A balance between elongation and trimming regulates telomere stability in stem cells

Teresa Rivera1, Candy Haggblom1, Sandro Cosconati2 & Jan Karlseder1

Telomere length maintenance ensures self-renewal of human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs); however, the mechanisms governing telomere length homeostasis in these cell types are unclear. Here, we report that telomere length is determined by the balance between telomere elongation, which is mediated by telomerase, and telomere trimming, which is controlled by XRCC3 and Nbs1, homologous recombination proteins that generate single-stranded C-rich telomeric DNA and double-stranded telomeric circular DNA (T-circles), respectively. We found that reprogramming of differentiated cells induces T-circle and single-stranded C-rich telomeric DNA accumulation, indicating the activation of telomere trimming pathways that compensate telomerase-dependent telomere elongation in hiPSCs. Excessive telomere elongation compromises telomere stability and promotes the formation of partially single-stranded telomeric DNA circles (C-circles) in hESCs, suggesting heightened sensitivity of stem cells to replication stress at overly long telomeres. Thus, tight control of telomere length homeostasis is essential to maintain telomere stability in hESCs.

Telomeres are nucleoprotein structures at the end of linear chromosomes that consist of tandem TTAGGG repeats, bound by the shelterin complex. They are characterized by single-stranded terminal overhangs, known as G-tails or G-overhangs. The G-overhang can invade the double-stranded telomeric region forming a stable secondary structure called the T-loop, a conserved protective structure that prevents the chromosome ends from being recognized as DNA damage.

Telomeres shorten during each cell division, owing to the ‘end-replication problem’ as well as telomere end processing. Telomere shortening can be counteracted by lengthening mechanisms. Most cancer cells, as well as germline and stem cells, activate the ribonucleoprotein enzyme telomerase to compensate for telomere loss.

The telomerase core complex consists of the reverse transcriptase hTERT and the RNA component hTR, used as a template to synthesize telomeric DNA. A fraction of human cancer cells, referred to as ALT cells, maintain telomere length by telomerase-independent mechanisms, referred to as alternative lengthening of telomeres (ALT). ALT cells rely on recombination pathways, showing high incidence of telomere sister chromatid exchange events (t-SCEs) and a number of distinctive characteristics, such as ALT-associated promyelocytic leukemia (PML) bodies (APBs) containing telomeric chromatin, heterogeneous telomere length, elevated frequency of 5′ C-rich telomeric overhangs and abundance of extrachromosomal telomeric repeats (ECTRs), including linear dsDNA, T-circles and C-circles. However, whether accumulation of ALT-related telomeric features is restricted to ALT activity is still under debate.

Telomere length homeostasis dictates cellular proliferative potential and becomes determinant in stem cells, where it ensures tissue homeostasis and affects age-related deterioration of stem cell function. Telomere length is established during embryogenesis such that telomeres are long enough to support extended series of regulated cell divisions during the developmental program but short enough to limit cell proliferation in the adult to suppress cancer initiation. The stable telomere length in hESCs suggests that defined mechanisms have evolved to promote the telomere length that is optimal for genomic stability.

Telomere length maintenance also becomes of special interest for the reprogramming of somatic cells, as it directly affects reprogramming efficiency and determines the maintenance of the pluripotent phenotype.

We aimed to gain insight into the mechanisms controlling telomere length homeostasis in hESCs and hiPSCs. Here we find that telomere length is regulated by active telomere trimming mechanisms in addition to telomerase-dependent elongation. We show that XRCC3 and Nbs1 mediate telomere attrition by catalyzing the formation of T-circles and C-rich overhangs. Moreover, hESCs accumulate other hallmarks of ALT, such as C-circles, as a result of increased telomere instability, but independently of recombination-mediated telomere elongation. We show that reprogramming of human differentiated cells leads to the appearance of C-rich overhangs and ECTRs in hiPSCs, which represents a valuable marker to characterize reprogramming efficiency. Our results demonstrate that a fine balance of length-control pathways dictates telomere stability in pluripotent stem cells, which is essential for the understanding of stem cell biology for stem cell-based therapies.

1Molecular and Cellular Biology Department, The Salk Institute for Biological Studies, La Jolla, California, USA. 2DiSTABiF, Second University of Naples, Caserta, Italy. Correspondence should be addressed to J.K. (karlseder@salk.edu).

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RESULTS

C-rich overhangs and ECTRs in hESCs

C-rich overhangs of 3'–5' orientation were initially related to ALT activity16,24; however, some telomerase-positive human cancer cells with overly elongated telomeres25 and cells of germline origin26 also accumulate single-stranded C-rich telomeric DNA. To evaluate C-rich overhang in hESCs, we performed native and denaturing 2D gel electrophoresis on DNAs from three different hESC lines (HUES6, H1 and H9), enabling the separation of restriction fragments (TRFs) by size and structure18,27,28 (Fig. 1a). 2D analysis revealed G-rich telomeric single-stranded DNA (ssDNA) under native conditions (Fig. 1b, top) that resembled the arc corresponding to telomeric double-stranded DNA (dsDNA) under denaturing conditions. In-gel hybridizations using a G-rich probe allowed us to detect C-rich telomeric ssDNA of equivalent intensity to the canonical G-overhang that also followed the same path as the telomeric dsDNA under denaturing conditions, indicating C-rich overhangs in HUES6 cells (Fig. 1b, bottom). We also observed C-rich telomeric ssDNA in H1 and H9 cell lines, consistent with the presence of 5' C-overhang in hESCs (Supplementary Fig. 1a, right).

To confirm the polarity of both overhangs, we treated the samples with ssDNA-specific exonuclease 1 (Exo1), which has 3'–5' polarity, or the exonuclease RecJf, which has 5'–3' polarity, before DNA restriction digestion. G-overhangs were susceptible to Exo1 treatment but resistant to RecJf. C-overhangs were sensitive only to RecJf digestion (Fig. 1b). Thus, our results indicate that terminal C-rich ssDNA with 3'–5' orientation at telomeres in hESCs.

Circular telomeric dsDNA in the form of T-circles was clearly visible, with both strand-specific telomeric probes under denaturing conditions in the three hESC lines (Fig. 1b and Supplementary Fig. 1a, red arrows).

To further explore the presence of C-rich, partially single-stranded circular ECTRs (C-circles), we performed C-circle assays19 in hESCs (Fig. 1c). We included samples from ALT cells (U2OS) as a positive control and IMR90 human primary fibroblasts as a negative control. As expected, C-circles were abundant in U2OS cells and absent in IMR90 cells. Notably, all hESC lines showed significantly higher levels of C-circles than did IMR90 cells (Fig. 1d).

Considering that C-overhangs and ECTRs, in particular C-circles, are distinctive characteristics of ALT cells19, we investigated whether hESCs employ ALT mechanisms for telomere elongation. Telomere recombination was evaluated by chromosome-orientation-FISH (CO-FISH) analysis on metaphase chromosomes13 (Fig. 2a). We detected much lower rates of t-SCEs in hESCs than in U2OS cells (Fig. 2a). To test whether telomere recombination contributes to telomere elongation pathways, we generated hESCs in which telomerase activity was reduced through the expression of dominant-negative hTERT (Fig. 2b). Telomeres in these cells shortened progressively (Fig. 2c), indicating that telomere recombination cannot compensate for impaired telomerase activity. Similarly, we did not observe APBs in hESCs (Supplementary Fig. 1b). We conclude that hESCs do not rely on recombination mechanisms for telomere elongation, despite the presence of a subset of ALT markers in these cells.

Accumulation of ECTRs in hESCs

To understand the mechanism of ECTR formation in hESCs, we asked whether ECTR accumulation is associated with telomerase-mediated telomere lengthening. HUES6 cells in which the RNA component hTR is overexpressed (HUES6+hTR) (Supplementary Fig. 2a) showed a significant (P < 0.01) increase in telomerase activity (Supplementary Fig. 2b) and rapid telomere elongation (Fig. 3a). Elongated telomeres became homogeneous in length with continuous culture (Fig. 3b), and hESCs maintained stem cell renewal capacity (Supplementary Fig. 2c) and differentiation potential (Supplementary Fig. 2d,e). Thus, hESCs can establish a new telomere length set point when telomerase...
activity increases. To determine whether telomere elongation promotes accumulation of single-stranded telomeric repeats, we performed TRF analysis under native and denaturing conditions using a C-rich or G-rich telomeric probe. We found that over-lengthened telomeres showed similar ratios of G- and C-tails, suggesting that the number of telomeres bearing 3′G- and 5′C-overhangs is maintained (Fig. 3b).

In addition, we evaluated T-circle and C-circle production in HUES6+hTR cells. T-circle assays demonstrated a significant increase in T-circle formation in these cells relative to control cells (Fig. 3c). Excessive telomere elongation also generated a robust accumulation of C-circles (Fig. 3d). Given that long telomeres are required for ALT induction, we investigated whether excessive telomere elongation in hESCs facilitates the activation of ALT. CO-FISH analysis showed that t-SCE rates were similar in control and hTR-overexpressing cells (Fig. 3e). Second, we did not observe APB-like foci. We conclude that telomerase-dependent elongation generates a subset of ALT hallmarks in hESCs independently of ALT activation.

hiPSCs show accumulation of 5′ C-rich overhangs and ECTRs

We next asked whether the features identified for hESCs could be induced by reprogramming of differentiated cells. We generated hiPSC lines by retroviral transduction of primary human lung (IMR90) fibroblasts with Oct4, Sox2, Klf4 and c-Myc. We confirmed the activation of endogenous pluripotent factors (Supplementary Fig. 3a-c) and performed functional analysis by differentiation into the embryonic germ layers in vitro (Supplementary Fig. 3d,e).

To ascertain whether the hiPSCs acquired the same characteristics as hESCs, we analyzed DNA isolated from three different hiPSC lines by 2D gel electrophoresis. Hybridization with a G-rich and C-rich probe under native and denaturing conditions showed the presence of G- and C-overhangs, whereas only G-overhangs were detected in the parental fibroblasts (Fig. 4a). T-circles were clearly visible with both strand-specific probes under denaturing conditions in hiPSCs but not in parental cells (Fig. 4a, red arrows), at levels comparable to those observed in hESCs (Supplementary Fig. 4a).

We then examined the presence of C-circles in hiPSCs, and found that the abundance of C-circles was similar to that detected in hESCs (Fig. 4b). Because we found hallmarks of ALT cells in hiPSCs, and considering that telomere elongation has been observed in hiPSCs derived from telomerase-deficient cells, we evaluated the contribution of telomere recombination mechanisms for telomere elongation in hiPSCs. We observed very low t-SCE
Figure 3  Telomere elongation stimulates the formation of extrachromosomal telomeric repeats in hESCs. (a) Telomere restriction fragment length assay of HUES6 cells, HUES6 cells stably expressing vector control (HUES6 + vector) or HUES6 + hTR cells on passage 3 (p3) after selection. (b) 1D gels of restriction-digested genomic DNA from parental HUES6 and HUES6 + vector and HUES6 + hTR cells at p34 and p42 after transduction. Gels were probed for G- and C-rich telomeric DNA under native and denatured conditions. The overhang signal in native gels was normalized against the total amount of telomeric DNA in denatured gels. Data represent mean ± s.d. of 3 independent experiments. (c) T-circle assay (left) of 1 µg digested genomic DNA from HUES6, HUES6 + vector and HUES6 + hTR cells. The presence or absence of φ29 DNA polymerase (used for rolling circle elongation of C-circles) is indicated. Quantification of T-circle products (arrow) relative to untreated cells and normalized against the signal from the reaction lacking φ29 polymerase (right). Data represent mean ± s.e.m. of 3 independent experiments. *P < 0.05 (two-tailed Student’s t-test). (d) C-circle assay for 100 ng digested genomic DNA from HUES6, HUES6 + vector and HUES6 + hTR cells. Quantification of C-circle levels (bottom) from 3 independent experiments. Data represent mean ± s.d. **P < 0.01 (two-tailed Student’s t-test). (e) Representative images of CO-FISH metaphases (left) and quantification of t-SCE events (left) from HUES6, HUES6 + vector and HUES6+hTR cells. Scale bar, 10 µm. Data are mean ± s.e.m. from 3 independent experiments. NS, not significant (two-tailed Student’s t-test).
Reprogramming of human differentiated cells induces the accumulation of C-rich telomeric overhang and extrachromosomal telomeric repeats. (a) 2D electrophoresis of restriction-digested genomic DNA from parental IMR90 and hiPSC lines (IPS#1, IPS#4, IPS#16) probed for DNA of G-rich (left) or C-rich (right) telomeric sequence under native and denatured conditions. Red arrows indicate T-circles. (b) C-circle assay in IMR90, H9, HUES6 and hiPSC lines. The dot blot was hybridized with 32P-end-labeled (CCCTAA)5 oligonucleotide probe. (c) Representative images of CO-FISH metaphases (left) and quantification of t-SCEs (right) in IMR90 and iPSCs. U2OS cells were included as a positive control. White arrowheads indicate t-SCEs. Scale bar, 10 μm. Data represent mean ± s.d. of 3 independent experiments (≥25 metaphases per experiment). NS, not significant (two-tailed Student’s t-test).

Recent studies demonstrated the correlation between DNA replication stress and C-circle formation. To investigate the molecular mechanisms underlying C-circle formation in hESCs, we examined the contribution of factors previously implicated in C-circle production: TRF1, the RECQ helicases BLM and WRN, Exo1, the helicase/nuclease DNA2 and SMARCAL1, a member of the SNF2 family of DNA-dependent ATPases. Cells depleted of TRF1, BLM, WRN or Exo1 via small interfering RNA (siRNA)-mediated knockdown showed similar numbers of C-circles as did control cells, whereas DNA2- and SMARCAL1-depleted cells accumulated significantly more C-circles (Fig. 5b and Supplementary Fig. 6b). DNA2 has an essential role in preventing telomere replication defects by resolving G4 structures and promoting degradation of reversed replication forks after fork stalling. SMARCAL1 catalyzes fork regression and is required for successful replication through telomeric sequences. Thus, we speculate that accumulation of G4 structures and aberrant processing of unresolved replication intermediates within telomeres upon DNA2 or SMARCAL1 depletion drives the accumulation of C-circles. As this effect is exacerbated in cells with long telomeres, we reasoned that long telomeric tracks could be more prone to replication stress. We tested whether hESCs with long telomeres showed fragile telomere phenotypes arising from telomeric DNA replication defects and observed higher frequencies of metaphase chromosomes with multitelomeric signals in cells with overextended telomeres (Fig. 5c), indicating increased replication defects.

To determine whether telomerase has a role during telomere replication, we subjected HUES6 and HUES6+hTR cells to short hairpin

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RNA (shRNA)-mediated silencing of hTERT (Supplementary Fig. 6c) and assessed the frequency of MTS. The MTS levels in the resulting hTERT-deficient cells were comparable to that of control cells in both HUES6 and HUES6+hTR cells (Supplementary Fig. 6d), indicating that the presence of over-elongated telomeres, rather than changes in telomerase expression, leads to telomere fragility. Likewise, HUES6+hTR cells showed significantly more telomere dysfunction-induced foci (TIFs) than did control cells (Fig. 5d), indicating that excessively long repetitive sequences compromise telomere stability. We observed that telomere elongation is followed by augmented loading of telomeric proteins (Supplementary Fig. 6e), suggesting that the slight increase in TIFs is due to DNA replication defects rather than defective telomere capping.

These data suggest that tight control of telomere length homeostasis is required to prevent telomere fragility and DNA damage at telomeres in hESCs, ensuring telomere integrity and maintenance of genome stability.

XRCC3 and Nbs1 regulate C-overhang and T-circle formation

We next investigated the molecular mechanisms underlying 5′ C-rich overhang formation and T-circles in hESCs. Human 5′ C-rich telomeric overhangs have been proposed to accumulate in the context of T-loop resolution events mediated by XRCC3 (ref. 40). To evaluate the contribution of XRCC3 in overhang formation in hESCs, we used siRNA to silence XRCC3 and performed native TRF analysis (Fig. 6a and Supplementary Fig. 7a). The G-overhang signal was significantly altered; however, we detected a 40% reduction in the levels of C-overhangs. These results support a highly conserved role for XRCC3 in C-overhang generation.

XRCC3 has been implicated in T-circle formation in different scenarios26,28,41. However, its activity did not have a significant effect on T-circle accumulation in hESCs (Supplementary Fig. 7b), suggesting that XRCC3 is not sufficient to promote T-circle formation in those cells. We then focused on Nbs1, a member of the Mre11–Rad50–Nbs1 complex and potential candidate involved in T-circle formation28,41.

shRNA-mediated knockdown of Nbs1 expression (to ~25–45% that of control cells, depending on the shRNA construct used) preserved stem-cell renewal capacity of hESCs (Supplementary Fig. 7c,f). Nbs1-knockdown cells showed significant reduction of T-circles (Fig. 6b), and we therefore suggest that Nbs1 is at least partially responsible for T-circle accumulation in hESCs.

It has been hypothesized that T-circles and C-overhangs could act as the precursors of C-circles16,42. Given that our data demonstrated a direct role of XRCC3 and Nbs1 in C-overhang and T-circle formation, respectively, we evaluated the accumulation of C-circles in the absence of these factors, but we did not observe changes in C-circle abundance (Supplementary Fig. 7d,e). Thus, we conclude that different pathways contribute to the generation of both types of ECTR in hESCs.

XRCC3 and Nbs1 regulate telomere length in hESCs

Our findings indicate that formation of 5′ C-rich overhangs and T-circle generation depends on XRCC3 and Nbs1. If these structures accumulate in hESCs as an outcome of processing events at the ends of the chromosome, depletion of XRCC3 and Nbs1 should lead to changes in telomere length. We evaluated telomere length 7 d after transduction of cells with siRNAs and/or shRNAs targeting XRCC3 and Nbs1, respectively (Supplementary Fig. 7f). Whereas knockdown of XRCC3 or Nbs1 alone did not significantly modify the distribution of the telomeric signal, cells in which both XRCC3 and Nbs1 were knocked down showed telomere lengthening (Fig. 6c), indicating defective telomere trimming. These data strongly suggest that XRCC3 and Nbs1 mediate regulation of telomere length through partially distinct parallel pathways and influence homeostasis of telomere length in hESCs.

In summary, our results demonstrate that telomere length regulation in hESCs is mediated by telomerase-dependent elongation and by XRCC3 and Nbs1, which counteract excessive telomere elongation. We hypothesize that the activation of telomere trimming events involves the resolution of the T-loop into a T-circle and C-rich...
XRCC3 and Nbs1 contribute to the formation of 5′ C-rich telomeric DNA and T-circles and regulate telomere length in hESCs.

(a) Left, 1D gel electrophoresis under native and denatured conditions for HUES6 cells transfected with control siRNA (siControl) or siRNA targeting XRCC3 (siXRCC3) and analyzed for G-rich and C-rich telomeric DNA. Right, quantification of the overhang signal. The amount of G-rich and C-rich telomeric ssDNA in native gels was normalized against the total amount of telomeric DNA in denatured gels. Values are relative to that in untreated cells from 5 independent transfection experiments (means ± s.e.m.). *P < 0.05; ns, not significant (two-tailed Student’s t-test). (b) Left, T-circle assay from HUES6 cells infected with scramble shRNA (sh control) or shRNAs against Nbs1 (sh1 Nbs1 and sh2 Nbs1). Samples were analyzed 5 d after transduction. Right, relative T-circle levels normalized to the signal without φ29 DNA polymerase from 3 independent experiments (mean ± s.e.m.). *P < 0.05, **P < 0.01 (two-tailed Student’s t-test). (c) Left, telomere length analyzed by TRF analysis 7 d after knockdown of XRCC3 and Nbs1 with the indicated constructs. Mean telomere length for each condition was calculated with TeloTool58. Right, quantification of telomere length (fold change relative to control samples). Data represent mean ± s.e.m. of 4 independent experiments. ****P < 0.0001, *P < 0.05 (two-tailed Student’s t-test).
After replication fork stalling, that C-circles are generated by resolution of replication intermediates (Fig. 7a). We have discovered that hESCs tightly control telomere length by trimming mechanisms mediated by XRCC3 and Nbs1 (Fig. 7b). The activity of XRCC3 and Nbs1 is required to promote the resolution of T-loops giving rise to 5′ C-rich telomeric ssDNA and T-circles. Both HR factors are essential to compensate for excessive telomere elongation. (c) Long telomeres are prone to replicative stress resulting in the formation of C-circles. We speculate that C-circles are generated by resolution of replication intermediates after replication fork stalling.

We observe that telomere length homeostasis is crucial to prevent telomere instability in hESCs and establish that reprogramming of differentiated cells induces the accumulation of T-circle and C-rich telomeric ssDNA, indicating the activation of telomere trimming pathways and emphasizing the requirement of tight telomere length homeostasis for telomere stability in hESCs and hiPSCs.

**DISCUSSION**

We have discovered that hESCs tightly control telomere length by establishing a proper equilibrium between elongation and trimming. Telomere elongation is achieved by telomerase and counteracted by HR factors that promote the generation of C-rich telomeric ssDNA and ECTRs in the form of T-circles. Failure to maintain length homeostasis leads to telomere instability and accumulation of C-circles. Given that these features are also acquired by reprogramming of human somatic cells, we suggest that such characteristics represent reliable markers for pluripotency.

**Telomere elongation in hESCs and hiPSCs**

The contribution of telomerase-independent telomere maintenance pathways in stem cell populations remains controversial. Initial studies in telomerase-deficient mouse embryonic stem cells (mESCs) showed telomere length maintenance in long-term cultures of proliferative survivors. High frequencies of t-SCEs were observed in telomerase-deficient mESCs, and telomere recombination was proposed to compensate the loss of telomerase in mESCs. In contrast, our data suggest that telomere elongation in hESCs is mediated exclusively by telomerase and that recombination-dependent pathways are not activated when telomerase activity is reduced. Consistently, a recent study found that telomerase-deficient hESCs showed progressive telomere shortening in continuous culture, concomitantly with reduced proliferation and increased cell death. Likewise, although studies in iPSCs derived from telomerase-deficient mice suggest that ALT pathways contribute to telomere maintenance, ALT activity was not detected in telomerase-mutant hiPSCs, consistent with our model. Analysis of hiPSCs derived from patients with telomere shortening disorders revealed that the severity of defects in telomere elongation correlated with the degree of telomerase deficiency. We observed comparable telomerase expression and telomere lengths in hiPSCs derived from different sources of primary cells, and these results also resembled those in hESCs. However, variability in telomere expression and telomere elongation among hiPSC clones has been reported, suggesting that hiPSCs are not homogeneous clonal populations and that culture conditions affect telomerase expression and function. We propose that telomerase activity regulates telomere length and that trimming mechanisms directly contribute to the proper telomere length set point. We suggest that future studies of the components that dictate telomere length homeostasis will facilitate the development of stem cell–based therapies for telomere biology disorders.

**XRCC3 and Nbs1 regulate telomere length in hESCs**

The accumulation of ECTRs in the form of T-circles after excessive telomere elongation indicated the activation of telomere trimming events in hESCs. Telomere trimming involves the conversion of a T-loop into a T-circle and a 5′ C-rich telomeric ssDNA intermediate. The observed maintenance of the ratio between G- and C-rich telomeric ssDNA after telomere elongation suggests that telomere deletion events are followed by nuclease degradation that compensates for the excess of 5′ C-rich telomeric ssDNA, indicating the presence of tightly controlled regulation of the balance between overhangs. Given that previous in vitro studies demonstrated that G- and C-rich telomeric ssDNA after telomere elongation suggests that telomere deletion events are followed by nuclease degradation that compensates for the excess of 5′ C-rich telomeric ssDNA, indicating the presence of tightly controlled regulation of the balance between overhangs.

Resolution of the T-loop as a Holliday junction by HR is mediated by Nbs1 and XRCC3 (Fig. 7a,b). We cannot exclude the possibility that both XRCC3 and Nbs1 contribute to the formation of C-rich telomeric ssDNA and T-circles. Complete deletion of these factors may be necessary to address this question; however, loss of HR factors leads to embryonic lethality, reduced growth rate and increased cell death, hindering the analysis.

The presence of trimming pathways in hESCs suggests that excessive telomere elongation could compromise genome integrity. Telomeres are known to be fragile sites prone to accumulate stalled replication forks and secondary structures, including G-quadruplexes, challenging...
Furthermore, telomere replication stress could promote genome instability and cancer. Our data indicate that hESCs with long telomeres show increased telomeric DNA damage, probably owing to augmented telomere replication stress. Given that the number of dysfunctional telomeres in cancer cells does not increase after excessive telomere elongation, these results suggest that hESCs harbor more stringent mechanisms to sense inappropriate telomere replication, as the maintenance of telomere stability is of critical importance for tissue homeostasis and to prevent cancer development. We also propose that tight regulation of telomere trimming mechanisms exists in hESCs to prevent excessive telomere attrition, which could compromise their pluripotent potential and contribute to age-related deterioration in stem cell function.

**Replication stress causes C-circle accumulation in hESCs**

A high frequency of t-SCEs and the presence of C-circles were considered the best indicators for ALT. Nevertheless, a recent study demonstrated the induction of C-circles in telomerase-positive cancer cells upon depletion of SMARCAL1, which led to telomere replication stress. No other ALT-related phenotypes were induced. The first observation of C-circle accumulation in response to perturbations in DNA replication was reported in telomerase-positive cancer cells, as a result of defective chromatin assembly after depletion of the histone chaperone ASF1. ALT was activated and C-circle levels reached the amount detected in ALT cells. Therefore, it is conceivable that C-circle levels beyond some threshold may be indicative of ALT. Our data also support this hypothesis, as the amount of C-circles detected in hESCs was not as high as that in U2OS cells, consistent with the lack of ALT activity in hESCs. Elongation of telomeres caused a dramatic increase in C-circle abundance; likewise, the aforementioned phenotypes were observed in cells with very long telomeres.

We propose that telomere length determines the formation of these telomeric DNA structures and that replication stress, which is exacerbated in the presence of long telomeres, triggers their accumulation (Fig. 7c). Consistently, we show an increase of C-circles after DNA replication stress in hESCs, suggesting that severe abrogation of DNA replication activity boosts C-circle formation. Remarkably, C-circle metabolism might differ between cell types, as treatment with DNA replication inhibitors has a different effect in cancer cells and immortal human cells without detectable telomerase activity. Supporting our data, we show that depletion of DNA2 and SMARCAL1 led to elevated C-circle accumulation consistent with their active role in S-phase progression, probably by facilitating repair and restart of stalled replication forks.

While the molecular mechanism governing C-circle formation remains elusive, we suggest that very long telomeres trigger C-circle accumulation. The increased telomere instability in this scenario would result in C-circle generation as a by-product of resolution of telomeric DNA replication intermediates. Excessive telomere elongation in primary fibroblasts also induced C-circle accumulation, albeit to a much lesser extent than in hESCs with long telomeres. We did not detect changes in the levels of single-stranded C-rich linear telomeric DNA after replication stress, demonstrating that resolution of replication intermediates specifically enriches for C-rich telomeric DNA in circular conformation. We suggest that hESCs are more prone to replication stress than differentiated cells, which is consistent with the finding that replication intermediates are frequently observed in ESCs but lost after differentiation.

Our study establishes that very tight control of telomere length is maintained by telomerase-dependent elongation and telomere trimming events, is required to ensure telomere stability in hESCs. This balance is also established in hiPSCs, providing insight into the characterization of faithfully reprogrammed cells, which represent an attractive tool in regenerative medicine owing to their therapeutic potential.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

T.R. designed and performed the experiments and wrote the manuscript. C.H. carried out experiments. S.C. provided RHP34. J.K. designed experiments, supervised the work and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Cell lines. Human H1 (WA01) and H9 (WA09) embryonic stem cell lines were obtained from WiCell Research Institute and HUES6 from the Harvard Stem Cell Institute. Human diploid IMR90 fibroblasts were obtained from ATCC. hESCs and hiPSCs were maintained on a mitotically inactive mouse embryonic fibroblast (MEFs, Millipore) feeder layer in hES medium, DMEM/F12 (Invitrogen) supplemented with 20% knockout serum replacement (Invitrogen), 1 mM L-glutamine, 0.1 mM nonessential amino acids, 55 µM β-mercaptoethanol and 10 ng/ml bFGF (Joint Protein Central). hESCs and hiPSCs were also cultured in Matrigel (BD Biosciences) using mTeSR™.

Human diploid IMR90 fibroblasts were cultured in Glutamax-DMEM (Life Technologies) supplemented with 15% FBS and 0.1 mM nonessential amino acids. U2OS and 293T cells were cultured in Glutamax-DMEM (Invitrogen) supplemented with 10% FBS and 0.1 mM nonessential amino acids at 7.5% CO2 and 3% O2.

For differentiation into embryoid bodies (EBs), pluripotent cell colonies growing on MEFs were loosely detached by dispase treatment, resuspended in DMEM/F12 supplemented with 10% FBS (Invitrogen), 0.5 mM L-glutamine, 0.1 mM nonessential amino acids and maintained on low attachment plates for 4 d. The EBs were then plated on gelatin-coated plates and allowed to differentiate for another 10 d.

hiPSC generation. IMR90 fibroblasts were infected with an equal ratio of retroviruses encoding Oct4, Klf4, Sox2 and C-Myc by spinfection at 800 × g for 1 h at room temperature in the presence of polybrene (4 µg/ml Cells). Cells were passaged onto fresh MEFs and switched to hESC medium 4 d after infection. For the derivation of hiPSC lines, colonies were manually picked and transferred onto MEF feeder cells for several passages before growth in Matrigel + mTeSR conditions.

Cytogenetics. Cells were sent to Cell Line Genetics for routine karyotype analysis.

Plasmids and virus preparation. pMX-Oct4, pMX-SOX2, pMX-KLF4 and pMX-C-Myc were obtained from Addgene (plasmids 17217, 17218, 17219 and 17220, respectively). Retroviral vectors were co-transfected with packaging plasmids (pCMV-gag-pol-PA and pCMV-VSVG, kindly provided by G. Pao) in pMX-Oct4, pMX-SOX2, pMX-KLF4 and pMX-C-Myc. Lentiviral vectors were cotransfected with packaging plasmids pMDL, Rev and pCMV-DVR (kindly provided by O. Singer) in 293T cells using Lipofectamine (Invitrogen) in accordance with manufacturer’s recommendations.

pBABE-U3-H1R was kindly provided by K. Collins. U3-H1R was cloned into third-generation lentiviral vector kindly provided by R.A. Rodriguez. pBABE-TERT and TERT-DN were subcloned into pLV-FU-TetO vector. Lentiviral vectors were cotransfected with packaging plasmids pMDL, Rev and VSVG (kindly provided by O. Singer) in 293T cells using Lipofectamine (Invitrogen) in accordance with manufacturer’s recommendations.

Lentiviral supernatants were collected 36 h after transfection, concentrated by lentivirus infections of hESCs were performed as previously described. Lentiviral infections of hESCs were performed as previously described. hESCs and hiPSCs were maintained on a mitotically inactive mouse embryonic fibroblast (MEFs, Millipore) feeder layer in hES medium, DMEM/F12 (Invitrogen) supplemented with 20% knockout serum replacement (Invitrogen), 1 mM L-glutamine, 0.1 mM nonessential amino acids, 55 µM β-mercaptoethanol and 10 ng/ml bFGF (Joint Protein Central). hESCs and hiPSCs were also cultured in Matrigel (BD Biosciences) using mTeSR™.

DNA isolation and qPCR analysis. DNA was extracted using TRizol Reagent (Invitrogen) according to the manufacturer’s instructions and purified using RNeasy mini kits (Qiagen). cDNA was synthesized using the Superscript III Reverse Transcriptase kit (Life Technologies) and qRT-PCR was performed using the SYBR Green master mix (ABI, Life Technologies). Values of gene expression were normalized using GAPDH expression and are shown as fold change relative to the value of the sample control. The list of the primers used in the RT-PCR analysis is provided in Supplementary Table 1.

Chromatin immunoprecipitation (ChIP). ChIP was performed as described.

Immunofluorescence and telomere FISH on metaphase spread. Cyto-centrifugation, immunofluorescence and telomeric FISH were carried out as described. For metaphase analysis, 225 metaphases were quantified for each metaphase–TIF experimental replicate.

Immunofluorescence. Immunostaining was performed as described. Antibodies used for immunofluorescence were as follows: anti-TRF2 (Genlantis AB1124); anti-PML (sc-5621); anti-Sox2 (Chemicon 5603); anti-SEAA4 (ab16287); anti-Oct4 (Cell Signaling 2750); anti-TRA-1-60 (MAB4360); anti-AFP (Dako A0008); anti-SMA (Sigma-Aldrich A5228); anti-tubulin-β3 (Covance, MMS-435P); anti-Cdx2 (Dako M3636).

Chromosome orientation FISH. CO-FISH was performed as described previously. Metaphase chromosomes were visualized by conventional fluorescence using a 63× objective lens on a Zeiss Axioskop II microscope, and ≥25 metaphases were analyzed in each experimental replicate.

Native and denaturing in-g gel hybridization. Telomere restriction fragments were analyzed as described. Briefly, genomic DNA was purified, digested with AluI and Mbol (New England BioLabs) and separated by gel electrophoresis on 0.6% agarose gels for 1 h at 38 V overnight in 0.5x TBE buffer. After electrophoresis, DNA was stained with ethidium bromide in 0.5× TBE buffer. M-aphidicolin (AG Scientific) or 0.5 M β-mercaptoethanol was added to the gel at final concentrations of 1 and 0.5 mM, respectively. The DNA was denatured and neutralized by boiling in 0.04 M NaOH at 95°C for 10 min followed by neutralization in 0.5 M Tris–HCl (pH 8.0). The DNA was then resolved on a 3% agarose gel in 0.5× TBE buffer (pH 8.0) for 2 h at 120 V. After electrophoresis, the gel was stained with ethidium bromide and photographed. The resulting gel images were analyzed using ImageQuant Software.

Two-dimensional gel electrophoresis. 2D analysis was carried out as described. C-circle assay. The method employed for the assay probing for C-circles was slightly modified from that performed in Henson et al. Briefly, genomic DNA was prepared as described above. Digested DNA was cleaned up by phenol–chloroform extraction and precipitation. DNA was diluted and measured using a Nanodrop spectrophotometer. Generally, 100 and 50 ng of DNA was used for each sample (10 µl). DNA was combined with 10 µl 0.2 mg/ml BSA (NEB), 0.1% Tween, 1 mM each dATP, dGTP and dTTP, 1× d29 Buffer (NEB) and 7.5 U d29 DNA polymerase (NEB) and incubated at 30°C for 12 h then at 65°C for 20 min. Reaction products were diluted to 100 µl with 2× SSC and dot-blotted onto a 2×-SSC-soaked nylon membrane. DNA was UV-cross-linked onto the membrane then hybridized at 50°C with end-labeled [32P]CCCTAA oligo probe. Blots were washed, exposed and scanned using a Typhoon 9400 Phospholimager (Amersham, GE Healthcare) and analyzed using ImageQuant Software.

T-circle assay. T-circle assay was conducted as described.

Flow cytometry analysis. For cell cycle analysis, HUES6 cells were individualized using accutase 1:4 in PBS and fixed in 70% ethanol overnight. For BrdU detection, cells were incubated with 10 µM BrdU for 30 min before collection. After incubation with 0.5 mg/ml pepsin, cells were treated with 2 N HCl for 20 min. Cells were then resuspended in PBS containing 50 µg/ml propidium iodide and 0.5 mg/ml of RNase A. Flow cytometry analyses were conducted using a LSRII cytometer (BD Bioscience), and data were analyzed using FlowJo software.
Telomerase assay. Telomerase activity was analyzed by PCR-based telomeric repeat amplification assay. hESCs were collected in cold PBS. Cell pellets were resuspended in telomerase buffer (TB) (10 mM Tris, pH 8.5, 3 mM KCl, 1 mM MgCl₂, 1 mM DTT) containing protease and RNase inhibitors. Cells were incubated in TB buffer on ice for 15 to 20 min and lysis was monitored taking small aliquots under the microscope. Cytoplasmic extracts were collected by centrifugation, adjusted to 20% glycerol and 0.1 M NaCl, aliquoted and stored at −80 °C. Protein concentration was determined and cytoplasmic extracts were diluted accordingly with TB containing 10% glycerol, protease and RNase inhibitors.

Telomerase elongation reaction was performed using different concentrations of extracts. The diluted extract was added to the elongation mix containing 0.1 mM each dATP, dGTP, dTTP, 0.25 mM spermidine, 25 µM spermine, 5 mM MgCl₂, and 17.5 ng/µl of primer (GCACATGCATCGAGAGTT) in TB and incubated during 30 min at 30 °C. A mixture of proteinase K, SDS and Tris-CDTA was added to stop the reaction and incubated for 1 h at 37 °C. After addition of 0.2 M LiCl, the reaction was extracted with phenol–chloroform and precipitated in ethanol overnight. Pellets were resuspended in 10 mM Tris, pH 8, and incubated with the PCR reaction mix: 1.5 mM MgCl₂, 0.2 mM each dATP, dGTP, dTTP, 2.5 µM dCTP, 0.125 µl [³²P]dCTP, 0.5 µl primary primer (GCACATGCATCGAGAGTT), 0.55 µl secondary primer (CGACTTGCCTAACCCTAA) and 0.2 µl AmpliTaq (Life Technologies) in AmpliTaq buffer. The amplification reaction was carried out in a thermal cycler as follows: 4 min at 94 °C followed by 29 cycles of PCR reaction (94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min) and 5 min at 72 °C. The amplified product was analyzed in 8% acrylamide–urea denaturing gel. The gel was dried at 75 °C for 1 h and exposed to Phospholmager screen overnight, scanned in a Typhoon 9400 Phospholmager (Amersham, GE Healthcare) and analyzed using ImageQuant Software.

Statistical methods. All statistical analysis was done with Prism 6 software. The number of independent experiments analyzed is shown in each figure legend. The significance between means was determined with a two-tailed unpaired Student’s t-test when Gaussian distribution was assumed and with a two-tailed Mann-Whitney test when not assumed. Statistically significant values are depicted as follows: ****P < 0.0001, ***P < 0.001, **P < 0.01 *P < 0.05; NS, not significant.

Data availability. All data supporting the findings of this study are available within the paper and its supplementary information files.