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Telomeric Noncoding RNA TERRA Is Induced by Telomere Shortening to Nucleate Telomerase Molecules at Short Telomeres

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SUMMARY

Elongation of a short telomere depends on the action of multiple telomerase molecules, which are visible as telomerase RNA foci or clusters associated with telomeres in yeast and mammalian cells. How several telomerase molecules act on a single short telomere is unknown. Herein, we report that the telomeric noncoding RNA TERRA is involved in the nucleation of telomerase molecules into clusters prior to their recruitment at a short telomere. We find that telomere shortening induces TERRA expression, leading to the accumulation of TERRA molecules into a nuclear focus. Simultaneous time-lapse imaging of telomerase RNA and TERRA reveals spontaneous events of telomerase nucleation on TERRA foci in early S phase, generating TERRA-telomerase clusters. This cluster is subsequently recruited to the short telomere from which TERRA transcripts originate during S phase. We propose that telomere shortening induces noncoding RNA expression to coordinate the recruitment and activity of telomerase molecules at short telomeres.

INTRODUCTION

Telomeres are dynamic nucleoprotein structures that constitute the ends of linear chromosomes in eukaryotic cells. Their function is to ensure genome stability by preventing chromosome end-to-end fusions and protecting the chromosome ends from degradation and erroneous recombination events (Jain and Cooper, 2010). Due to the end replication problem, telomeres progressively shorten in dividing cells, and continual loss of telomeric repeats eventually generates dysfunctional telomeres, producing genome instability and ultimately leading to cellular senescence (de Lange, 2009). In order to counteract telomere shortening, most organisms express the enzyme telomerase, a ribonucleoprotein complex that adds telomeric repeats to the 3′ end of chromosomes by reverse transcription of the template region of its associated RNA moiety (Greider and Blackburn, 1985; Hug and Lingner, 2006).

In *Saccharomyces cerevisiae*, telomeric DNA consists of approximately 300 nt irregular $C_{1-2}A/TG_{1-3}$ telomeric repeats, terminating with a 15 nt single-strand overhang (Wellinger and Zakian, 2012). Budding yeast telomerase is composed of an RNA molecule (*TLC1*), a catalytic reverse transcriptase (Est2), and associated proteins (Est1, Est3, yKu). Telomerase activity is regulated at individual chromosome ends by the action of telomere binding proteins (Shore and Bianchi, 2009). As a result, in a given cell, telomerase is not active at each chromosome end in every cell cycle but preferentially elongates the shortest telomeres (Teixeira et al., 2004). Telomerase activity is regulated during the cell cycle and is predominantly detected during the late S/G2 phases, coupled with the replication of telomeric DNA (Marcand et al., 2000). In agreement with these observations, live-cell imaging analyses of *TLC1* RNA revealed that telomerase nucleates during S phase, generating telomerase clusters named T-Recs (*t*elomerase *rec*ruitment *c*lusters), which colocalize with a few telomeres (Gallardo et al., 2011). Formation of T-Recs depends on factors involved in telomerase-dependent telomere elongation, suggesting that these clusters reflect the active form of telomerase. How these telomerase clusters are formed on telomeres in S phase is still unknown.

Telomeres are transcribed in a strand-specific manner, generating G-rich telomeric transcripts known as telomeric repeat-containing RNA or TERRA (Azzalin et al., 2007; Schoeftner and Blasco, 2008). The transcription of TERRA proceeds from the subtelomeric regions toward the chromosome ends and terminates within the telomeric repeat tract, producing long noncoding RNA heterogeneous in length. While several functions have been proposed for TERRA, its biological role remains largely elusive. The 3′ end of the TERRA sequence is complementary to the template region of telomerase RNA, and TERRA transcripts have been shown to interact with telomerase in mammalian cells (Redon et al., 2010). In vitro experiments, using TERRA-mimicking oligonucleotides, have suggested that TERRA may inhibit telomerase activity (Redon et al., 2010; Schoeftner and Blasco, 2008). In mammalian cells, TERRA molecules have been proposed to regulate telomere structural maintenance and heterochromatin formation (Arnoult et al., 2012; Deng et al., 2009). Finally, recent evidence indicates that human TERRA can mediate the exchange of single-strand telomere binding proteins during the cell cycle, thereby promoting telomere capping after DNA replication (Flynn et al., 2011).

In *Saccharomyces cerevisiae*, the transcription of TERRA is negatively regulated by the activity of telomere binding proteins,

and TERRA transcripts are actively degraded by the 5′-3′ exonuclease Rat1 (Iglesias et al., 2011; Luke et al., 2008). For these reasons, TERRA levels are very low in wild-type (WT) yeast cells and are not easily detectable by biochemical assays. This condition has hampered its characterization, and studies of TERRA in yeast have often required the use of mutant strains or the integration of inducible promoters at telomeres in order to sustain the expression of TERRA. In particular, induction of TERRA expression via activation of a telomere-integrated artificial promoter results in telomere shortening in a telomerase-independent manner (Maicher et al., 2012; Pfeiffer and Lingner, 2012). Yet, the functions of endogenous TERRA in yeast cells are still unclear.

Herein, we use RNA fluorescence in situ hybridization (FISH) and live-cell imaging analyses to study endogenous TERRA expression in wild-type yeast at the single-cell level. This approach allowed us to detect TERRA molecules transcribed from a single telomere in a small population of yeast cells. We provide evidence that TERRA expression is induced by telomere shortening, leading to the accumulation of TERRA molecules into a single perinuclear focus. Coimmunoprecipitation and live-cell imaging suggest that the TERRA foci may act as scaffolds for the recruitment of telomerase molecules in S phase and trigger the formation of telomerase clusters (T-Recs). The TERRA-telomerase cluster is subsequently recruited to the telomere from which TERRA molecules originate, suggesting that TERRA plays a role in the spatial organization of telomerase activity at telomeres.

RESULTS

An In Vivo Assay to Study TERRA Expression at the Single-Cell Level

In order to study the expression of endogenous TERRA in WT yeast cells, we used a cell biology approach to detect TERRA transcripts generated from single telomeres at the single-cell level. First, expression of TERRA was analyzed by RNA FISH. To this aim, DNA oligonucleotide probes were designed to detect TERRA molecules transcribed from telomere 1L (Tel1L-TERRA), telomere 6R (Tel6R-TERRA), or from all the Y′-element containing telomeres (Y′-TERRA). As shown in Figure 1A, RNA FISH analyses revealed TERRA signal as a discrete perinuclear focus, sensitive to ribonuclease (RNase) treatment. Interestingly, while Tel6R-TERRA and Tel1L-TERRA formed a single focus, Y′-TERRA were mostly detected as double or multiple foci. Cell counting analyses showed that about 15% of cells express TERRA from either telomere 1L or telomere 6R, while a higher percentage (28%) displayed Y′-TERRA foci. These results indicate that a small population of WT yeast cells expresses TERRA from a specific telomere at a given time.

In order to get further insight in the localization of TERRA transcripts and the regulation of TERRA expression, we developed a live-cell assay to detect endogenous TERRA molecules generated from a single telomere. To this aim, we used the MS2-GFP system, which relies on the high-affinity binding between the 21 nt stem-loop MS2 RNA and the MS2 phage coat protein. This system has been widely used for the detection of endogenous RNAs in several organisms, including budding yeast (Bertrand et al., 1998; Gallardo and Chartrand, 2011). In order to detect TERRA, a cassette containing either two or six MS2 stem loops was integrated at telomere 1L or telomere 6R, in both cases 80 nts upstream from the telomeric repeats tracts, generating four different clones containing MS2 stem loops (TERRA-MS2). Following integration of the MS2 cassette, the selection marker was removed by Cre-mediated recombination. Sequencing analyses confirmed that integration of the MS2 repeats did not generate deletions or mutations of the subtelomeric regions and that every TERRA-MS2 clone retained full-length native subtelomeric sequences (Figure S1A available online; data not shown). Furthermore, the integration of the MS2 repeats did not significantly affect the length of the telomeric-repeat tracts of the specific chromosome end, as confirmed by Southern blot analyses (Figure S1B).

TERRA-MS2 transcripts were visualized by expressing the MS2-GFP fusion protein, while the expression of a Rap1-mCherry fusion protein allowed the detection of both nucleoplasm and telomere clusters (Gallardo et al., 2011). In agreement with the data obtained by RNA FISH analyses on WT cells (Figure 1A), microscopy analyses of TERRA-MS2 clones revealed the formation of a single TERRA-MS2-GFP focus at the nuclear periphery in every clone analyzed (Figure 1B). As expected, GFP foci were not detected in WT cells expressing MS2-GFP alone. Cell counting analyses showed that only a small percentage of cells, corresponding to about 10% of the population, expressed TERRA-MS2-GFP foci from either telomere 1L or telomere 6R.

In order to confirm that the MS2-GFP foci correspond to TERRA foci detected by RNA FISH, an MS2-specific probe was used in an RNA FISH assay to determine if TERRA-MS2 RNA colocalizes with the MS2-GFP foci. These experiments confirmed that an MS2-GFP focus colocalizes with TERRA-MS2 RNA in every TERRA-MS2 clone analyzed (Figure S1C). Furthermore, RNA FISH performed in TERRA-MS2 clones not expressing MS2-GFP shows that TERRA transcripts localize at the nuclear periphery, confirming that expression of MS2-GFP does not influence TERRA localization (Figure S1D). Quantitative RT-PCR analysis confirmed that the integration of 2xMS2 repeats at telomere 1L or telomere 6R did not influence TERRA expression levels, while the integration of 6xMS2 resulted in an increased expression of Tel6R- and Tel1L-TERRA (Figure S1E). We therefore used the strains expressing 2xMS2-tagged TERRA for all the imaging analyses.

In budding yeast, TERRA expression is repressed by the activity of telomere binding proteins, including Rif1 and Rif2 (Iglesias et al., 2011), and TERRA transcripts are degraded by the Rat1 5′-3′ exonuclease (Luke et al., 2008). To further confirm that the MS2-GFP foci correspond to TERRA, TERRA-MS2 clones were generated in a *rat1* temperature-sensitive strain (*rat1-1*) as well as in *rif1* and *rif2* mutant strains. Microscopy analyses revealed an increased number of cells expressing TERRA-MS2-GFP foci in the *rat1-1* strain when grown at semipermissive temperature (30°C) and in both *rif1* and *rif2* mutant strains, as compared to TERRA-MS2 WT strain (Figures S1F and S1G). These results indicate that expression of TERRA-MS2-GFP foci is sensitive to Rat1 and Rif proteins, as expected if the formation of these foci depends on TERRA expression. Taken

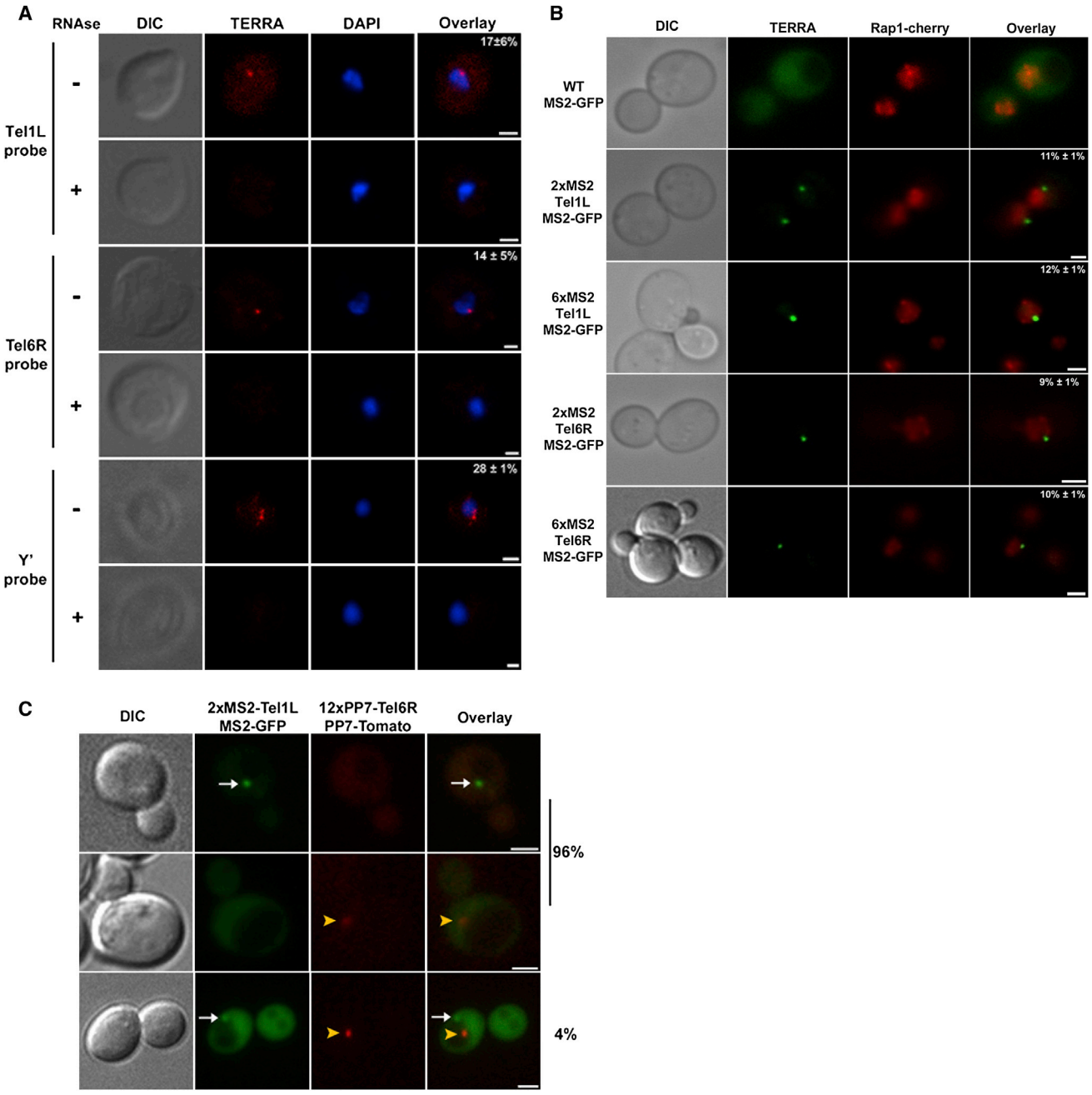

Figure 1. Endogenous TERRA Accumulate as a Focus in WT Yeast Cells

(A) Fluorescent in situ hybridization on endogenous Tel6R, Tel1L, and Y′ TERRA transcripts shows TERRA signal detected as a focus in WT yeast cells. Cells were treated (+) or not (−) with RNase prior to in situ hybridization. The percentage of cells in which a focus is detected is indicated (mean ± SD; n = 4). At least 400 cells were analyzed in each experiment. Nuclei are stained with DAPI. Scale bar = 1 μm.

(B) Live-cell microscopy analysis of Tel6R and Tel1L TERRA-MS2 clones shows TERRA-MS2-GFP focus localizing at the nuclear periphery. Nuclei were visualized using Rap1-mCherry. The percentage of cells in which a focus is detected is indicated (mean ± SD; n = 3). At least 300 cells were counted in each experiment. Scale bar = 1 μm.

(C) Expression of Tel1L-TERRA-MS2 and Tel6R-TERRA-PP7 in the yeast population. Representative images of cells expressing a Tel1L-TERRA-MS2-GFP focus (white arrow) or a Tel6R-TERRA-PP7-Tomato focus (yellow arrowhead), or coexpressing both foci, are shown. Numbers on the right represent the percentage of cells with the indicated phenotype (n = 2, where >50 cells were counted in each analysis). Scale bar = 1 μm. See also Figure S1.

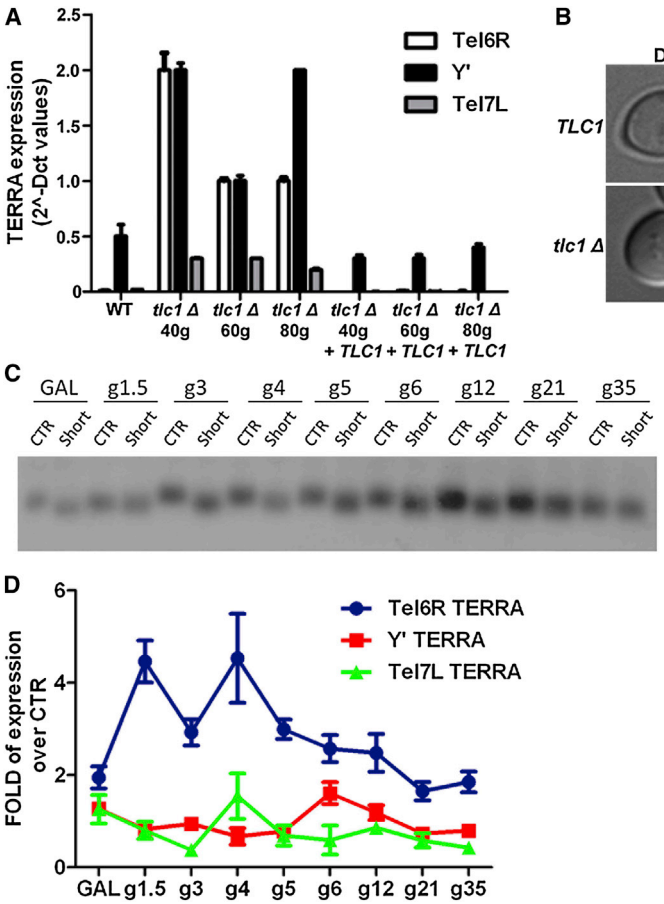

Figure 2. TERRA Expression Is Induced at Short Telomeres

(A) RT-qPCR analyses of Tel7L, Tel6R, and Y′ TERRA expression in *tlc1*Δ cells. g, generations. Data shown represent mean ± SD (n = 2).

(B) Examples of TERRA-MS2-GFP foci expressed in cells with normal (*TLC1*) or shorter (*tlc1Δ*) telomeres.

(C) Southern blot analysis of telomere 6R length in short inducible telomere strain (short) and control strain (CTR). GAL, galactose; g, generations after telomere shortening.

(D) RT-qPCR analyses of TERRA expression. Values shown represent fold of induction of 2^{-Dct} TERRA values detected in the short inducible strain over the control (CTR) strain. Data shown represent mean ± SD from triplicate samples of the experiment in (C) out of three independent experiments. See also Figure S2.

together, these data indicate that the expression and localization of TERRA-MS2-GFP is identical to endogenous TERRA detected by FISH. Therefore, the MS2-tagged TERRA can be used as a marker of TERRA expression and trafficking in single living cells.

RNA FISH and live-cell imaging analyses have shown that a similar percentage of cells express TERRA from either telomere 1L or telomere 6R (Figures 1A and 1B). This raises the question of whether both telomeres are transcribed independently in different cells or if a small population of cells transcribes several telomeres simultaneously. For this reason, we investigated whether Tel1L-TERRA and Tel6R-TERRA are coexpressed in the same cells by tagging both telomeres using different in vivo detection systems. To this aim, yeast clones containing 2xMS2 repeats at telomere 1L and 12xPP7 repeats at telomere 6R were produced. PP7 repeats are made up of 25 nt RNA stem loops, which are bound by the PP7 phage coat protein (Larson et al., 2011). This specific number of PP7 repeats was used to allow a similar sensitivity of the two in vivo detection systems (Figure S1H). Live-cell imaging analyses revealed that among the cells expressing a TERRA focus (either Tel1L-TERRA or Tel6R-TERRA), 96% contained only a single TERRA focus, while 4% showed both Tel6R-TERRA and Tel1L-TERRA foci, detected as distinct single perinuclear foci, which did not colocalize (Figure 1C).

TERRA Expression Is Induced at Short Telomeres

Our results suggest that TERRA expression might be regulated by mechanisms acting in *cis* at the single chromosome end. We formulated the hypothesis that expression of TERRA could be induced at short telomeres. To investigate this possibility, we analyzed TERRA levels in *tlc1*Δ cells containing short telomeres due to the absence of telomerase activity. Using these cells, total RNA was extracted at different generations, and TERRA expression was analyzed by RT-qPCR. Elevated levels of TERRA were detected in *tlc1Δ* cells, compared to WT cells, for all the telomeres analyzed (Figure 2A). Moreover, exogenous expression of *TLC1* RNA in a *tlc1*Δ strain restores TERRA expression to WT levels (Figure 2A), suggesting that the increased levels of TERRA detected in *tlc1*Δ cells are due to the absence of telomerase activity. In order to investigate TERRA expression in living cells in a *tlc1*Δ background, the *TLC1* gene was deleted in TERRA-MS2 clones. Live-cell imaging of *tlc1*Δ TERRA-MS2 clones revealed an increased number of cells exhibiting TERRA foci, as well as an increased number of foci per cell, as compared to *TLC1* WT cells (Figure 2B; Figures S2A and S2B). Altogether, these results suggest that telomere shortening is associated with increased levels of TERRA.

To confirm the link between TERRA expression and telomere length, we measured TERRA expression from a single short telomere using a short inducible telomere assay (Marcand et al., 1999). In this assay, the galactose-dependent induction of Flp1 recombinase results in the elimination of a telomere 6R-integrated cassette, which generates a suddenly short telomere (Figures S2C and S2D). Importantly, the shortened telomere 6R retains the complete subtelomeric sequence, thus maintaining native sequences of TERRA and its promoter regions. Using this strain, time course experiments were performed, inducing telomere 6R shortening and analyzing TERRA expression over

time. As shown in Figure 2C, growth in galactose-containing rich medium for 3 hr generated a short telomere 6R in the short inducible strain (short strain) compared to the control strain (CTR strain) (see also Figure S2D). After telomere shortening, the cells were grown in glucose-rich medium and maintained in exponential growth ($OD_{600} < 2$) by regular dilutions. During the time course, the short telomere 6R was progressively relengthened by the activity of telomerase, reaching the same length as the control after 21 generations (Figure 2C). These data are consistent with a previous study on the kinetics of telomere elongation (Marcand et al., 1999). Interestingly, RT-qPCR analyses revealed that Tel6R-TERRA levels increased after telomere 6R shortening (Figure 2D). Moreover, during the course of the experiment, Tel6R-TERRA expression clearly correlated with the length of the telomere 6R, with the RNA levels progressively decreasing during the relengthening of the telomere. Conversely, the expression levels of TERRA from other telomeres (Tel7L-TERRA and Y′-TERRA) remained stable and were not influenced by the shortening of telomere 6R. Altogether, these results indicate that TERRA expression is induced at short telomeres and that TERRA levels inversely correlate with the length of their telomere of origin. Thus, TERRA expression is regulated in *cis* at each chromosome end by telomere length-dependent mechanisms.

A TERRA Focus Preferentially Associates with its Telomere of Origin during Mid-Late S Phase

Our previous results show that TERRA foci localize at the nuclear periphery (Figure 1). As telomeres cluster at the nuclear periphery in interphase cells (Gotta et al., 1996), we investigated whether a TERRA focus may localize at its telomere of origin. To this aim, 112-TetO repeats were integrated at telomere 6R in Tel6R- and Tel1L-TERRA-MS2 clones (TetO-TERRA-MS2 clones), and both TetR-RFP and MS2-GFP fusion proteins were expressed to detect telomere 6R and TERRA transcripts in the same cells. Live-cell imaging analyses of TetO-TERRA-MS2 clones did not reveal a stable colocalization between either the Tel6R-TERRA focus and telomere 6R, or the Tel1L-TERRA focus and telomere 6R in the asynchronous population (data not shown). Therefore, TERRA focus and telomere 6R foci were monitored over time in cells from early to late S phase by two-color time-lapse confocal microscopy. These analyses revealed that the Tel6R-TERRA focus transiently colocalizes with telomere 6R during the mid-late S phase of the cell cycle (Figure 3A and Movie S1). Notably, while the colocalization between the Tel6R-TERRA focus and telomere 6R was detected in about 70% of the time-lapse analyses, colocalization between the Tel1L-TERRA focus and telomere 6R was detected in only 9% of the time courses (Figure 3B and Movie S2). To further support these results, the localization of the Tel6R-TERRA focus was monitored with respect to a 112-TetO/TetR-RFP-labeled telomere 7L using time-lapse imaging. These analyses revealed that Tel6R-TERRA did not colocalize with telomere 7L, as a single partial colocalization was observed in only 1 out of 11 cells analyzed (Figure S3A). In agreement with these observations, neither Tel1L-TERRA nor Tel6R-TERRA foci stably colocalize with Rap1-mCherry foci, indicating that TERRA transcripts do not stably associate with telomeres (data not shown). These results show that a TERRA focus preferentially and transiently colocalizes with its telomere of origin during S phase.

In order to confirm the recruitment of TERRA at telomeres, chromatin immunoprecipitation (ChIP) experiments were performed using the 6xMS2-Tel6R-TERRA strain. Cells were arrested in G1 by α factor synchronization, and samples were collected at 15 min intervals after release from G1 arrest (Figure 3C). After formaldehyde crosslinking and chromatin sonication, TERRA-MS2-GFP complexes were immunoprecipitated using anti-GFP antibody, and telomere 1L or telomere 6R chromatin enrichment was quantified by qPCR. As shown in Figure 3D, specific enrichment of telomere 6R, but not telomere 1L, was detected in Tel6R-TERRA immunoprecipitates at the 30 min time point, during S phase. Importantly, RT-qPCR analyses confirmed that Tel6R-TERRA levels do not significantly vary during the different time points analyzed (Figure S3B), suggesting that the enrichment of Tel6R-TERRA at telomere 6R during S phase is not due to the detection of nascent Tel6R-TERRA transcripts. Altogether, both live-cell imaging and ChIP indicate that TERRA transcripts associate preferentially with their telomere of origin only during S phase.

The TERRA Focus May Act as a Seed to Trigger the Formation of a Telomerase Cluster

Since TERRA is expressed from short telomeres (Figure 2) and interacts with its telomere of origin in S phase, when the TERRA focus relocates from the nuclear periphery to the telomere (Figure 3), we hypothesized that TERRA may associate with telomerase, which also acts preferentially on short telomeres in S phase (Bianchi and Shore, 2007; Sabourin et al., 2007). To this aim, we employed TERRA-MS2 clones to immunoprecipitate TERRA-MS2-GFP complexes and measured *TLC1* RNA enrichment by RT-qPCR. As expected, immunoprecipitation of the MS2-GFP protein efficiently pulls down TERRA-MS2 transcripts (Figure S4A). Interestingly, endogenous *TLC1* RNA was found specifically enriched in both Tel1L-MS2-TERRA and Tel6R-MS2-TERRA pull-down samples (Figure 4A). *TLC1* RNA was not detected in WT cells expressing MS2-GFP alone or in TERRA-MS2 clones not expressing MS2-GFP. Enrichment of *TLC1* RNA was also detected upon immunoprecipitation of Tel1L-TERRA or Tel6R-TERRA in the presence of DNase I, indicating that TERRA-*TLC1* interaction is DNA independent (Figure S4B). In order to confirm the interaction between TERRA and *TLC1* RNA, the reverse experiment was performed by pulling down the *TLC1* RNA and analyzing TERRA enrichment by RT-qPCR. To this aim, a strain expressing the endogenous *TLC1* RNA containing 10xMS2 repeats (*TLC1-MS2* cells) was used (Gallardo et al., 2011). In *TLC1-MS2* cells, MS2-GFP immunoprecipitation allowed the efficient pull down of the endogenous *TLC1-MS2* RNA (Figure S4C). As shown in Figure 4B, *TLC1-MS2* RNA pull-down resulted in a consistent enrichment of TERRA from various telomeres (Tel1L-TERRA, Tel6R-TERRA, and Y′-TERRA), indicating that *TLC1* RNA interacts with TERRA transcripts generated from different telomeres in vivo.

These findings prompted us to investigate if TERRA and *TLC1* RNA colocalize, using live-cell imaging. To this aim, a strain expressing endogenous *TLC1-MS2* RNA and a PP7-tagged Tel6R-TERRA was used, and both *TLC1-MS2* and Tel6R-TERRA-PP7

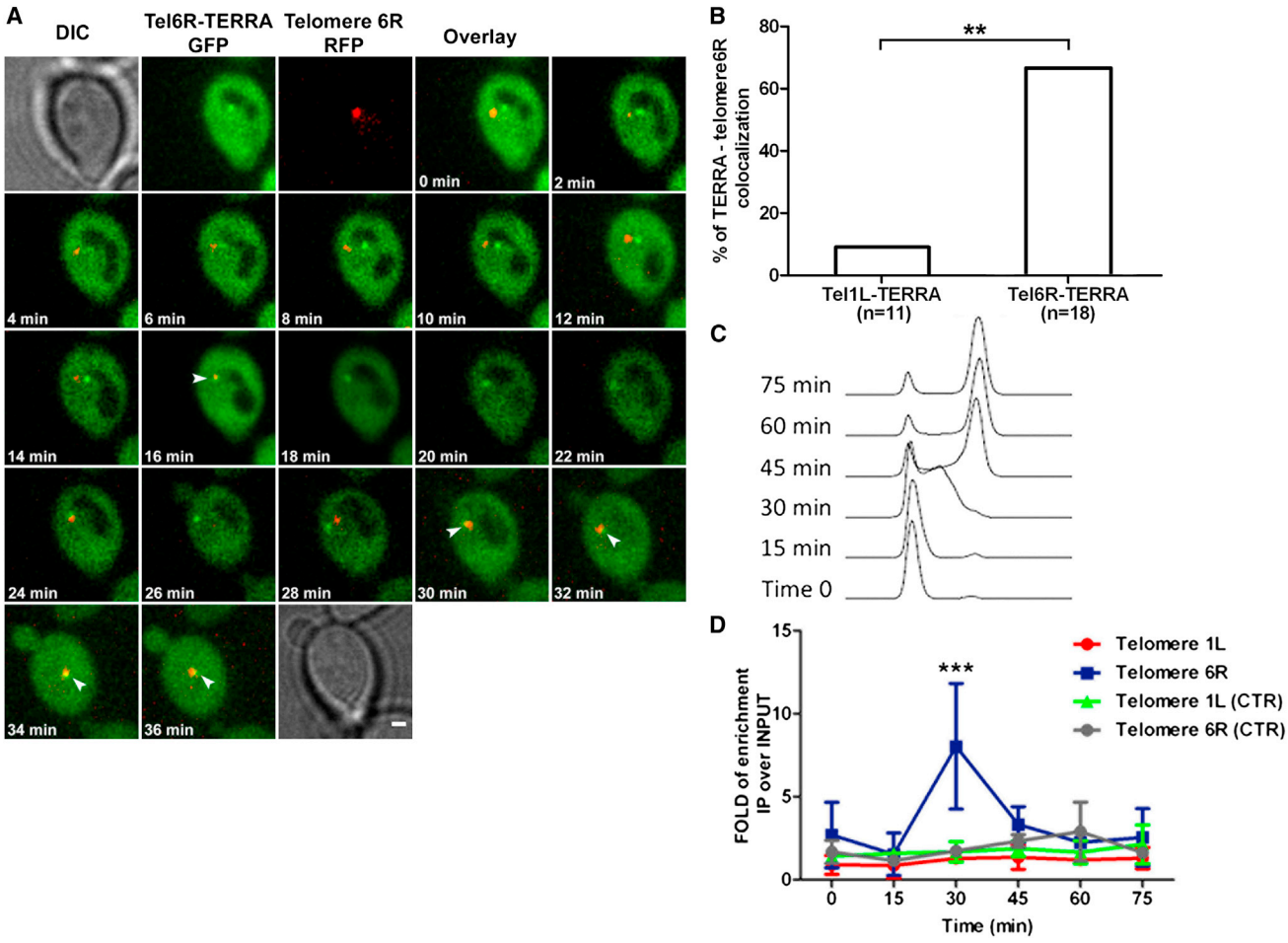

Figure 3. TERRA Associates with its Telomere of Origin during S Phase

(A) Time-lapse microscopy analysis shows transient colocalization between Tel6R-TERRA-MS2-GFP and telomere 6R-TetR-RFP during S phase. The localization of Tel6R-TERRA and telomere 6R was analyzed by performing two-color z stack acquisitions every 2 min. Tel6R-TERRA focus is in green, and telomere 6R focus is in red. White arrows indicate the time points at which colocalization is detected. Scale bar = 1μm. See also Movie S1.

(B) Quantification of colocalization occurrence between TERRA-MS2-GFP foci and telomere 6R-TetR-RFP in time-lapse experiments. See also Movies S1 and S2. **$p < 0.01$ using unpaired t test.

(C) Cell-cycle synchronization analyses by FACS after G1 arrest by α factor and subsequent release of the cell cycle.

(D) Chromatin immunoprecipitation (ChIP) experiment. Tel6R-TERRA-MS2-GFP was immunoprecipitated using anti-GFP antibody. Telomere 1L or 6R DNA enrichment was determined by qPCR. IgG antibody was used as control (CTR). Data shown represent mean ± SD (n = 3). ***$p < 0.001$ using unpaired t test.

transcripts were visualized by expressing MS2-CFP and PP7-GFP fusion proteins, respectively. Importantly, the two fluorescent proteins used in these analyses did not show bleed-through occurrences, as determined in control microscopy experiments (Figure S4D). Two-color acquisition analyses revealed that 93% of Tel6R-TERRA foci colocalized with a *TLC1* RNA cluster (T-Recs) in S phase (26 out of 28 TERRA foci; data not shown). These results lead us to monitor *TLC1* RNA and TERRA localization over time by performing time-lapse experiments. Surprisingly, imaging cells expressing Tel6R-TERRA foci over time revealed spontaneous events of *TLC1* RNA nucleation onto the TERRA foci, starting as early as late G1/early S phase. Such an event is shown in Movie S3 and Figure 4C, in which the spontaneous nucleation of *TLC1* RNA molecules on a Tel6R-TERRA focus can be observed over time. Postacquisition analyses confirmed that the area of the *TLC1* RNA cluster increases over time, as *TLC1* RNA molecules nucleate on the TERRA focus, until it reaches the same area as the TERRA focus (Figure 4D). The analysis of 13 nucleation events reveals that this process is stochastic, with a half-time of foci formation varying between 1.5 and 11 min, with a median value of 5.2 min (Figure 4E). Altogether, these results indicate that TERRA transcripts associate with *TLC1* RNA in vivo. More importantly, these data suggest that TERRA foci are involved in the nucleation of *TLC1* RNA molecules and generate TERRA-*TLC1* clusters in S phase.

TERRA-*TLC1* RNA Clusters Colocalize with the Telomere of Origin of TERRA during S Phase

Our results show the formation of clusters containing both TERRA and *TLC1* RNA (TERRA-*TLC1* clusters) during S phase

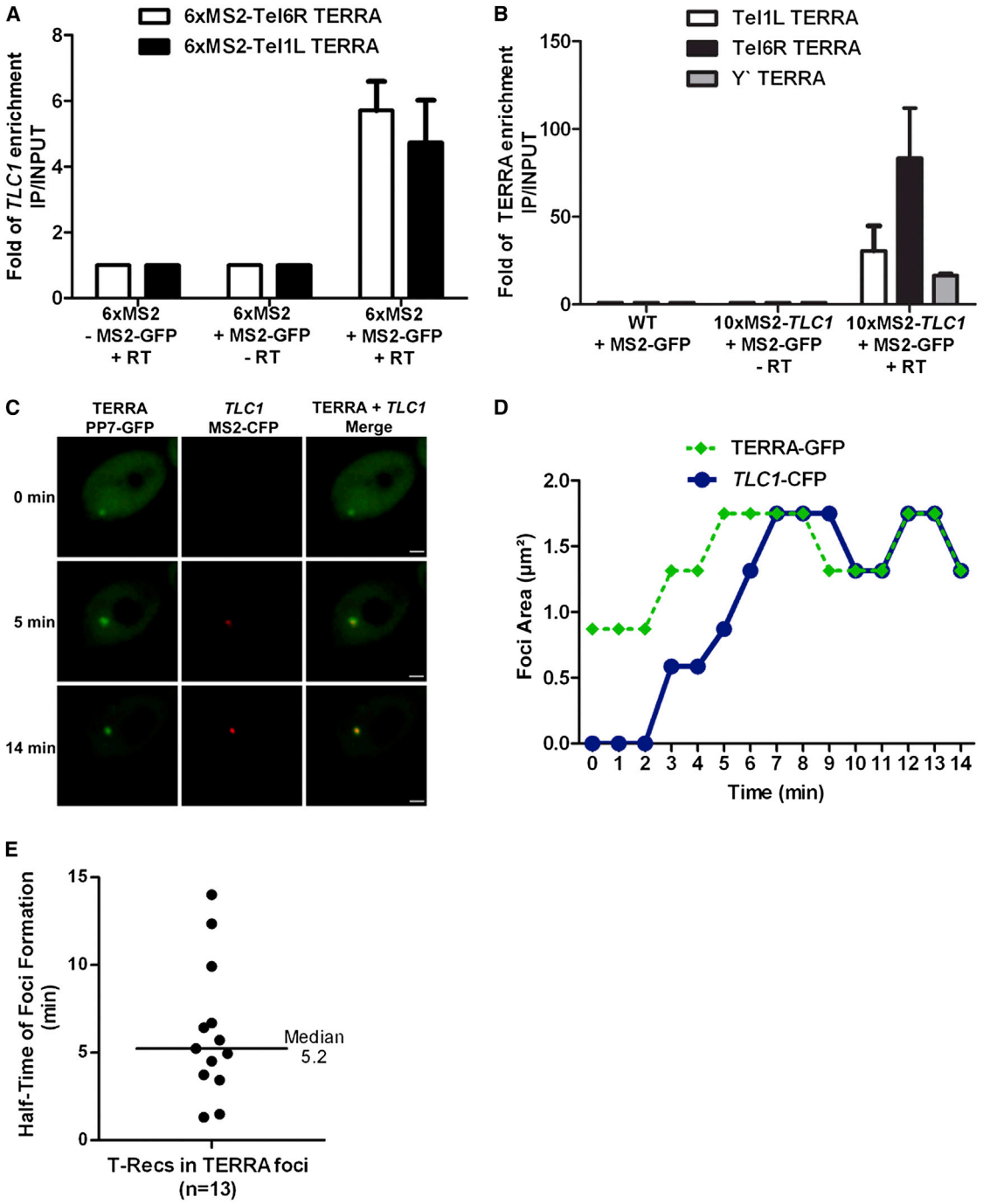

Figure 4. TERRA Foci May Act as a Scaffold to Trigger the Formation of Telomerase Clusters

(A) Enrichment of *TLC1* RNA in TERRA-MS2-GFP immunoprecipitates. *TLC1* RNA enrichment is shown as fold of enrichment in IP samples versus input. Data shown represent mean ± SD (n = 3).

(B) Enrichment of TERRA transcripts in *TLC1*-MS2-GFP immunoprecipitates. TERRA enrichment is shown as fold of enrichment in IP samples versus input. Data shown represent mean ± SD (n = 2).

(C) Nucleation of *TLC1* RNA cluster on a TERRA focus. Confocal microcopy images of *TLC1*-MS2-CFP and Tel6R-TERRA-PP7-GFP from Movie S3 at indicated time points. Tel6R-TERRA-GFP and *TLC1*-CFP were monitored over time by performing sequential two-color acquisitions. The *TLC1*-CFP focus (in red) appears directly over the Tel6R-TERRA-GFP focus (in green). Scale bar = 1µm.

(D) Quantification analysis of *TLC1*-CFP and TERRA-GFP foci areas. *TLC1* RNA and TERRA foci area quantifications were analyzed by ImageJ software. The graph shows the analysis of the time course shown in (C).

(E) Half-time of formation of *TLC1* RNA clusters (T-Recs). See also Figure S4.

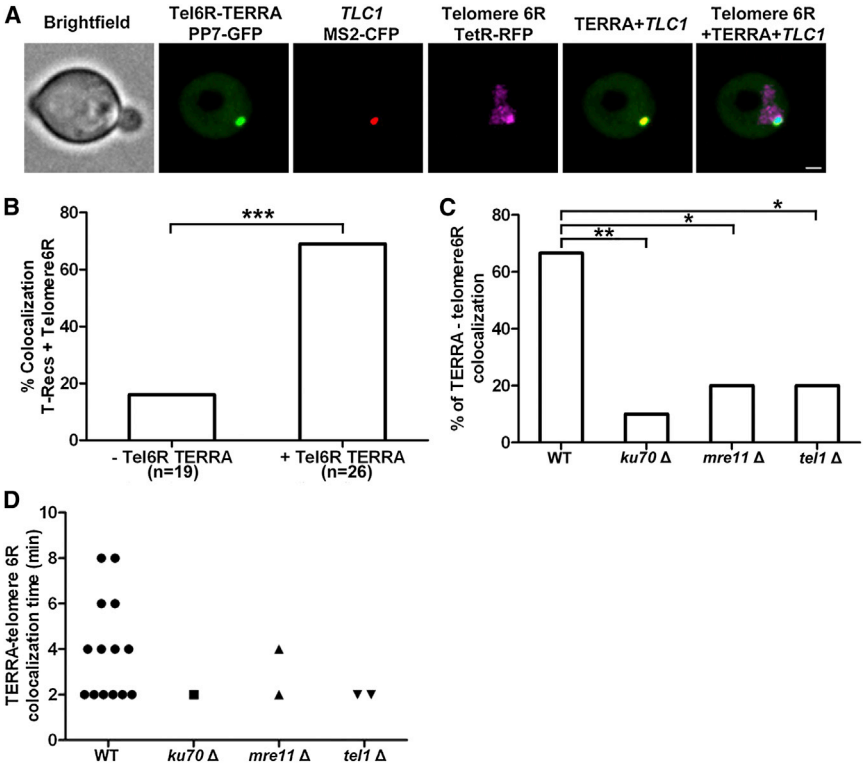

Figure 5. TERRA Foci and *TLC1* Clusters Localize at the Telomere of Origin of TERRA during S Phase

(A) Colocalization between Tel6R-TERRA-PP7-GFP focus, *TLC1*-MS2-CFP cluster, and telomere 6R-TetR-RFP focus in S phase cell. See also Movie S4.

(B) Occurrence of T-Recs localization at telomere 6R during S phase in cells not expressing Tel6R-TERRA foci (– Tel6R TERRA) or in cells that express a Tel6R-TERRA focus (+ Tel6R-TERRA). ***$p < 0.001$ using unpaired t test.

(C) Telomeric localization of TERRA is impaired in mutants that disrupt the recruitment of telomerase at telomeres. Time-lapse microscopy experiments were performed using the indicated mutant strains expressing Tel6R-TERRA-MS2-GFP and telomere 6R labeled with TetR-RFP. At least 10 time courses were performed for each clone. *$p < 0.05$, **$p < 0.01$ using unpaired t test.

(D) Residence time of Tel6R-TERRA focus at the telomere 6R in WT and mutant strains. Each symbol represents a single time course experiment.

(Figure 4). Since TERRA transcripts are expressed from short telomeres (Figure 2) and are recruited to their telomere of origin during S phase (Figure 3), when telomerase is also recruited at short telomeres (Bianchi and Shore, 2007; Sabourin et al., 2007), we investigated whether the TERRA-*TLC1* RNA clusters colocalize with the telomere-expressing TERRA. To this aim, multicolor live-cell imaging analyses were performed to monitor the localization of PP7-Tel6R-TERRA, *TLC1-MS2* RNA, and 112-TetO repeat-containing telomere 6R by imaging PP7-GFP, MS2-CFP, and TetR-RFP fusion proteins, respectively. Importantly, control experiments revealed that no bleed-through events occur during the in vivo imaging acquisitions of the three different fluorescent proteins (Figure S4D). For time-lapse experiments, three-color z stack images were acquired at one minute intervals. Interestingly, in 70% of the cells analyzed, *TLC1* RNA clusters and Tel6R-TERRA foci simultaneously colocalize with telomere 6R during S phase (Figures 5A and 5B; and Movie S4). In these cells, the colocalization of *TLC1* RNA with TERRA occurred prior to their recruitment on telomere 6R. On the other hand, in cells that do not express a Tel6R-TERRA focus, the *TLC1* RNA cluster colocalizes with telomere 6R in only 15% of the cells analyzed (Figure 5B). Formation of *TLC1* RNA clusters in these cells may be due to the expression of TERRA from telomeres other than telomere 6R.

Since it is a Tel6R-TERRA-*TLC1* RNA cluster that is recruited at telomere 6R, we investigated whether factors involved in the recruitment of telomerase at telomeres, such as Mre11, Tel1, and yKu70 (Fisher et al., 2004; Goudsouzian et al., 2006), might influence the localization of TERRA foci at telomeres. Time-lapse microscopy experiments were performed to quantify the localization of Tel6R-TERRA foci at 112-TetO-telomere 6R in *yku70Δ*, *mre11Δ*, and *tel1Δ* strains. As shown in Figures 5C and 5D, in all knockout strains analyzed, the colocalization between Tel6R-TERRA and telomere 6R was significantly impaired. Indeed, Tel6R-TERRA and telomere 6R colocalized in 65% of WT cells that were imaged, but only in 10%–20% of the cells in the mutants analyzed (Figure 5C). Furthermore, the residence time of Tel6R-TERRA foci at telomere 6R was significantly decreased in the few instances of colocalization in knockout strains (2 min) compared to colocalization events in WT cells (median of 4 min) (Figure 5D). These results show that TERRA-*TLC1* RNA clusters are recruited preferentially to the telomere of origin of the TERRA molecules and that the factors controlling telomerase recruitment at telomeres are involved in the telomeric localization of TERRA.

DISCUSSION

An In Vivo System to Study TERRA Expression and Localization

In this study, we used cytological approaches to detect TERRA transcripts expressed from a unique telomere in single cells. These approaches allowed us to investigate the expression and localization of TERRA in budding yeast cells, in which endogenous TERRA levels are low and poorly amenable to biochemical studies (Luke et al., 2008). Our results indicate that in a small population of wild-type cells, TERRA transcripts escape repression and degradation and are expressed from a specific chromosome end. In these cells, TERRA molecules assemble into a discrete nuclear focus at the nuclear periphery, which is not stably associated with telomeres. TERRA foci are not specific to yeast, as they have also been described in mammalian cells (Azzalin et al., 2007; Schoeftner and Blasco,

2008). Large TERRA foci have been observed in the nucleus of highly proliferating cells and in a variety of cancers, suggesting that aggregation of TERRA into large foci is a conserved feature between yeast and humans (Deng et al., 2012). Interestingly, RNA FISH experiments carried out in mammalian cells revealed that TERRA foci colocalize with only a subset of telomeres at any given time (Azzalin et al., 2007; Lai et al., 2013). Moreover, in vitro experiments have shown that in human cells at least a fraction of TERRA is nucleoplasmic and not associated with chromatin (Porro et al., 2010). These findings indicate that in mammalian cells, as in yeast, TERRA molecules are not stably associated with chromosome ends and that they can relocate from telomeres to other nuclear compartments.

TERRA Expression Is Induced at Short Telomeres

By being able to observe TERRA expression from a single telomere, we were able to show that only a small fraction of cells express TERRA from a chromosome end. Why do these few cells express TERRA from that specific telomere, compared to the majority of the cells in the population? Our results suggest that in this small population, the TERRA-expressing telomere is short, which leads to TERRA expression and accumulation. Indeed, we show that TERRA levels are upregulated when telomeres shorten in a *tlc1*Δ strain. Moreover, live-cell imaging analyses revealed that *tlc1*Δ TERRA-MS2 clones show a higher number of cells expressing a TERRA focus and an increased number of foci per cell compared to *TLC1* WT cells. These findings were confirmed by a time course experiment using a strain containing a short inducible telomere, which showed that shortening of this single chromosome end induces TERRA expression in *cis*. Conversely, telomere relengthening by telomerase results in a progressive decrease of TERRA levels. These results indicate that TERRA expression is regulated in a telomere length-dependent manner and that TERRA levels inversely correlate with the length of their telomere of origin. These findings would point to TERRA expression as a marker of telomere shortening in a cell population.

It has been shown that the yeast telomeric repeat DNA-binding protein Rap1 recruits inhibitory factors to telomeres, such as Sir and Rif proteins that repress TERRA expression (Iglesias et al., 2011). We can thus hypothesize that short telomeres, containing a smaller number of Rap1 binding sites, exhibit a weaker repressive effect on TERRA expression. Similarly, telomere relengthening gradually reestablishes TERRA repression by restoring the missing number of telomere binding proteins. This hypothesis is supported by the fact that TERRA levels are upregulated in *rif1*, *rif2*, and *rap1-17* mutant strains (Iglesias et al., 2011). Interestingly, studies in mammalian cells have shown that TERRA levels are elevated in cancer cells and autosomal-recessive immunodeficiency, centromeric region instability, and facial anomalies syndrome (ICF) cells, which contain short telomeres (Yehezkel et al., 2008). Moreover, a recent study shows that telomere elongation represses TERRA expression in mammalian cells, due to the increased density of heterochromatic marks present at longer telomeres (Arnoult et al., 2012). Although it is likely that in mammalian cells more complex mechanisms regulate the expression of TERRA, these findings are in agreement with our results.

TERRA Focus as a Scaffold to Promote the Nucleation of a Telomerase Cluster Prior to Telomerase Recruitment at Telomeres

Using coimmunoprecipitation, we show that endogenous TERRA transcripts interact with the yeast telomerase RNA *TLC1* in vivo. While it is not yet clear if this interaction is direct, these results are in agreement with previous findings obtained in mammalian cells in which TERRA molecules interact with both human telomerase RNA (hTR) and human telomerase reverse transcriptase (hTERT) in vivo (Redon et al., 2010). In mammalian cells, TERRA-mimicking oligonucleotides inhibit telomerase activity in vitro (Redon et al., 2010; Schoeftner and Blasco, 2008). While these findings are suggestive of a potential role of TERRA as a telomerase inhibitor, direct evidence of this function in vivo is still missing (Farnung et al., 2012). Our data suggest instead that, at least in yeast, TERRA interacts with the active telomerase complex since (1) TERRA is expressed when a telomere shortens; (2) TERRA is recruited on its telomere of origin in S phase, when telomere elongation occurs; (3) TERRA interacts with *TLC1* RNA, and this complex is recruited simultaneously and preferentially on the telomere of origin of TERRA (i.e., a short telomere); and (4) association of TERRA with its telomere depends on factors involved in telomerase recruitment at telomeres (Mre11, Tel1, and yKu70). Altogether, these results suggest a positive role for TERRA during telomere elongation by telomerase.

Using live-cell imaging, the interaction between TERRA and *TLC1* RNA was found to occur, at least in part, within TERRA foci. We were able to capture, in real time, spontaneous nucleation events of *TLC1* RNA molecules on TERRA foci. These nucleation events lead to the formation of the telomerase clusters or T-Recs, which have been previously described as the active form of telomerase at telomeres in S phase (Gallardo et al., 2011). These results support the hypothesis that TERRA may act as a scaffold for the spatial organization of telomerase molecules into a T-Rec. This scaffold-like function of telomeric noncoding RNA may be similar to what has been proposed for a growing number of long noncoding RNAs (lncRNAs). Indeed, several studies have shown that lncRNAs play important roles in the recruitment of protein complexes to specific chromosome loci in order to control chromatin structure (Tsai et al., 2010; Wang and Chang, 2011) or induce the formation of nuclear bodies (Mao et al., 2011; Shevtsov and Dundr, 2011). While most of these nuclear lncRNAs act cotranscriptionally to recruit protein cofactors, TERRA acts in *trans* to assemble a telomerase cluster, forming a TERRA-telomerase complex, which is then recruited to the telomere from which TERRA originates.

The formation of a TERRA-telomerase complex could explain the preferential recruitment of TERRA transcripts to their telomere of origin. Indeed, telomerase is preferentially recruited to short telomeres (Bianchi and Shore, 2007; Sabourin et al., 2007), and mutant strains, such as *tel1*, *mre11*, and *yku70*, where this recruitment is compromised, show an impaired telomeric localization of TERRA. Thus, the recruitment of telomerase at short telomeres may direct TERRA to its (short) telomere of origin. If so, it is (in principle) possible that TERRA transcripts might also relocate to other short telomeres as a result of their association with telomerase. Because of the low frequency of

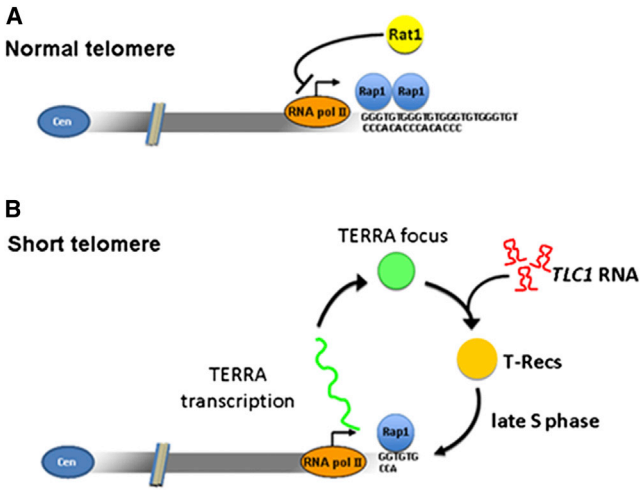

Figure 6. Model of TERRA Expression and Function at Telomeres

(A) At a normal telomere, TERRA expression is repressed by Rap1-associated factors and degradation by the exonuclease Rat1.

(B) At a short telomere, TERRA expression is induced, leading to the accumulation of TERRA transcripts as a nuclear focus (TERRA focus). In S phase, the TERRA focus acts as a scaffold to promote the nucleation of *TLC1* RNA molecules and generate a T-Rec. In late S phase, the T-Rec is recruited on the short telomere from which TERRA molecules originate.

multiple short telomeres in a single cell, these events will be relatively rare and difficult to determine.

In summary, we propose a model in which a threshold of telomere erosion induces TERRA expression (Figure 6). Upon transcription, TERRA molecules are rapidly displaced from their telomere of origin and accumulate into a focus. In early S phase, a TERRA focus acts as seed to nucleate the *TLC1* RNA, directly or via binding to different telomerase components, leading to the formation of a T-Rec. Later in S phase, the T-Rec is recruited to the telomere from which TERRA originates, which would then be elongated by telomerase. Thus, TERRA expression might represent a signal induced by short telomeres to trigger telomerase clustering and subsequent telomere elongation. Interestingly, in cancer cells, formation of telomerase RNA (hTR) foci at telomeres has been linked to a distributive action of telomerase at telomeres in which several telomerase molecules elongate a short telomere (Zhao et al., 2011). Whether TERRA also acts as a scaffold for the formation of such foci remains to be established. Our data reveal that lncRNAs do not only act as regulators of gene expression, but also actively organize catalytic activities involved in genome maintenance.

EXPERIMENTAL PROCEDURES

Yeast Strains

All the strains used in this study were generated in a FY23 background, with the exception of the strains used in the ChIP experiments, which are derived from W303 (see Table S1). Insertion of 2xMS2 and 6xMS2 at telomere 1L or telomere 6R was performed by the integration of a PCR cassette containing the MS2 sequences and a G418 resistance (*KAN*) gene, flanked by *loxP* sites. *KAN* marker gene was eliminated by expression of the Cre recombinase.

Fluorescence In Situ Hybridization

FISH experiments using oligonucleotide DNA probes have been described in (Gallardo and Chartrand, 2011), and the detailed modified protocol used in this study is provided in the Supplemental Experimental Procedures.

Live-Cell Imaging

Yeast cells were grown overnight at 30°C in the appropriate selection medium, diluted to optical density 600 (OD_{600}) = 0.1 and subsequently grown until an OD_{600} of 0.3–0.4. For TERRA-MS2-GFP foci imaging and TERRA-telomere 6R colocalization experiments, cells were imaged with a Zeiss Cell Observer SD spinning disk confocal microscope, using a 100×/1.46 NA objective and an Evolve 512 EM-CCD camera (Photometrics). For simultaneous TERRA-PP7-GFP, *TLC1*-MS2-CFP, and telomere 6R-TetR-RFP imaging, acquisition was performed at the Cell Imaging and Analysis network (McGill University) using a Quorum WaveFX-X1 Spinning Disk Confocal System, which is based on a Leica DMI6000B inverted microscope, with an ASI MS-2000 Piezo Stage, a 63×/1.40NA Leica PL APO objective, a Hamamatsu C9100-13 EM-CCD camera, and a Live Cell Instruments Chamlide TC environmental control system. Detailed acquisition and image analysis procedures are described in the Supplemental Experimental Procedures.

Telomere Shortening Experiment

Cells were grown overnight at 30°C in YP medium containing raffinose. The following day, expression of Flp1 recombinase was induced by diluting the cells to OD_{600} = 0.2 in 30°C prewarmed YP medium containing 3% galactose. Cells were grown in galactose for 3 hr and then collected, washed twice in yeast extract peptone dextrose (YPD), and diluted in YPD medium at OD_{600} = 0.2. Cells growing in logarithmic phase at an OD_{600} < 2 were collected at the time points corresponding to the indicated generations. Two pellets per time point were frozen for subsequent RNA and DNA extraction.

Chromatin Immunoprecipitation

Cells were grown in selection medium at 30°C until an OD_{600} of 0.7–0.8. Then, α factor was added to 2.5 μM final concentration for 2 hr. The cells were subsequently collected and washed twice in YPD medium, resuspended in YPD, and grown at room temperature (23°C) for the indicated time. At each 15 min time point, 50 ml aliquots of cells were fixed in 1% formaldehyde. Cells were collected and washed twice in ice-cold PBS. Pellets were resuspended in 400 μl lysis buffer (50 mM HEPES [pH 8], 140 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100) containing protease inhibitors (EDTA-free protease inhibitor mix and 1 mM PMSF, Roche) and RNase inhibitor (2 mM vanadyl ribonucleoside complex [VRC]). Cells were lysed using glass beads by vortexing 6 times × 45 s. Lysates were eluted in new tubes by needle puncturing and centrifuged 5 min at 3,000 rpm and 4°C, and supernatant was transferred into a new tube. Pellets were resuspended in 200 μl lysis buffer, centrifuged again, and supernatant was added to the corresponding tube. Samples were sonicated on ice 4 times for 20 s each and subsequently precleared for 1 hr at 4°C with 45 μl of equilibrated magnetic beads (Millipore). After a preclearing step, immunoprecipitation was performed overnight using monoclonal anti-GFP antibody (Roche) or mouse immunoglobulin G (IgG). The following day, 45 μl magnetic beads were added to each sample, and binding to the antibodies was allowed for 1 hr at 4°C. Protein bead complexes were collected and washed 4 times for 5 min each at room temperature using the following wash buffers: wash 1, 700 μl of lysis buffer; wash 2, 1 ml lysis buffer containing 500 mM NaCl; wash 3, 700 μl LiCl buffer (10 mM Tris [pH 8], 1 mM EDTA [pH 8], 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate); wash 4, 700 μl of wash buffer 4 (10 mM Tris [pH 8], 1mM EDTA [pH 8], 100 mM NaCl). Protein bead complexes were eluted and decrosslinked in 200 μl elution buffer (10 mM Tris [pH 8], 1 mM EDTA [pH 8], 0.5% SDS) at 65°C overnight. The following day, 225 μl of solution containing 10 mM Tris (pH 8), 1 mM EDTA (pH 8), and 25 μg proteinase K was added to each tube, and samples were incubated for 6 hr at 37°C. Phenol/chloroform and chloroform extractions were performed, followed by DNA precipitation overnight at −20°C. DNA was resuspended in 40 μl of H_2O, and qPCR experiments were performed in triplicates using 3 μl of sample per reaction. Telomere 1L and 6R DNA enrichment was assessed by calculating 2^{-Dct} values using *ACT1*

gene for normalization in both immunoprecipitate (IP) and input samples and expressed as fold of enrichment IP over input.

RNA Extraction and RT-qPCR Analyses

Cells were harvested during logarithmic phase, washed in ice-cold PBS, and resuspended in 400 μl of ice-cold Tris/EDTA/SDS (TES) solution (10 mM Tris [pH 7.5], 10 mM EDTA, 0.5% SDS). A total of 400 μl of acid phenol were added to each sample, and tubes were incubated for 1 hr at 65°C with vortexing for 5 sec every 10 min. Samples were then chilled in ice for 5 min and centrifuged at 13,000 rpm for 5 min at room temperature. After chloroform extraction, RNA was precipitated by addition of 1/10 volume of 3 M NaAc (pH 5.2) and 2 volumes of 100% ethanol (ETOH). RNA was subsequently collected by centrifugation and washed with 500 μl of 70% ETOH. Pellets were resuspended in diethylpyrocarbonate (DEPC) water and run on 1× MOPS agarose gel for quantification and quality control. A total of 3 μg of RNA was treated with DNase I (Fermentas) for 1 hr at 37°C, and 200 ng of DNase-treated RNA was reverse transcribed by using H-MULV-H enzyme (Fermentas) at 42°C for 1 hr. For TERRA, a C-rich primer (primer sequence: CACCACACCCACACAC CACACCCACA) was used for reverse transcription (RT), while a specific primer was used for *TLC1* RNA (primer sequence: CGAAGGCATTAGGA GAAGTAGC). A total of 3 μl of the RT reaction was used for the qPCR experiments. qPCR experiments were performed in triplicates using the qPCR master mix SsoFAST EvaGreen Supermix from Bio-Rad. qPCR was carried out on a Roche LightCycler 480 Sequence Detection System. Analyses were performed by calculating the averages and SDs of the 2^{-Dct} values, normalized on *ACT1* messenger RNA (mRNA), for each triplicate.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, one table, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.08.029.

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REFERENCES

Arnoult, N., Van Beneden, A., and Decottignies, A. (2012). Telomere length regulates TERRA levels through increased trimethylation of telomeric H3K9 and HP1α. Nat. Struct. Mol. Biol. *19*, 948–956.

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

Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S.M., Singer, R.H., and Long, R.M. (1998). Localization of ASH1 mRNA particles in living yeast. Mol. Cell *2*, 437–445.

Bianchi, A., and Shore, D. (2007). Increased association of telomerase with short telomeres in yeast. Genes Dev. *21*, 1726–1730.

de Lange, T. (2009). How telomeres solve the end-protection problem. Science *326*, 948–952.

Deng, Z., Norseen, J., Wiedmer, A., Riethman, H., and Lieberman, P.M. (2009). TERRA RNA binding to TRF2 facilitates heterochromatin formation and ORC recruitment at telomeres. Mol. Cell *35*, 403–413.

Deng, Z., Wang, Z., Xiang, C., Molczan, A., Baubet, V., Conejo-Garcia, J., Xu, X., Lieberman, P.M., and Dahmane, N. (2012). Formation of telomeric repeat-containing RNA (TERRA) foci in highly proliferating mouse cerebellar neuronal progenitors and medulloblastoma. J. Cell Sci. *125*, 4383–4394.

Farnung, B.O., Brun, C.M., Arora, R., Lorenzi, L.E., and Azzalin, C.M. (2012). Telomerase efficiently elongates highly transcribing telomeres in human cancer cells. PLoS ONE *7*, e35714.

Fisher, T.S., Taggart, A.K.P., and Zakian, V.A. (2004). Cell cycle-dependent regulation of yeast telomerase by Ku. Nat. Struct. Mol. Biol. *11*, 1198–1205.

Flynn, R.L., Centore, R.C., O'Sullivan, R.J., Rai, R., Tse, A., Songyang, Z., Chang, S., Karlseder, J., and Zou, L. (2011). TERRA and hnRNPA1 orchestrate an RPA-to-POT1 switch on telomeric single-stranded DNA. Nature *471*, 532–536.

Gallardo, F., and Chartrand, P. (2011). Visualizing mRNAs in fixed and living yeast cells. Methods Mol. Biol. *714*, 203–219.

Gallardo, F., Laterreur, N., Cusanelli, E., Ouenzar, F., Querido, E., Wellinger, R.J., and Chartrand, P. (2011). Live cell imaging of telomerase RNA dynamics reveals cell cycle-dependent clustering of telomerase at elongating telomeres. Mol. Cell *44*, 819–827.

Gotta, M., Laroche, T., Formenton, A., Maillet, L., Scherthan, H., and Gasser, S.M. (1996). The clustering of telomeres and colocalization with Rap1, Sir3, and Sir4 proteins in wild-type Saccharomyces cerevisiae. J. Cell Biol. *134*, 1349–1363.

Goudsouzian, L.K., Tuzon, C.T., and Zakian, V.A. (2006). S. cerevisiae Tel1p and Mre11p are required for normal levels of Est1p and Est2p telomere association. Mol. Cell *24*, 603–610.

Greider, C.W., and Blackburn, E.H. (1985). Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell *43*, 405–413.

Hug, N., and Lingner, J. (2006). Telomere length homeostasis. Chromosoma *115*, 413–425.

Iglesias, N., Redon, S., Pfeiffer, V., Dees, M., Lingner, J., and Luke, B. (2011). Subtelomeric repetitive elements determine TERRA regulation by Rap1/Rif and Rap1/Sir complexes in yeast. EMBO Rep. *12*, 587–593.

Jain, D., and Cooper, J.P. (2010). Telomeric strategies: means to an end. Annu. Rev. Genet. *44*, 243–269.

Lai, L.-T., Lee, P.J., and Zhang, L.-F. (2013). Immunofluorescence protects RNA signals in simultaneous RNA-DNA FISH. Exp. Cell Res. *319*, 46–55.

Larson, D.R., Zenklusen, D., Wu, B., Chao, J.A., and Singer, R.H. (2011). Real-time observation of transcription initiation and elongation on an endogenous yeast gene. Science *332*, 475–478.

Luke, B., Panza, A., Redon, S., Iglesias, N., Li, Z., and 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.

Maicher, A., Kastner, L., Dees, M., and Luke, B. (2012). Deregulated telomere transcription causes replication-dependent telomere shortening and promotes cellular senescence. Nucleic Acids Res. *40*, 6649–6659.

Mao, Y.S., Sunwoo, H., Zhang, B., and Spector, D.L. (2011). Direct visualization of the co-transcriptional assembly of a nuclear body by noncoding RNAs. Nat. Cell Biol. *13*, 95–101.

Marcand, S., Brevet, V., and Gilson, E. (1999). Progressive cis-inhibition of telomerase upon telomere elongation. EMBO J. *18*, 3509–3519.

Marcand, S., Brevet, V., Mann, C., and Gilson, E. (2000). Cell cycle restriction of telomere elongation. Curr. Biol. *10*, 487–490.

Pfeiffer, V., and Lingner, J. (2012). TERRA promotes telomere shortening through exonuclease 1-mediated resection of chromosome ends. PLoS Genet. *8*, e1002747.

Porro, A., Feuerhahn, S., Reichenbach, P., and Lingner, J. (2010). Molecular dissection of telomeric repeat-containing RNA biogenesis unveils the

presence of distinct and multiple regulatory pathways. Mol. Cell. Biol. *30*, 4808–4817.

Redon, S., Reichenbach, P., and Lingner, J. (2010). The non-coding RNA TERRA is a natural ligand and direct inhibitor of human telomerase. Nucleic Acids Res. *38*, 5797–5806.

Sabourin, M., Tuzon, C.T., and Zakian, V.A. (2007). Telomerase and Tel1p preferentially associate with short telomeres in S. cerevisiae. Mol. Cell *27*, 550–561.

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

Shevtsov, S.P., and Dundr, M. (2011). Nucleation of nuclear bodies by RNA. Nat. Cell Biol. *13*, 167–173.

Shore, D., and Bianchi, A. (2009). Telomere length regulation: coupling DNA end processing to feedback regulation of telomerase. EMBO J. *28*, 2309–2322.

Teixeira, M.T., Arneric, M., Sperisen, P., and Lingner, J. (2004). Telomere length homeostasis is achieved via a switch between telomerase- extendible and -nonextendible states. Cell *117*, 323–335.

Tsai, M.-C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J.K., Lan, F., Shi, Y., Segal, E., and Chang, H.Y. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. Science *329*, 689–693.

Wang, K.C., and Chang, H.Y. (2011). Molecular mechanisms of long noncoding RNAs. Mol. Cell *43*, 904–914.

Wellinger, R.J., and Zakian, V.A. (2012). Everything you ever wanted to know about Saccharomyces cerevisiae telomeres: beginning to end. Genetics *191*, 1073–1105.

Yehezkel, S., Segev, Y., Viegas-Péquignot, E., Skorecki, K., and 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.

Zhao, Y., Abreu, E., Kim, J., Stadler, G., Eskiocak, U., Terns, M.P., Terns, R.M., Shay, J.W., and Wright, W.E. (2011). Processive and distributive extension of human telomeres by telomerase under homeostatic and nonequilibrium conditions. Mol. Cell *42*, 297–307.