencing) pathway and the major telomere-binding protein Taz1 (ortholog of human TRF1 and TRF2) establish Swi6 (ortholog of HP1) heterochromatin at telomeres in parallel pathways (Kanoh *et al*, 2005). Two lines of evidence suggest a possible function for RNAi in maintaining the telomeric structure and TERRA regulation in mammals. First, upon transfection of mouse cells with synthetic siRNAs, irrespective of the siRNA sequence used, the Argonaute 1 protein associates with telomeres and TERRA expression increases (Ho *et al*, 2008). Second, the inactivation of Dicer had effects on TERRA levels in mouse ES cells in two different studies, although it needs to be resolved whether levels increase or decrease, as the reports were conflicting on this issue (Schoeftner and Blasco, 2008; Zhang *et al*, 2009). It will be interesting to determine whether TERRA also base pairs with other cellular RNAs and whether it is directly processed into small RNA species by Dicer, which cleaves long dsRNA molecules into short fragments as a first step in the RNAi pathway. Intriguingly, telomeric antisense transcripts, termed 'ARRET', have been detected in budding yeast (Houseley *et al*, 2007; Luke *et al*, 2008), although the canonical RNAi machinery does not exist in this organism.

Development

Recent reports have also shown that TERRA may be regulated developmentally and, in turn, may be important for orchestrating some aspects of the complex chromosome transactions that occur during cellular differentiation (Schoeftner and Blasco, 2008; Marion *et al*, 2009; Zhang *et al*, 2009). Interestingly, in undifferentiated ES cells, TERRA can be found to be associated with both X chromosomes in females and with both X and Y chromosomes in males (Zhang *et al*, 2009). After differentiation, only one sex chromosome is marked with TERRA (Xi in females and Y in males,), indicating that TERRA localization is developmentally regulated. Consistently, when differentiated fibroblasts are induced to form pluripotent stem cells with the reprogramming factors, Oct3/4, Sox2, Klf4 and cMyc, TERRA levels are greatly increased (Marion *et al*, 2009). It is not yet clear whether the reprogramming itself is responsible for increased TERRA levels or whether the reprogramming factors are direct transcriptional regulators of TERRA.

TERRA dysfunction

Replication

Human cells depleted for UPF1, hEST1A and SMG1 increase the number of TERRA foci at chromosome ends and they lose entire telomeric tracts at a high rate. UPF1 depletion also causes severe DNA damage elsewhere in the genome and ATR-dependent checkpoint activation in the S phase (Azzalin and Lingner, 2006). The association of NMD factors with chromatin and their enrichment at telomeres suggest a causal link between proper TERRA displacement from telomeres and telomere integrity (Azzalin *et al*, 2007), and possibly the removal of other chromatin-associated RNA molecules by NMD factors elsewhere in the genome. Removal of TERRA and potentially other RNAs seems to be important for proper DNA replication and S-phase progression also in *S. cerevisiae*,

as DNA replication defects were apparent in the *rat1-1* mutant cells that upregulate TERRA (Luke *et al*, 2008). Replication might be impeded when TERRA hybridizes to single-stranded telomeric DNA, leading to the arrest of the replication fork and to the generation of double-stranded breaks in the telomeric tract (Figure 2). Interestingly, human UPF1 also physically interacts with DNA polymerase δ (Carastro *et al*, 2002; Azzalin and Lingner, 2006). Thus, one could speculate that DNA polymerase δ recruits UPF1 in conjunction with other SMG factors to displace TERRA from telomeres during progression of the replication fork. One speculative hypothesis would be that upon fork stalling and SMG recruitment to the replication fork, telomerase, through EST1A/SMG6, is also recruited in the event that *de novo* telomere addition is required after replication fork collapse to heal the broken telomere, or simply to ensure that telomerase is correctly deposited at the chromosome end in a timely manner (Figure 2). This model, as well as other models that may explain the observed phenotypes, needs to be tested in the future.

Several features seen in SMG-depleted cells were recently also detected in cells derived from ICF (Immunodeficiency, Centromeric region instability, Facial anomalies) patients, who carry defective genes for DNA methyltransferase 3b (DNMT3b). DNMT3b defects lead to hypomethylation of CpG dinucleotides in heterochromatic regions, including pericentromeric satellites, the inactive X chromosome and in subtelomeric regions. Intriguingly, ICF patient-derived cells also harbour short telomeres and display increased TERRA levels (Yehezkel *et al*, 2008). Furthermore, many chromosome ends in ICF patient-derived cells lacked detectable telomeric DNA signals altogether. Thus, as in the case of SMG depletion, TERRA upregulation correlates with a sudden telomere loss in ICF patient-derived cells.

Perspective

The notion that the ends of our chromosomes are transcribed into TERRA and that telomeric chromatin contains RNA molecules adds another fascinating level of complexity and provides another example of a structural RNA molecule that may prove to be of utmost importance. TERRA and other chromatin-associated RNA molecules have the unique feature that they are synthesized at the site of their action. Thus, their synthesis rate is closely related to changes in the chromatin structure. Therefore, TERRA seems ideally suited to initiate an appropriate response at a telomere in reaction to other local changes at a telomere, which may involve length

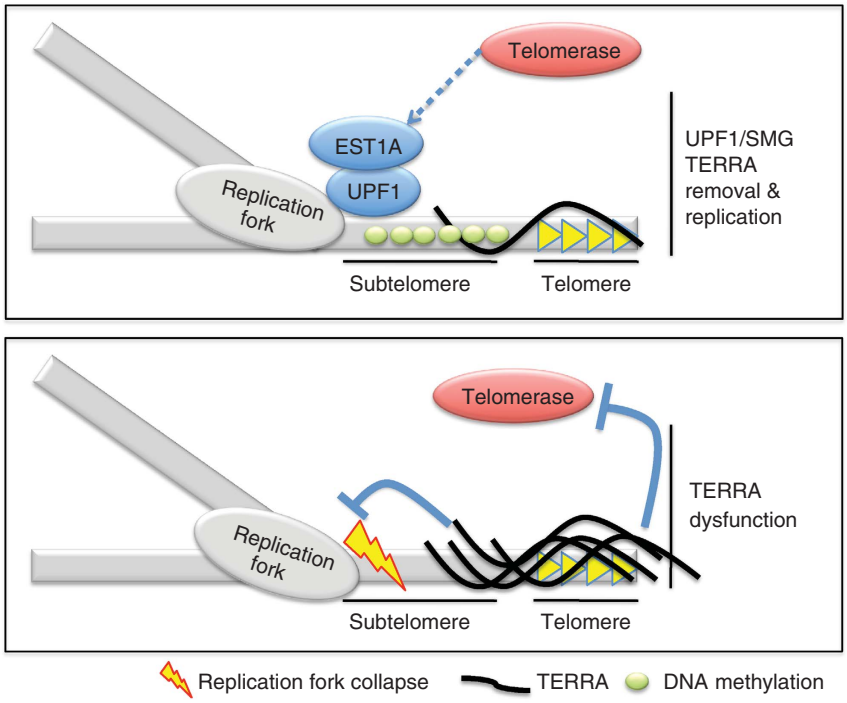

Figure 2 Telomere dysfunction associated with TERRA. TERRA removal upon DNA replication (upper panel): As the replication fork passes into the subtelomeric and telomeric regions, TERRA may potentially create an obstacle through the formation of an RNA/DNA hybrid. DNA methylation within the subtelomeric tract is one potential means of ensuring that the levels of the telomeric transcript remain low. hUPF1, which associates with the replicative polymerase, may encounter such hybrids and be involved in their active displacement through its helicase activity. EST1A/SMG6 may also be involved in actively removing TERRA molecules, perhaps through its endonuclease activity. Moreover, EST1A could potentially be involved in the recruitment of telomerase to irreversible fork pausing/collapse events in which rapid *de novo* telomere addition is required. TERRA accumulation and telomere dysfuntion (lower panel): In ICF patient cells, levels of DNA methylation in the subtelomeric regions are greatly reduced because of mutations in the DNA methyltransferase, DNMT3b. These cells consequently express higher levels of TERRA and may, as a result, experience an increased frequency of replication fork collapse resulting in the observed increase in telomere loss events. These effects would potentially be augmented in these cells because of the inherent ability of TERRA to inhibit telomerase activity and therefore to prevent telomere healing at the collapsed fork. These phenotypes are re-capitulated in cells in which SMG protein function has been experimentally reduced, in that TERRA accumulates at telomeres and cells lose large tracts of telomeric DNA. In this instance, the combination of TERRA accumulation and lack of SMG proteins may together inhibit telomerase activity and prevent its recruitment, respectively, to collapsed forks within the telomeric tracts.

changes or perhaps cell-cycle-dependent changes. It has been proposed for the centromeric heterochromatin in fission yeast that a boost of transcription occurs in the S phase of the cell cycle in order to trigger assembly of the heterochromatin, which, when fully assembled, prevents further transcription at the centromere during other phases of the cell cycle (Kloc and Martienssen, 2008). Similarly, TERRA may trigger heterochromatin assembly at the telomere, contribute to the regulation of telomerase at individual chromosome ends in a length-dependent manner and, perhaps, even be involved in DNA damage response at short telomeres.

Several of the above-discussed biological functions of TERRA are still speculative, and the underlying mechanisms of action remain to be discovered. The telomeric dysfunctions of TERRA that can lead to sudden telomere loss are also very ill defined. Clearly, it is important to analyze the mechanisms of TERRA biogenesis and turnover, identify the protein factors that interact and collaborate with TERRA and elucidate their mechanisms of action. These studies may also uncover or show possible functions of TERRA during tumourigenesis or animal development. These are exciting times on planet earth (TERRA)—a feel of change is coming on for our views on how chromosome ends are regulated and how they function.

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References

Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB (1992) Telomere length predicts replicative capacity of human fibroblasts. *Proc Natl Acad Sci USA* **89:** 10114–10118

Azzalin CM, Lingner J (2006) The human RNA surveillance factor UPF1 is required for S phase progression and genome stability. *Curr Biol* **16:** 433–439

Azzalin CM, Lingner J (2008) Telomeres: the silence is broken. *Cell Cycle* **7:** 1161–1165

Azzalin CM, Reichenbach P, Khoriauli L, Giulotto E, Lingner J (2007) Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. *Science* **318:** 798–801

Benetti R, Schoeftner S, Munoz P, Blasco MA (2008) Role of TRF2 in the assembly of telomeric chromatin. *Cell Cycle* **7:** 3461–3468

Blackburn EH, Greider CW, Szostak JW (2006) Telomeres and telomerase: the path from maize, *Tetrahymena* and yeast to human cancer and aging. *Nat Med* **12:** 1133–1138

Blasco MA (2007) The epigenetic regulation of mammalian telomeres. *Nat Rev* **8:** 299–309

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* **279:** 349–352

Burd CG, Dreyfuss G (1994) RNA binding specificity of hnRNP A1: significance of hnRNP A1 high-affinity binding sites in pre-mRNA splicing. *EMBO J* **13:** 1197–1204

Carastro LM, Tan CK, Selg M, Jack HM, So AG, Downey KM (2002) Identification of delta helicase as the bovine homolog of HUPF1: demonstration of an interaction with the third subunit of DNA polymerase delta. *Nucleic Acids Res* **30:** 2232–2243

Cech TR (2004) Beginning to understand the end of the chromosome. *Cell* **116:** 273–279

Chan A, Boule JB, Zakian VA (2008) Two pathways recruit telomerase to *Saccharomyces cerevisiae* telomeres. *PLoS Genet* **4:** e1000236

Chawla R, Azzalin CM (2008) The telomeric transcriptome and SMG proteins at the crossroads. *Cytogenet Genome Res* **122:** 194–201

Ciaudo C, Bourdet A, Cohen-Tannoudji M, Dietz HC, Rougeulle C, Avner P (2006) Nuclear mRNA degradation pathway(s) are implicated in Xist regulation and X chromosome inactivation. *PLoS Genet* **2:** e94

Dahlseid JN, Lew-Smith J, Lelivelt MJ, Enomoto S, Ford A, Desruisseaux M, McClellan M, Lue N, Culbertson MR, Berman J (2003) mRNAs encoding telomerase components and regulators are controlled by UPF genes in *Saccharomyces cerevisiae*. *Eukaryot Cell* **2:** 134–142

Dejardin J, Kingston RE (2009) Purification of proteins associated with specific genomic loci. *Cell* **136:** 175–186

Eberle AB, Lykke-Andersen S, Muhlemann O, Jensen TH (2009) SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells. *Nat Struc Mol Biol* **16:** 49–55

Enomoto S, Glowczewski L, Lew-Smith J, Berman JG (2004) Telomere cap components influence the rate of senescence in telomerase-deficient yeast cells. *Mol Cell Biol* **24:** 837–845

Harley CB, Futcher AB, Greider CW (1990) Telomeres shorten during ageing of human fibroblasts. *Nature* **345:** 458–460

Ho CY, Murnane JP, Yeung AK, Ng HK, Lo AW (2008) Telomeres acquire distinct heterochromatin characteristics during siRNA-induced RNA interference in mouse cells. *Curr Biol* **18:** 183–187

Houseley J, Kotovic K, El Hage A, Tollervey D (2007) Trf4 targets ncRNAs from telomeric and rDNA spacer regions and functions in rDNA copy number control. *EMBO J* **26:** 4996–5006

Huntzinger E, Kashima I, Fauser M, Sauliere J, Izaurralde E (2008) SMG6 is the catalytic endonuclease that cleaves mRNAs containing nonsense codons in metazoan. *RNA* **14:** 2609–2617

Kanoh J, Sadaie M, Urano T, Ishikawa F (2005) Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres. *Curr Biol* **15:** 1808–1819

Kloc A, Martienssen R (2008) RNAi, heterochromatin and the cell cycle. *Trends Genet* **24:** 511–517

LaBranche H, Dupuis S, Ben-David Y, Bani MR, Wellinger RJ, Chabot B (1998) Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase. *Nat Genet* **19:** 199–202

Levy MZ, Allsopp RC, Futcher AB, Greider CW, Harley CB (1992) Telomere end-replication problem and cell aging. *J Mol Biol* **225:** 951–960

Luke B, Panza A, Redon S, Iglesias N, Li Z, Lingner J (2008) The Rat1p 5′ to 3′ exonuclease degrades telomeric repeat-containing RNA and promotes telomere elongation in *Saccharomyces cerevisiae*. *Mol Cell* **32:** 465–477

Lundblad V, Szostak JW (1989) A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell* **57:** 633–643

Marion RM, Strati K, Li H, Tejera A, Schoeftner S, Ortega S, Serrano M, Blasco MA (2009) Telomeres acquire embryonic stem cell characteristics in induced pluripotent stem cells. *Cell Stem Cell* **4:** 141–154

McKay SJ, Cooke H (1992) hnRNP A2/B1 binds specifically to single stranded vertebrate telomeric repeat TTAGGGn. *Nucleic Acids Res* **20:** 6461–6464

Michishita E, McCord RA, Berber E, Kioi M, Padilla-Nash H, Damian M, Cheung P, Kusumoto R, Kawahara TL, Barrett JC, Chang HY, Bohr VA, Ried T, Gozani O, Chua KF (2008) SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. *Nature* **452:** 492–496

Morcillo G, Barettino D, Carmona MJ, Carretero MT, Diez JL (1988) Telomeric DNA sequences differentially activated by heat shock in two *Chironomus* subspecies. *Chromosoma* **96:** 139–144

Munoz P, Blanco R, de Carcer G, Schoeftner S, Benetti R, Flores JM, Malumbres M, Blasco MA (2009) TRF1 controls telomere length and mitotic fidelity in epithelial homeostasis. *Mol Cell Biol* **29:** 1608–1625

Ng LJ, Cropley JE, Pickett HA, Reddel RR, Suter CM (2009) Telomerase activity is associated with an increase in DNA methylation at the proximal subtelomere and a reduction in telomeric transcription. *Nucleic Acids Res* **37:** 1152–1159

Ottaviani A, Gilson E, Magdinier F (2008) Telomeric position effect: from the yeast paradigm to human pathologies? *Biochimie* **90:** 93–107

Palm W, de Lange T (2008) How shelterin protects mammalian telomeres. *Annu Rev Genet* **42:** 301–334

Pennock E, Buckley K, Lundblad V (2001) Cdc13 delivers separate complexes to the telomere for end protection and replication. *Cell* **104:** 387–396

Redon S, Reichenbach P, Lingner J (2007) Protein RNA and protein protein interactions mediate association of human EST1A/SMG6 with telomerase. *Nucleic Acids Res* **35:** 7011–7022

Reichenbach P, Hoss M, Azzalin CM, Nabholz M, Bucher P, Lingner J (2003) A human homolog of yeast Est1 associates with telomerase and uncaps chromosome ends when overexpressed. *Curr Biol* **13:** 568–574

Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY (2007) Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* **129:** 1311–1323

Rudenko G, Van der Ploeg LH (1989) Transcription of telomere repeats in protozoa. *EMBO J* **8:** 2633–2638

Sandell LL, Gottschling DE, Zakian VA (1994) Transcription of a yeast telomere alleviates telomere position effect without affecting chromosome stability. *Proc Natl Acad Sci USA* **91:** 12061–12065

Schoeftner S, Blasco MA (2008) Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. *Nat Cell Biol* **10:** 228–236

Snow BE, Erdmann N, Cruickshank J, Goldman H, Gill RM, Robinson MO, Harrington L (2003) Functional conservation of the telomerase protein Est1p in humans. *Curr Biol* **13:** 698–704

Solovei I, Gaginskaya ER, Macgregor HC (1994) The arrangement and transcription of telomere DNA sequences at the ends of lampbrush chromosomes of birds. *Chromosome Res* **2:** 460–470

Ulaner GA, Giudice LC (1997) Developmental regulation of telomerase activity in human fetal tissues during gestation. *Mol Hum Reprod* **3:** 769–773

Xu Y, Kimura T, Komiyama M (2008) Human telomere RNA and DNA form an intermolecular G-quadruplex. *Nucleic Acids Symp Ser (Oxf)* **52:** 169–170

Yehezkel S, Segev Y, Viegas-Pequignot E, Skorecki K, Selig S (2008) Hypomethylation of subtelomeric regions in ICF syndrome is associated with abnormally short telomeres and enhanced transcription from telomeric regions. *Hum Mol Genet* **17:** 2776–2789

Zhang L, Ogawa Y, Ahn J, Namekawa S, Silva S, Lee JT (2009) Telomeric RNAs mark sex chromosomes in stem cells. *Genetics*; e-pub ahead of print 24 February 2009; doi: genetics. 109.103093 [pii]10.1534/genetics.109.103093

Zhang QS, Manche L, Xu RM, Krainer AR (2006) hnRNP A1 associates with telomere ends and stimulates telomerase activity. *RNA* **12:** 1116–1128

Zhao J, Sun BK, Erwin JA, Song JJ, Lee JT (2008) Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. *Science* **322:** 750–756

