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Identification of chromatin marks at TERRA promoter and encoding region

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ABSTRACT

TERRA is a long non-coding RNA that is essential for telomere integrity. Although it is transcribed from subtelomeres and telomeres, how it is expressed in heterochromatic region is currently unknown. In this study, we focused our analysis on TERRA-encoding region TelBam3.4 and TelBam3.4-like sequences, and determined their transcription start sites, as well as enrichment of RNA polymerase II and histone modifications. We found that H3K4me3 and H3K9me3 are present at TERRA promoters, whereas H3K27ac and H3K9me3 are present at telomeric repeats. Consistently, we show that presence of active histone modifications H3K4me3 and H3K27ac are correlated to TERRA expression. These results mark an important step towards understanding telomere maintenance and transcription.

1. Introduction

Histone modifications play a fundamental role in gene regulation. Characteristic patterns of histone modifications are observed at different genomic regions. For example, histone H3 acetylation, H3K4me3 and H3K4me2 are associated at highly expressed gene promoters and are involved in transcription initiation [1–3]. H3K36me3 and H3K79me2 are associated with gene bodies of actively transcribed genes and support transcription elongation, as well as repressing intragenic transcription initiation [4]. In contrast, H3K9me3, H3K27me3 and H4K20me3 are known as transcriptionally silent marks and are observed at inactive genes [5]. Although these active and silent histone modifications are normally mutually exclusive, they are found together in some cases, for example, H3K4me3 and H3K27me3/H3K9me3 at developmental genes in mouse embryonic cells, and are referred to as bivalent chromatin [6,7].

Telomeres are terminal regions of chromosomes that are essential to prevent chromosome fusion and DNA erosion during replication, thus essential for genome stability [8]. They are heterochromatic, consist of many TTAGGG repeats, and are enriched for H3K9me3 and H4K20me3 [9]. These histone modifications are essential for telomere integrity and loss of histone methyltransferase Suv3-9h or Suv4-20h cause aberration of telomere length regulation and recombination frequency [10,11]. Since telomeres and its adjacent regions subtelomeres are enriched with transcriptionally silent histone modifications, it had been believed for a long time that there is no transcription at subtelomeres or telomeres. Recent studies, however, showed that long non-coding RNA called TERRA is transcribed from subtelomere to telomere of most or all chromosome ends, containing UUAGGG repeats [12,13], and that they are required for heterochromatin formation at telomeres [14]. Since telomeres and subtelomeres are enriched with transcriptionally silent marks, an intriguing and unanswered question is how TERRA expression is regulated.

Terminal sequences on human chromosomes X/Y (Xq/Yq) were originally identified by sequencing of a clone called TelBam3.4, which has been particularly studied for TERRA expression mechanism, since it contains both telomere TTAGGG repeats and sequences ~3.5 Kbp upstream of them containing unique sequences (GENBANK accretion#: M57752.1, [15]). It has been shown that DNA methylation of a CpG island in TelBam3.4 negatively regulates TERRA expression and the transcription factor CTCF is located upstream of TTAGGG repeats that is required for activating TERRA expression [16,17]. However, it is still unknown what chromatin marks are present and required for TERRA expression. The repetitive sequence nature of subtelomeres and telomeres pose difficulties in chromatin marks analysis since it is difficult to align the

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reads to repetitive sequences unambiguously.

Here, by generating a custom reference genome, we focused our analysis on TERRA-encoding regions on several chromosome arms altogether. We identified precise TSSs[1] of TERRA in the CpG island, and show TERRA promoters are enriched with chromatin marks for active promoters. An active transcription mark H3K27ac is also present at the telomeric repeats. Finally, we show that TERRA expression is positively correlated with these active histone modifications. These results will contribute towards understanding the regulation of TERRA expression and thus telomere maintenance.

2. Materials & methods

2.1. Generation of a custom reference genome

TelBam3.4 sequence was obtained from NCBI (GenBank ID: M57752.1). TelBam3.4-like sequences were identified by running BLAT implemented in the UCSC genome browser with TelBam3.4 sequence as query against the human genome hg19. Top seven TelBam3.4-like regions found in hg19 by BLAT were masked in the hg19 assembly with 'N' by bedtools [18]. TelBam3.4 and masked hg19 were combined to generate the custom reference genome.

2.2. NGS[2] datasets

All analyzed datasets of ChIP-Seq[3], RNA-Seq and CAGE[4], were obtained from ENCODE (Supplementary Table 1), with the exception of the H3K27ac_long, which was generated for this work.

2.3. Analysis of transcriptome data

Poly(A)+ and whole cell fractions datasets were used for CAGE and RNA-Seq (see Supplementary Table 1 for the list of cells).

For CAGE data, linker and EcoP15i recognition site (CAGCAG) in CAGE reads were trimmed with FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Trimmed CAGE reads were compared with human ribosomal DNA sequences by rRNAdust program and the rRNA-derived sequences were discarded in MOIRAI system [19]. Remaining reads were aligned to the custom reference genome by BWA-backtrack 0.7.9 with default setting [20]. Frequencies of 5′-end of CAGE read alignments are counted and displayed along the TelBam3.4 sequences by using Integrative Genomic Viewer (IGV) [21].

For RNA-Seq data, individual reads were mapped to the custom reference genome by bowtie2 2.1.0 with default setting [22], which were visualized with IGV in bedgraph format. Custom gene annotation based on GENCODE v19 that contains annotation of TERRA exon (TelBam3.4: 2960–3400 bp) was generated for quantification of TERRA expression [23]. The number of reads overlapped to exon region defined by custom gene annotation file was counted by GenomicRanges (R package; Overlap resolution modes for counting was "union" and strand was considered) as raw signals of gene expression levels [24]. The read counts were subsequently normalized as reads per kilobase of exon model per million mapped reads (RPKM).

2.4. Definitions of promoters, transcribed region and centromeres

TERRA locus promoter was defined as 300 bp upstream and 100 bp downstream from the most downstream active TSS observed by CAGE (2660–3060 bp on TelBam3.4). Pre-telomere was defined as a region between TERRA promoter and telomere (3060–3400 bp). Telomere region in TelBam3.4 that was defined previously (3400–4408 bp) [17] was further divided into telomeric repeat-poor and -rich regions based on TTAGGG repeat density (3400–3839 bp and 3839–4408 bp, respectively). For euchromatic genes, promoters were defined as 300 bp upstream and 100 bp downstream from TSSs, and gene bodies were defined as 100 bp downstream of TSSs to transcription terminal sites of GENCODE v19 gene model. Transcription activity levels of genes were classified based on gene expression measured by RNA-Seq into active (RPKM $\geq$ 1), weak (0 < RPKM < 1) and inactive promoter (RPKM = 0) in K562 cells. Alpha satellite repeat and Satellite III repeat were determined based on RepeatMasker track in the UCSC Genome Browser [25].

2.5. ChIP-Seq analysis and enrichment calculation

ChIP-Seq data were mapped to the custom reference genome by BWA-backtrack 0.7.9 with the default setting and visualized with IGV. Enrichments of histone modifications were calculated as proportion of the number of reads aligned to the target region, which was subsequently normalized by that of input. The P-value was calculated with Fisher's exact test.

2.6. ChIP-Seq for H3K27ac_long

ChIP was performed as described previously [25] with following modifications: (i) 6×10^6 cells were fixed by 1% formaldehyde for 5 min in PBS, (ii) LB3 was substituted with SDS lysis buffer (50 mM Tris–HCl, pH 8.0, 1% SDS, 10 mM EDTA and Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland)), (iii) chromatin was sheared with Covaris S220 to 200–500 bp, (iv) 6 μg of anti-H3K27ac (Cosmo bio, Tokyo, Japan; MABI0309) antibody was used for immunoprecipitation, and (v) washing after antibody binding were performed with Wash buffer I twice and Wash buffer II three times, with no Wash buffer III.

Sequencing libraries were prepared by Mondrian™ SP system with Ovation® SP + Ultralow DR Multiplex System (NuGEN, CA, USA) from 2 ng DNA with 11 cycles of PCR amplification, which were sequenced on HiSeq 2500 (Illumina, CA, USA) by 150 bp paired-end. Sequenced data was processed in the same way as the ENCODE ChIP-Seq data as described above, except for using BWA-mem 0.7.9 for alignments with the custom reference genome. The sequence data was deposited to DDBJ Sequence Read Archive (http://trace.ddbj.nig.ac.jp/dra/index_e.html) under Accession Number DRA003999.

2.7. Correlation analysis between TERRA expression level and histone modification enrichment

Correlation coefficient r and P values were calculated by Pearson correlation. 0.1 was added to all of the RPKM values of TERRA and enrichment value of histone modification prior to taking binary logarithms to avoid negative infinity.

3. Results

3.1. Generation of a custom reference genome for TERRA analysis

Transcriptome and epigenome analysis of TERRA-encoding regions are challenging due to not only the presence of repetitive sequences at each telomere, but regions upstream of the telomere repeats are also found on several chromosome ends. Thus, typical

[1] TSSs, transcription start sites.
[2] NGS, next generation sequencing.
[3] ChIP, chromatin immune-precipitation.
[4] CAGE, cap analysis of gene expression.

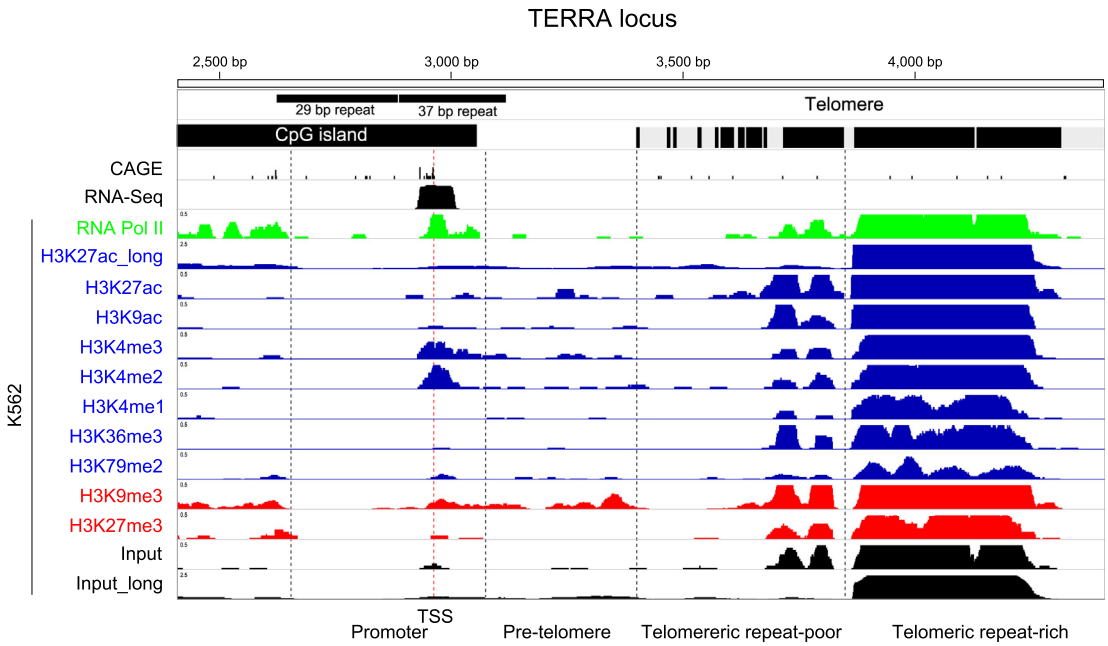

Fig. 1. Genomic view of transcriptome and chromatin at the TERRA locus. A browser view of the transcriptome (CAGE and RNA-Seq) and chromatin signals (ChIP-Seq of listed marks: RNA polymerase II (green), transcriptionally active (blue) and silent (red) histone modifications) at the TERRA locus is shown. CAGE (only first bases of CAGE tags are shown to illustrate TSSs positions) and RNA-Seq tracks show combined data from 9 cell lines of mapped reads in the plus strand only. ChIP-Seq tracks are of K562 cells. The reads aligned to multiple loci are included. The numbers at left side in tracks indicate track height with tag counts in CAGE and RNA-Seq and with normalized count per million in ChIP-Seq tracks. Borderlines (vertical black lines) between regions and the most downstream active TSS (vertical red line) are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

strategy for NGS data analysis of using only uniquely aligned reads would be inadequate to analyze the TERRA locus. To overcome this problem, we generated a modified hg19 human genome assembly by adding TelBam3.4 sequence, which contains unique sequences upstream of the telomeric repeats. Additionally, seven TelBam3.4-like regions identified in hg19 by BLAT (Supplementary Table 2) were hard masked as "N" (see Materials and methods). By masking the TelBam3.4-like sequences, the reads that would otherwise map to these regions result in aligning to the TelBam3.4. Thus, this strategy essentially allowed us to map TelBam3.4 and −like sequences to a unique location we refer to as the 'TERRA locus', rather than these sequences being randomly mapped over different chromosomes. It is of note that telomere repeats TTAGGG are still repetitive in the TERRA locus, thus we show two results in this study, one that allows for uniquely mapped reads only ('uniquely aligned reads'), and one that allows reads aligned to multiple loci ('all aligned reads').

3.2. Transcription initiation activities of TERRA at a high resolution

Although the TERRA promoter was previously identified within the CpG islands in 29 bp repeats in TelBam3.4 [17] (Fig. 1), the precise position of TSSs have not been clearly assessed yet. CAGE data provides positions and levels of transcription initiation at 1 base-pair resolution by monitoring frequencies of 5′-end capped transcripts by sequencing, whereas RNA-Seq data provides positions and levels of transcribed exons that are long and poly(A)+ by capturing randomly fragmented exons [26,27]. To assess TSSs of TERRA in depth, we examined ENCODE datasets [28]. We took CAGE and RNA-Seq datasets for nine cell lines for which both datasets were available for, and aligned to our custom reference genome. Our analysis shows that there are signals of CAGE and RNA-Seq from the 37 repeat region for most cell lines (Fig. 1 shows CAGE tags from all cells and Supplementary Fig. 1 for each cell line), which are consistent with the presence of RNA polymerase II in the region (Fig. 1). Many cell lines show multiple CAGE tags at multiple positions in the vicinity (Supplementary Table 3), suggesting they

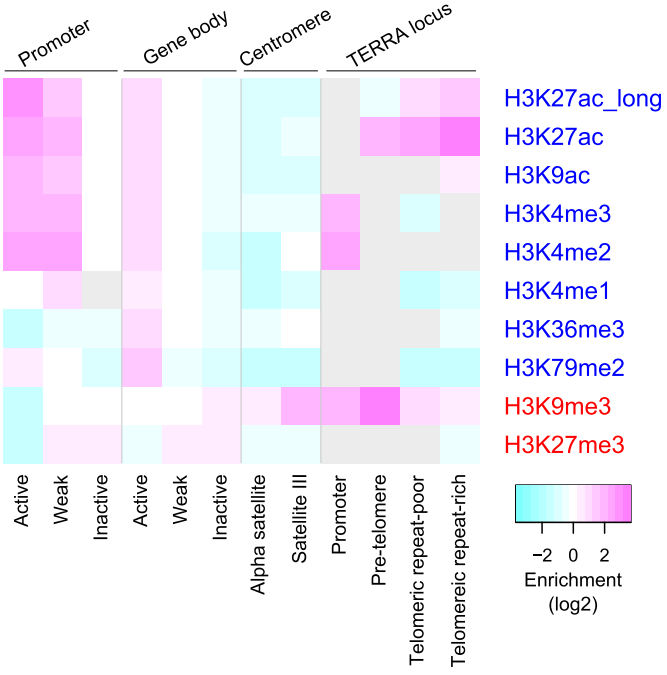

Fig. 2. Histone modification enrichments over TERRA locus in K562 cells. The color represents significant enrichment level of histone modification in log2 scale, whereas gray indicates statistically insignificant samples ($P > 0.05$) due to low number of sequence reads. Transcription activities of promoter and gene bodies were determined by RNA-Seq and categorized into active, weak and inactive. Reads aligned to multiple loci are included. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

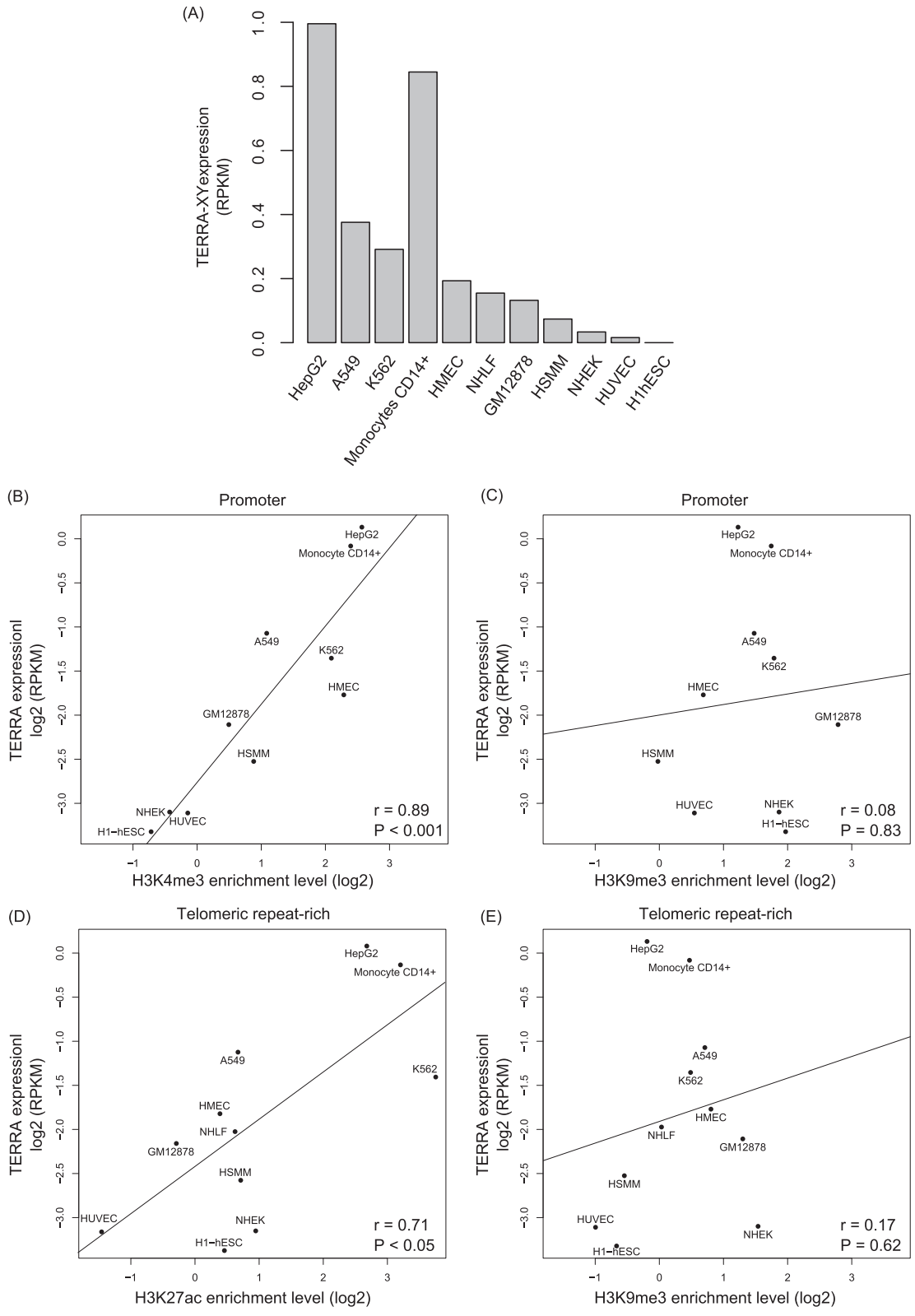

Fig. 3. TERRA expression level is correlated with active histone modifications. (A) TERRA expression analyzed by RNA-Seq. Bar graph indicates mean value of TERRA expression levels from 2 replications in 11 human cell lines. (B–E) Correlation between TERRA expression and enrichment levels of histone modification. Scatter plot shows relationship between TERRA expression and H3K4me3 at TERRA promoter (B), H3K9me3 at TERRA promoter (C), H3K27ac at telomeric repeat-rich (D) and H3K9me3 at telomeric repeat-rich region (E). Vertical axis and horizontal axis indicate TERRA expression and histone modification enrichments in log2 scale, respectively. TERRA expression and histone modification enrichments were calculated from the number of all aligned reads.

are broad promoters. We conclude that the CAGE tags at the 37 bp repeats represent a series of TERRA TSSs.

3.3. Active histone modifications are present at the TERRA locus

We next examined the localization of 9 histone modifications at the TERRA locus in K562 cells. We found signals of active promoter marks H3K4me2 and H3K4me3 at the promoter, as well as heterochromatic mark H3K9me3 (Fig. 1). We also found telomeric TTAGGG repeats show signals of transcriptionally active marks such as H3K27ac and H3K9ac (Fig. 1). These observations were validated by ChIP-qPCR and ChIP-Southern hybridization (Supplementary Fig. 2). We next set out to verify H3K27ac signals are not originating from internal regions far from the chromosomal ends, which includes TTAGGG repeats. Based on the current human genome assembly, all TTAGGG repeats far from chromosomal ends are shorter than 100 bp (Supplementary Fig. 3). Thus, we carried out 150 bp paired-end ChIP-Seq with H3K27ac in K562 cells (H3K27ac_long, Fig. 1), classified reads that contain over 150 bp of tandem TTAGGG repeats to be telomeric, and found over 95% of TTAGGG repeats from H3K27ac ChIP-Seq are derived from telomeres (Supplementary Table 4). From these results, we conclude that most of the H3K27ac with TTAGGG repeats are originated from the telomere.

We next compared histone marks at the TERRA locus to other regions (Fig. 2). For comparison, euchromatic regions (promoters and gene bodies) and centromeric repeats (alpha satellites and satellite III, which are also transcribed heterochromatic regions) [29–31] were included. Promoters and gene bodies of euchromatic genes were categorized into three transcription activity levels (active, weak and inactive) based on RNA-Seq data. Compared to the actively and weakly expressed promoters in euchromatin that are enriched with H3K4me2, H3K4me3, H3K9ac and H3K27ac, TERRA promoter is enriched with H3K4me2 and H3K4me3 only. Telomeric repeat-poor and -rich regions are enriched with H3K27ac only unlike euchromatin gene bodies or the centromere (Fig. 2). However, it is of note that other modifications are insignificant in their enrichments (indicated by gray), which may be due to the low number of reads, and does not rule out the possibility of their enrichments. Taken together, active transcription marks are present at the TERRA locus, both its promoter and gene body, in K562 cells.

Among ten other human cell lines, H3K4me3 is enriched in three cell lines at the TERRA promoter (A549, HMEC and Monocyte CD14+) (Supplementary Figs. 1 and 4). Other cell lines did not show significant enrichments due to insufficient depth of reads. At the telomeric repeat-rich region, many cell lines have H3K27ac enrichment with varying degrees. Taken together, not all human cell lines have H3K4me3 at the TERRA promoter and H3K27ac at telomeric repeats.

3.4. TERRA expression is correlated with active histone modifications at TERRA promoter and telomere

Since histone modifications play an important role in gene regulation and are correlated with gene expression [1,32], we next asked whether TERRA expression is correlated with histone marks at the TERRA locus. We first determined expression levels of TERRA in the same 11 cell lines as before from RNA-Seq data. Among the cell lines we analyzed, HepG2 and Monocyte CD14+ cells have relatively high levels of TERRA, whereas it is undetectable in H1-hESC (Fig. 3A). These are consistent with the levels of H3K4me3 at the TERRA promoters (Supplementary Fig. 1).

We next analyzed correlation between TERRA expression level and each histone mark by Pearson's correlation coefficient for 11 cell lines (Supplementary Fig. 5; NHLF was excluded for promoter analysis, since no reads from inputs were identified). A significant correlation was observed between TERRA expression levels and H3K4me3 levels at the promoter ($r = 0.89$, $P < 0.001$, Fig. 3B). Correlation was also observed for H3K27ac and other active marks at telomeres to a less extent ($r = 0.71$, $P < 0.05$ at telomeric repeat-rich, Fig. 3D and Supplementary Fig. 5). Importantly, H3K9me3 is not correlated with TERRA expression levels ($r = 0.08$, $P = 0.83$ at TERRA promoter; $r = 0.17$, $P = 0.62$ at telomeric repeat-rich; Fig. 3C and E), as well as another silent chromatin mark H3K27me3 (Supplementary Fig. 5). These results suggest that TERRA expression may require active histone marks, such as H3K4me3 and H3K27ac at the promoter and transcribed regions.

4. Discussion

We found active histone modifications H3K4me3 and H3K27ac are present at the TERRA locus. Similar finding has also been reported for telomeres in other systems such as budding yeast and *Arabidopsis thaliana*, although histone modification patterns are distinct among the species [33–36]. Presence of active marks at the TERRA locus is consistent with other observations of the telomeres, such as early replication timing and containing histone H3 variant H3.3 [37–40], which are both hallmarks of euchromatic regions.

Although active and silent chromatin marks are mutually exclusive in general, transcriptionally active genes in pericentromeric heterochromatin in *Drosophila melanogaster* also show similarly mixed active and silent marks [41]. In fact, heterochromatin protein HP1 in *D. melanogaster* is required for expression of some genes that reside in the heterochromatin [42]. Thus, it is feasible that given the unique environment of the heterochromatin, a unique mechanism may be needed for transcription. Whether these active and silent marks at the TERRA locus are present in the same nucleosome, i.e. whether they are bivalent chromatin, however, is unknown [6,7]. Further experiments are needed to examine whether these active and silent marks are in the same nucleosomes, such as re-ChIP.

It is of note that although we have verified the presence of active marks at the TERRA locus, our method essentially analyses TERRA loci from seven different regions on different chromosome arms combined, and thus we cannot map the reads to unique region across the genome. Since not all chromosome ends may be transcribing TERRA, we may be looking at the mixture of signals; regions that are expressing TERRA and containing active marks only, and regions that are not expressing TERRA containing silent marks only. This is especially true for TTAGGG telomeric repeats that are on all chromosome ends, and there is currently no method that is capable of determining which chromosome end they originate from.

Finally, we also observed that different cell lines have different TERRA expression levels. This is consistent with the previous study that TERRA expression is developmentally regulated [13]. In fact, it is known that TERRA expression is altered according to cell states, such as development, cell cycle, and diseases such as cancer and ICF syndrome [43,44]. Further understanding of the mechanism of how TERRA expression is regulated at the chromatin level may lead to further understanding of development as well as diseases.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.09.176.

Transparency document

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