Methods 114 (2017) 39–45

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

A practical qPCR approach to detect TERRA, the elusive telomeric repeat-containing RNA

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ARTICLE INFO

Article history:
Received 27 May 2016
Received in revised form 1 August 2016
Accepted 7 August 2016
Available online 12 August 2016

Keywords:
Telomeres
Subtelomeres
TERRA
Transcriptional regulation
qRT-PCR

ABSTRACT

Telomeres, the heterochromatic structures that protect the ends of the chromosomes, are transcribed into a class of long non-coding RNAs, telomeric repeat-containing RNAs (TERRA), whose transcriptional regulation and functions are not well understood. The identification of TERRA adds a novel level of structural and functional complexity at telomeres, opening up a new field of research. TERRA molecules are expressed at several chromosome ends with transcription starting from the subtelomeric DNA proceeding into the telomeric tracts. TERRA is heterogeneous in length and its expression is regulated during the cell cycle and upon telomere damage. Little is known about the mechanisms of regulation at the level of transcription and post transcription by RNA stability. Furthermore, it remains to be determined to what extent the regulation at different chromosome ends may differ. We present an overview on the methodology of how RT-qPCR and primer pairs that are specific for different subtelomeric sequences can be used to detect and quantify TERRA expressed from different chromosome ends.

Contents

1. Introduction

Telomeres are nucleoprotein structures at the ends of eukaryotic chromosomes. They perform crucial functions as tumor suppressors and they protect chromosome ends from degradation and rearrangements. Telomere functions are mediated largely by the proteins that associate with them. In addition, certain telomere functions are linked to the long noncoding RNA TERRA (for telomeric repeat containing RNA) [1–3]. TERRA transcription starts from promoters in the subtelomeric regions of various chromosomes and proceeds towards chromosome ends [4]. Recent work suggests that TERRA sustains several important functions (reviewed in [2]).

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http://dx.doi.org/10.1016/j.ymeth.2016.08.004
1046-2023/

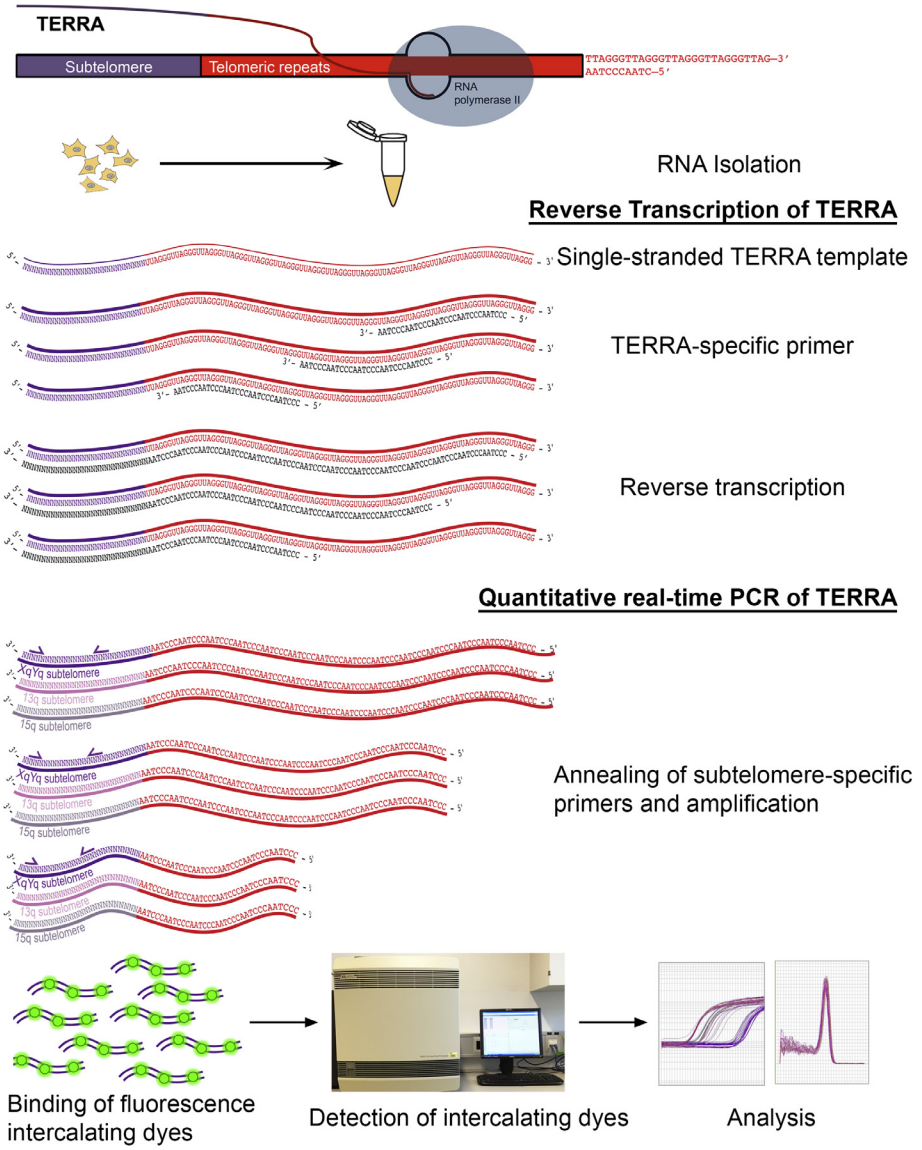

Fig. 1. Schematic representation of TERRA RT-qPCR assay. TERRA synthesis, which is mainly mediated by RNA polymerase II, derives from the subtelomeric region (purple) and extends towards the end of the chromosome into the telomeric repeats (red). A TERRA-specific oligonucleotide comprised of telomeric sequence will anneal and reverse transcribe TERRA from numerous positions in the telomeric tract (red), generating a pool of cDNA molecules with diverse subtelomeric sequences (purple) and length. In quantitative PCR specific primer pairs (purple arrows) amplify the desired subtelomeric sequence from the pool of transcribed TERRA molecules. Fluorescence intercalating dyes bind the double-stranded amplicon, which is detected and analyzed by the RT-qPCR system. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TERRA can regulate telomere length through modulation of exonuclease 1 and telomerase [5,6]. TERRA expression is upregulated at damaged telomeres where it sustains recruitment of the chromatin modifiers LSD1 (a lysine demethylase) and SUV39H1 (a histone H3 lysine 9 methylase) thereby enabling DNA end processing [4,7]. TERRA has also been proposed to promote telomere protein composition changes during cell cycle progression. In particular TERRA may favor the binding of the single strand telomeric DNA binding protein POT1/TPP1 to telomeres after their replication during which the single strand DNA binding and replication protein RPA is present at telomeres [8]. Consistent with a role during the cell cycle, human TERRA is expressed during G1 and G2 phases of the cell cycle but is repressed during late S-phase [9]. Finally, recent evidence suggests that TERRA, when engaged into RNA/DNA hybrid structures at chromosome ends, may promote homologous recombination of telomeres in so-called ALT cells (alternative lengthening of telomeres). ALT cells maintain telomeric DNA by recombination instead of telomerase and express high TERRA levels [10,11]. Abnormally high levels of TERRA at telomeres can also interfere with telomere maintenance in human cells as seen in RNA surveillance mutants [1]. In ICF (immunodeficiency, centromeric instability, facial anomalies) patient-derived cell lines which lack DNA methyl transferase 3b [12], subtelomeric CpG islands are undermethylated, which presumably leads to TERRA overexpression in these patients. ICF patients have extremely short telomeres and it remains to be determined whether this phenotype is linked to TERRA overexpression. As opposed to classical analysis of TERRA by Northern blots, RT-qPCR allows accurate quantification of TERRA molecules expressed from individual chromosome ends (Fig. 1). This type of analysis will be most critical to elucidate TERRA regulation and better understand its functions in normal development and its dysfunctions in disease.

2. Challenges of TERRA isolation and quantification

2.1. RNA isolation

A highly efficient RNA purification protocol is required to detect the low levels of TERRA. Most commercially available RNA isolation kits are based on a silica-based membrane technology that allows purification of high-quality RNA suitable for TERRA analysis. However, although silica-based membranes are designed to efficiently remove most of the DNA from the samples, some residual DNA may still contaminate the final RNA solution, which will be amplified during qPCR along with TERRA cDNAs. Therefore, to eliminate residual DNA contamination from the sample, it is highly recommended to perform three DNase treatments during RNA purification, two on-column digestions and one in solution.

2.2. RNA purity

The concentration and the quality of the RNA can greatly affect the outcome of the RT-qPCR. Contaminations of the RNA with salt, ethanol or proteins strongly perturb the performance of downstream applications. In addition, it is important for RT-qPCR that the RNA starting concentrations of samples to be compared are very similar in order to obtain reliable quantitative data. RNA purity and concentration can be measured by NANOdrop spectrophotometers (Thermo Scientific) or Qubit Fluorometric Quantitation (ThermoFisher Scientific). In NANOdrop, UV absorbance is measured at 230 nm, 260 nm and 280 nm. The A260/A280 ratio of pure RNA should be between 1.8 and 2.0 and the A260/A230 ratio in the range of 2.0–2.2. Isolation of intact RNA is crucial for accurate gene expression analysis. RNA integrity should be assessed on a denatured agarose gel stained with ethidium bromide. Intact total RNA will present two sharp, clear bands corresponding to the 28S and 18S rRNA. The upper 28S band should be twice as intense as the lower 18S rRNA band.

2.3. qPCR primer design

Good primer design is most crucial for RT-qPCR experiments. Reliable and accurate qPCR results require efficient and specific amplification of the target that must be validated. The amplicon size should be 80–120 bp in length and be composed of a unique sequence to distinguish unspecific amplification. qPCR primers are usually 19–22 bp long and they are purified using standard desalt purification methods. The sequence of the primers should be chosen to avoid intramolecular or intermolecular interactions resulting in hairpins, self dimerization or cross dimerization, which will reduce the availability of the primers for the reaction. qPCR primers should also have a 50–60% GC content with Gs or Cs at the 3′ ends of the primer to facilitate specific binding of the 3′ end of the primer to the target sequence. The GC content of the primer will dictate the melting temperature, which plays a major role in the specificity of the method. The optimal melting temperature of the qPCR primer pair should be between 59 °C and 64 °C with no more than 1 °C difference between them.

Primer design for TERRA quantification follows the same basic guidelines; however, due to the repetitive nature of the target sequence additional parameters and validation is applied. As TERRA transcripts start in the subtelomeric regions and extend towards the ends of chromosomes they are comprised of subtelomeric derived sequence and 5′-UUAGGG-3′ repeats at their 3′ ends (Fig. 1). TERRA quantification derives from quantification of the subtelomeric part of the RNA molecule, where the sequence is significantly different from the $(UUAGGG)_n$ repeats. Nevertheless, subtelomeric sequences also contain long repetitive sequences, many of which are shared between different chromosomal subtelomeres. Therefore, designing unique primers to amplify a specific subtelomeric sequence is challenging and requires rigorous testing through PCR and sequencing. Until recently, the complete human subtelomeric sequences were not available and Northern blot analysis was the sole approach for TERRA quantification. However, Riethman et al. and Stong et al. generated complete assemblies of most of the human subtelomeres [13,14], which have been used to analyze ChIP-Seq data and map the binding sites of RNAPII. Furthermore, the genome wide TERRA transcriptome analysis defined possible TERRA start sites in the subtelomeric sequences at different chromosome ends [4,15]. The most updated subtelomere assemblies can be found at the Riethman laboratory website in the Wistar Institute (https://www.wistar.org/sites/default/files/protected/htel_1-500K_1_10_12_v4_3_12fasta.TXT). TERRA primer pairs are designed downstream of possible TERRA transcription start sites and RNAPII binding sites of each subtelomere sequence. New primer pairs are subjected to extensive BLAST analysis against the genome sequence database (i.e. Primer-BLAST: http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and against the human subtelomere database (custom made databases) to ensure unique alignment with the target sequence.

Newly designed subtelomeric primer pairs are first used to amplify the desired specific target sequence in a conventional PCR (ideally using a high fidelity polymerase i.e. Phusion Green Hot Start ThermoScientific #F537L) followed by gel electrophoresis. Primer pairs that produce unspecific bands or numerous bands on agarose gels are excluded from further analysis. The amplification products that correspond to the expected size are cloned into a vector (i.e. Zero Blunt TOPOPCR Cloning Kit, Invitrogen #K287540) for sequence analysis. The primer pairs that amplify only the desired subtelomeric target sequence are chosen for analysis in qPCR assays. Functional primer pairs are subjected to post-amplification melting curve analyses to ensure the absence of primer-dimer artifacts and unspecific amplifications. Finally, amplification efficiency should be determined for newly synthesized primer pairs to ensure robust and precise qPCR analysis of TERRA levels relative to a reference gene.

For some subtelomeric sequences that are highly conserved it is not possible to find specific primer pairs. Table 1 summarizes published and unpublished primer pairs that have been validated and successfully used for TERRA quantifications from distinct chromosome ends. The primer efficiency of each primer was calculated in HeLa cells and incorporated in Table 1. In addition, we performed a titration of the RNA concentration in the qPCR assays to evaluate the lowest concentration that can produce a reliably measured Ct value for some TERRA molecules (Fig. S1).

3. Description of the method

3.1. Isolation and preparation of RNA from desired samples

Total RNA is isolated from human tissue cell lines maintained at low to medium confluency (70–80%). If an adherent cell line is used it is preferred to wash, lyse, and homogenize the cells directly on the plate to minimize exposure to stressful conditions. The following protocol was performed on HeLa cell lines, grown in 10-cm dishes at 50–60% confluency. RNA is isolated from HeLa or other human cell lines using the RNeasy Mini Kit (QIagen #74106) or NucleoSpin RNA II (Macherey-Nagel # 740955.250), following the manufacturer's protocol. Both kits have been tested and proven to isolate, clean, high quality RNA samples. The RNA samples are always subjected to an on-column DNase treatment. Purified RNA is treated again with DNase in solution, re-purified using the kit, and digested a third time with DNase on the column

Table 1
List of oligonucleotides used for RT-qPCR and siRNAs.

Primer	Sequence (5′–3′)	Distance from TTAGGG repeats	Efficiency (%) in HeLa	Reference
TERRA-specific RT-primer	CCCTAACCCTAACCCTAACCCTAA-CCCTAA			
GAPDH-specific RT-primer	GCCCAATACGACCAAATCC			
GUSB-specific RT-primer	AATACAGATAGGCAGGGCGTTCG			
1q TERRA – forward	GCATTCCTAATGCACACATGAC	213 bp	111	This study
1q TERRA – reverse	ACCCTAACCCGAACCCTA	137 bp		This study
2q TERRA – forward	AAAGCGGGAAACGAAAAGC	247 bp	112	[15]
2q TERRA – reverse	GCCTTGCCTTGGGAGAATCT	287 bp		[15]
7p TERRA 1 – forward	CAATCTCGGCTCACCACAATC	825 bp	183	[15]
7p TERRA 1 – reverse	GGAGGCTGAGGCAGGAGAA	785 bp		[15]
7p TERRA 2 – forward	GAGAGAGGGTTTCACTCTGTTG	568 bp	116	This study
7p TERRA 2 – reverse	GGTGGTTCACGCCTGTAAT	481 bp		This study
9p TERRA – forward	GAGATTCTCCCAAGGCAAGG	237 bp	122	This study
9p TERRA – reverse	ACATGAGGAATGTGGGTGTTAT	138 bp		This study
10q TERRA 1 – forward	AAAGCGGGAAACGAAAAGC	449 bp	114	[15]
10q TERRA 1 – reverse	GCCTTGCCTTGGGAGAATCT	409 bp		[15]
10q TERRA 2 – forward	ATGCACACATGACACCCTAAA	332 bp	90	This study
10q TERRA 2 – reverse	TACCCGAACCTGAACCCTAA	284 bp		This study
13q TERRA 1 – forward	CCTGCGCACCGAGATTCT	204 bp	110	[15]
13q TERRA 1 – reverse	GCACTTGAACCCTGCAATACAG	167 bp		[15]
13q TERRA 2 – forward	CTGCCTGCCTTTGGGATAA	342 bp	99	This study
13q TERRA 2 – reverse	AAACCGTTCTAACTGGTCTCTG	262 bp		This study
15q TERRA 1 – forward	GCGTGGCTTTGGGACAACT	317 bp	93	[15]
15q TERRA 1 – reverse	TGCAACCGGGAAAGATTTTATT	280 bp		[15]
15q TERRA 2 – forward	CAGCGAGATTCTCCCAAGCTAAG	176 bp	120	[9]
15q TERRA 2 – reverse	AACCCTAACCACATGAGCAACG	62 bp		[9]
15q TERRA 3 – forward	GCAAATGCAGCAGTCCTAATG	112 bp	111	This study
15q TERRA 3 – reverse	GACCCTGACCCTAACCCTAA	48 bp		This study
17p TERRA 1 – forward	CTTATCCACTTCTGTCCCAAGG	365 bp	91	This study
17p TERRA 1 – reverse	CCCAAAGTACACAAAGCAATCC	307 bp		This study
17p TERRA 2 – forward	GATCCCACTGTTTTTATTACTGTTCCT	431 bp	129	[15]
17p TERRA 2 – reverse	GGGACAGAAGTGGATAAGCTGATC	371 bp		[15]
17q TERRA 3 – forward	GTCCATGCATTCTCCATTGATAAG	940 bp	112	[15]
17q TERRA 3 – reverse	AGCTACCTCTCTCAACACCAAGAAG	889 bp		[15]
XqYq TERRA 1 – forward	GAAAGCAAAAGCCCCTCTGA	156 bp	95	[15]
XqYq TERRA 1 – reverse	CCCCTTGCCTTGGGAGAA	120 bp		[15]
XqYq TERRA 2 – forward	TCCTAATGCACACATGATACCC	51 bp	106	This study
XqYq TERRA 2 – reverse	CCCTAAGCACATGAGGAATGT	15 bp		This study
XpYp TERRA 1 – forward	GCGCGTCCGGAGTTTG	305 bp	129	[15]
XpYp TERRA 1 – reverse	CCACAACCCCACCAGAAAGA	255 bp		[15]
XpYp TERRA 2 – forward	GCAAAGAGTGAAAGAACGAAGCTT	136 bp	126	[9]
XpYp TERRA 2 – reverse	CCCTCTGAAAGTGGACCAATCA	21 bp		[9]
XpYp TERRA 3 – forward	AAGAACGAAGCTTCCACAGTAT	125 bp	94	This study
XpYp TERRA 3 – reverse	GGTGGGAGCAGATTAGAGAATAAA	56 bp		This study
GAPDH – forward	AGCCACATCGCTCAGACAC	N/A	110	
GAPDH – reverse	GCCCAATACGACCAAATCC	N/A		
GusB – forward	CAGCGTGGAGCAAGACAGTGG	N/A	108	
GusB – reverse	AATACAGATAGGCAGGGCGTTCG	N/A		
CTCF – forward	GGGTCTGCTATCAGAGGTTAATG	N/A		This study
CTCF – reverse	GCTCACACTGGAATGTCTTCT	N/A		This study
siCTCF6	GUGGACGAUACCCAGAUUAUA			This study
siCTCF7	AGGGUGAUUAUGAGUGGUUCA			This study

(Qiagen RNase-Free DNase Set #79254, following the manufacturer's protocol), followed by final elution and storage at −70 °C. RNA isolation from 5×10^6 cells yields ~50–75 μg of total RNA. RNA isolation from 6-well plates (~1.2×10^6 cells at 80% confluency) yields up to 30 μg of total RNA.

3.2. First strand cDNA synthesis

RNA samples from three biological replicates are converted to cDNA using Invitrogen's SuperScript III Reverse Transcriptase (#18080044) following a modified protocol. For most applications, oligo(dT) primers are the favorite choice for cDNA synthesis because of their binding to the polyA tail of mRNAs, allowing the reverse transcription of multiple different targets in one reaction. However, only 7% of TERRA is polyadenylated [9]. Therefore, TERRA is reverse transcribed using the TERRA specific oligonucleotide 5′-$(CCCTAA)_5$-3′ (Fig. 1). A housekeeping gene-specific primer is included in the same reaction for normalization purposes (Table 1). The RNA concentration used in the reverse transcription reaction is contingent on the cell type. In U2OS cells TERRA levels are relatively high and 1 μg of RNA is sufficient for RT-qPCR. In HeLa cells TERRA levels are lower and 3 μg of RNA was used for reverse transcription. Reactions are prepared on ice using RNase-free materials. TERRA is first partially denatured at 65 °C before reverse transcription at 55 °C. In 200 μl PCR tubes a 13 μl reaction is assembled containing: 2 pmol each of gene specific oligonucleotides, 3 μg of total RNA, 0.5 mM dNTPs mix. The samples are incubated in a PCR thermocycler at 65 °C for 5 min and immediately transferred on ice (or 4 °C). An extension reaction pre-mix is prepared on ice for both reverse transcription and no reverse transcription (no-RT control to detect genomic DNA contaminations) reactions containing: 1× First-Strand Buffer, 5 mM DTT, 20 U SUPERase IN (Ambion #AM2696) and 200 U SuperScript III RT (200 U/μl) or H_2O for no RT-control to 7 μl total. Upon addition of the extension reaction pre-mix to the TERRA sample, reverse transcription is carried in a thermocycler at 55 °C for 60 min followed by enzyme inactivation

at 70 °C for 15 min. The cDNA is diluted to a final volume of 40 µl and stored at −20 °C.

3.3. Quantitative PCR

Quantitative PCR is performed in an Applied Biosystems 7900HT Fast Real-Time System using Power SYBR Green PCR Master Mix (Applied Biosystems #4368708) in a 384-well reaction plate (Applied Biosystems MicroAmp Optical 384-well reaction Plate with Barcode #4309849). Utilization of an automated pipetting robot or calibrated multichannel pipets reduces pipetting discrepancies. For each qPCR sample three independent biological replicates and two technical replicates should be carried out. For each primer pair a no-template control (NTC) samples was included to detect the formation of a primer dimers. The mastermix for each reaction is prepared on ice as follows: 2 µl diluted cDNA, 5 pmol of forward primer, 5 pmol reverse primer, 1× Power SYBR Green PCR Master Mix and H_2O to a total volume of 10 µl. The SYBR green master mix already contains the DNA polymerase with the required reaction components along with the SYBR Green Dye and the passive reference dye (ROX). Thus, it is essential to keep all the components on ice and avoid direct exposure to light. The plate is sealed with optical adhesive film (Applied Biosystems #4311971), centrifuged briefly at 4000 rpm (2000×*g*), and loaded on the RT-qPCR instrument. The qPCR is performed according to the manufacturer: denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min for 40 cycles, followed by the dissociation stage for melting curve analysis. The melting curves for each primer pair used in this study are shown in Supplementary Fig. S2.

3.4. Data analysis

qPCR data for TERRA quantification are analyzed using the relative quantification method. GAPDH is used as a reference gene to normalize the gene expression data of TERRA between the calibrator and the test samples. To determine and analyze the relative changes in TERRA expression between samples the $2_t^{-\Delta\Delta C}$ method is applied [16,17]. This method feeds the Ct values obtained from the qPCR experiment into a series of subtractions to calculate the relative gene expression of the gene of interest (TERRA) normalized against a reference gene (GAPDH) in different conditions (shTRF2 OFF and shTRF2 ON). A reference gene exhibits stable levels of expression through different conditions and is used to correct for variations stemmed from DNA/RNA concentration, pipetting errors, or cycling conditions.

The first step is to calculate the average Ct value and the standard deviation (SD) for each sample from the three biological and two technical replicates. The difference between TERRA and GAPDH in shTRF2 OFF and shTRF2 ON are termed $\Delta Ct_{shTRF2\ OFF}$ and $\Delta Ct_{shTRF2\ ON}$. The difference between the two conditions $\Delta Ct_{shTRF2\ OFF}$ and $\Delta Ct_{shTRF2\ ON}$ is termed ΔΔCt.

$$\Delta Ct_{shTRF2\ OFF} = Ct_{TERRA} - Ct_{GAPDH} \text{ and } \Delta Ct_{shTRF2\ ON} = Ct_{TERRA} - Ct_{GAPDH}$$

$$\Delta\Delta Ct = \Delta Ct_{shTRF2\ ON} - \Delta Ct_{shTRF2\ OFF}$$

The normalized target gene expression levels in any given condition is equal to $2_t^{-\Delta\Delta C}$. The standard deviation is calculated from the three biological and two technical replicates and the error is propagated by taking the square root of the sum of the squares of the SD of TERRA and GAPDH. An example of TERRA quantification with the respective Ct values is presented in Supplementary Table 1.

Following the analysis the negative control samples were evaluated. The no-reverse transcriptase control, which monitors genomic DNA contamination, and the no-template control, which monitors the presence of primer dimers, produced undetermined Ct values. Moreover, analysis of the dissociation curves revealed a single peak, indicating the production and detection of a single amplicon for each primer pair (Fig. S1).

4. Results and discussion

Telomeric DNA is associated with specialized telomere binding proteins forming the shelterin complex, which regulate telomere homeostasis and mediate telomere functions. Depletion of the shelterin component TRF2 leads to de-protected telomeres triggering activation of the DNA damage response (DDR) pathway and chromosome-chromosome fusions [18]. In addition, in a recent study we found that depletion of TRF2 not only causes rampant genomic instability but it also leads to increased TERRA expression through transcriptional de-repression of the damaged telomere. At intact telomeres, the TRF2 homodimerization domain, which is involved in chromatin compaction also represses TERRA [4,19].

To assess the levels of TERRA upon TRF2 depletion we used a HeLa cell line with a doxycycline-inducible expression shRNA system against TRF2 [20]. In this system addition of doxycycline induces TRF2 depletion, leading to a DNA damage response and chromosome-chromosome fusions after five days of induction. We verified efficient depletion of TRF2 by western blot analysis upon doxycycline induction (Fig. 2A). RNA was isolated from this HeLa cell line in the absence and presence of doxycycline using the RNeasy Mini Kit with three DNase treatments. The RNA purity and concentration was measured by NANOdrop and the RNA integrity was determined by agarose formaldehyde gel electrophoresis. Total TERRA levels were evaluated by Northern blot analysis using a strand specific radiolabeled probe. Quantification of the hybridization signal for TERRA and actin (loading control), revealed accumulation of TERRA upon TRF2 depletion (Fig. 2B). Although Northern blot was able to detect a global increase in TERRA transcripts, it is ineffective in measuring minor changes in transcription or quantifying individual TERRA levels. To identify variations in individual molecules, TERRA levels were measured with RT-qPCR and normalized to the reference gene GAPDH. TRF2 depletion caused an upregulation of TERRA expression at most chromosome ends as examined with a panel of subtelomere-specific PCR-primers (Fig. 2C). In a separate experiment, TERRA transcripts quantified by RT-qPCR were also normalized to the reference gene GUSB. This analysis confirmed that depletion of TRF2 leads to a significant increase of TERRA expression at the subtelomeres examined (Fig. 2C). The values obtained from the normalization with the two reference genes differed only slightly. RT-qPCR was successful to detect subtle, as well as major changes in TERRA levels from numerous subtelomeric locations upon TRF2 depletion which were not distinguished in the Northern blot. Overall, RT-qPCR remained the fasted approach for accurately detecting and quantifying TERRA expression levels in different samples with high sensitivity.

Approximately a quarter of human telomeres contain three defined repetitive elements with remarkably high content of CpG dinucleotides that may drive transcription of TERRA [21]. Porro et al. [4] found that there is an overrepresentation of binding sites for multiple regulatory factors in HeLa telomeres, including the chromatin-organizing factor CTCF [4]. In the human osteosarcoma cell line U2OS, Deng et al. [15] showed that CTCF directly regulates TERRA expression and binds proximal to the 5′-end of CpG islands of multiple chromosome [15]. The U2OS cell line overcomes replicative senescence through the alternative lengthening of telomeres pathway (ALT), which is observed in 10–15% of all human cancers [22,23]. ALT cell lines are characterized by the loss of the chromatin-remodeling protein ATRX that leads to high

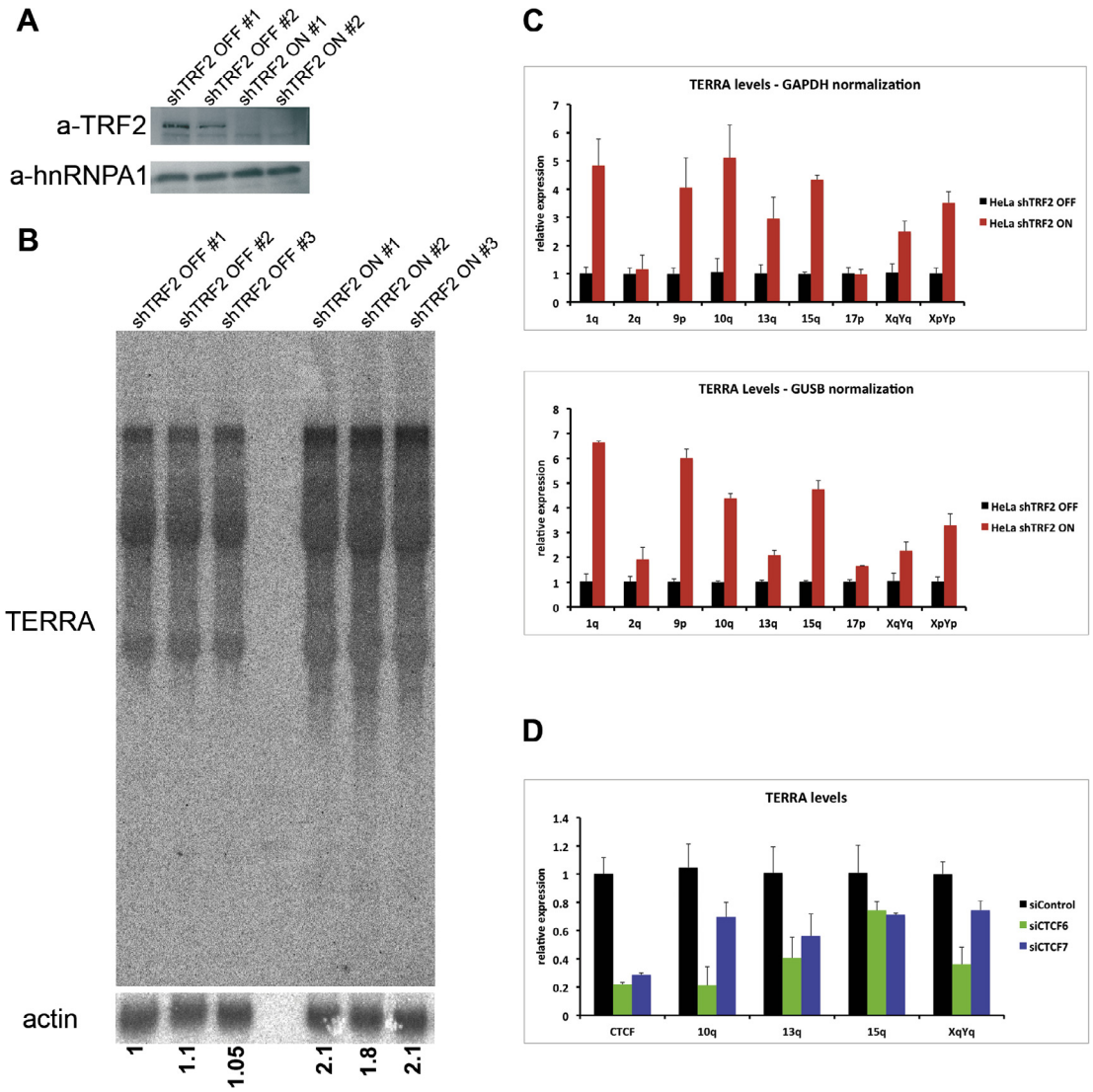

Fig. 2. TERRA levels upon depletion of TRF2 shelterin and CTCF transcription factor. (A) Western blot analysis to evaluate the depletion of TRF2 upon doxycycline induction of TRF2-directed shRNAs for 5 days. (B) Cells with and without doxycycline (HeLa shTRF2 ON and OFF respectively) were incubated for 5 days followed by total RNA isolation. 5 μg of total RNA were evaluated in a Northern blot analysis labeled with a TERRA specific probe and actin for loading control. (C) The RNA samples were subjected to RT-qPCR analysis and the levels of TERRA with different subtelomeric sequences stemming from different chromosome ends were measured and normalized to GAPDH and GusB reference mRNA and compared to siControl. (C) HeLa cells were transfected with siControl, siCTCF6, and siCTCF7 (using the calcium phosphate transfection approach) and incubated for 48 h. TERRA levels from individual subtelomeres were measured from total RNA and normalized to GAPDH and compared to shTRF2 OFF. The No-RT and NTC controls produced not measurable Ct values. The bars represent the average value from three biological and two technical replicates for each sample. Error bars represent the standard deviation. P-values were calculated by paired two-tailed Student *t*-test (n = 3).

TERRA abundance [24]. To investigate the role of CTCF in TERRA transcription in HeLa cells we identified two siRNAs to target CTCF (Fig. 2C). HeLa cells were transiently transfected with the two different siRNAs (siCTCF6 and siCTCF7) for 48 h, followed by RNA isolation. TERRA and CTCF levels were measured with RT-qPCR and normalized to the GAPDH reference mRNA. The two different siRNAs effectively depleted CTCF to similar levels (Fig. 2D). Depletion of CTCF significantly decreased TERRA transcripts as measured by chromosome end-specific RT-qPCR (Fig. 2D).

5. Conclusions

A remarkably large number of different functions in telomere biology have been linked to TERRA. Multiple functions for TERRA appear feasible if TERRA promotes telomere protein composition changes, serving as an assembly platform for a great variety of proteins. In addition, telomere transcription may facilitate protein turnover, loosening up telomere structures. To dissect the transcriptional network that regulates TERRA at different chromosome ends, accurate quantification of individual TERRA molecules will be indispensible. TERRA quantification will also be required to test systems that manipulate TERRA by genetic means, which will be instrumental to thoroughly test the already proposed functions for TERRA and possibly discover new ones.

Acknowledgements

Research in J.L.'s laboratory was supported by the Swiss National Science Foundation (SNSF), the SNSF funded NCCR RNA and disease network, an Initial Training Network (ITN) grant (CodeAge) from the European Commission's Seventh Framework Programme [grant agreement number 316354], the Swiss Cancer League and EPFL. M.F. was supported by a Marie Curie postdoctoral fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2016.08.004.

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