2D gel electrophoresis reveals dynamics of t-loop formation during the cell cycle and t-loop in maintenance regulated by heterochromatin state

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Running title: Study t-loop with 2D gel method

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Abstract

Linear chromosome ends are capped by telomeres that have been previously reported to adopt a t-loop structure. The lack of simple methods for detecting t-loops has hindered progress in understanding the dynamics of t-loop formation and its function in protecting chromosome ends. Here, we employed a classical two-dimensional agarose gel method (2D gel method) to innovatively apply to t-loop detection. Briefly, restriction fragments of genomic DNA were separated in a 2D gel, and the telomere sequence was detected by in-gel hybridization with telomeric probe. Using this method, we found that t-loops are present throughout the cell cycle and t-loop formation tightly couples to telomere replication. We also observed that t-loop abundance positively correlates with chromatin condensation, i.e. cells with less compact telomeric chromatin (ALT cells and trichostatin A [TSA]-TSA treated HeLa cells) exhibited fewer t-loops. Moreover, we observed that telomere dysfunction-induced foci (TIFs), ALT-associated PML bodies (APBs), and telomere sister chromatid exchanges (T-SCEs) are activated upon TSA-induced loss of t-loops. These findings confirm the importance of the t-loop in protecting linear chromosomes from damage or illegitimate recombination.

Introduction

Telomeres protect linear chromosomes from exonucleolytical degradation, undesirable illegitimate recombination events, and end-to-end fusions (1,2). In human cells, telomeres are composed of tandem repeats of dsDNA TTAGGG/AATCCC, a terminal ssDNA 3′-G-rich overhang, and a telomere binding complex called “shelterin” (3,4). The ends of telomeric DNA have a propensity to
form "t-loops", in which 3′-G-rich overhang invades and paired with the C-rich strand of the dsDNA telomeric repeat tract in cis to form D-loop (5). It has been proposed that t-loops may play a critical role in protecting linear chromosomes from nuclease-mediated end-resection and unscheduled DNA repair (6).

T-loops were first discovered in the nuclei of human and mouse cells (5). They were subsequently observed in other eukaryotic species including *Trypanosoma brucei* (7), *Pisum sativum* (8) and *Gallus gallus* (9) and *Caenorhabditis elegans* (10). T-loops are considered to be an evolution-conserved structure for protecting linear chromosome terminals. However, many questions regarding the establishment and maintenance of t-loops in cells remain to be elucidated (11). For example, very little is known about how t-loops are negotiated by DNA replication machinery during S phase. The mechanism underlying the formation and maintenance of t-loops through cell cycle is also poorly understood. Moreover, it is not yet known what happens to telomeres/chromosome ends if massive t-loops are disrupted in vivo. The answers to these questions are foundation for better understanding the function of t-loops in protection of chromosome ends.

One of the roadblocks to answering these critical questions is the lack of a reliable, sensitive, and commonly used method to detect t-loops in cells and/or cell-free extracts. Electron Microscope (EM) and recently developed stochastic optical reconstruction microscopy (STORM) are valuable tools for studying and visualizing t-loops, but they are low throughput labor-intensive that require equipments which are prohibitive for most research groups (5,12). Therefore, an accurate and sensitive biochemical method to detect t-loops is needed to stimulate progress in this important research area.

Based on the structure similarity between t-loop and well-characterized rolling circle replication intermediates (RCIs) and the RCIs migrate in a unique size- and shape-dependent sigmoid pattern in non-denatured two-dimensional (2D) agarose gel electrophoresis (13-15), we proposed that t-loops might segregate from linear telomere DNA during 2D gel electrophoresis. Here, we report the development of a non-denatured 2D agarose gel electrophoresis method (2D gel method) that readily detects t-loops from human cells. The method was carefully validated by a series of biochemical assays as well as conventional EM. Evidences presented here support the following four major conclusions about the formation and dynamics of t-loops in human cells: 1) t-loops are present throughout the cell cycle; 2) t-loop formation is tightly coupled with telomere replication; 3) less condensed telomeric chromatin showed fewer t-loops in human ALT cells; 4) trichostatin A (TSA) induced hyperacetylation of telomeric nucleosomes promotes disruption of t-loops that is associated with formation of APBs and increased frequency of T-SCE, indicating the homologue recombination occurring at unprotected telomeres.

**Results**

**Mobility of telomere-homologous structures during non-denatured 2D agarose gel electrophoresis**

Rolling circle replication intermediates (RCIs) are a unique and extensively characterized type of replication intermediate. RCIs and t-loops are similar to each other, both comprising a circular dsDNA and a linear dsDNA tail. Several methods are available to detect RCIs based on their structure, including those based on characteristic gel electrophoretic mobility. RCIs with a fixed loop size but different tail lengths are detected as an “eyebrow”-like shape (see inset in Fig. 1A) (13,16). The multiple “eyebrows” formed from RCIs with different loop sizes converged into a sigmoidal arc (Fig. 1A) (13).

Here, we exploit non-denatured 2-dimensional agarose gel electrophoresis followed by hybridization to a telomere-specific probe (2D gel method) to specifically detect t-loops in human cells.
Given the structural similarity between RCIs and t-loops, both consisting of loops and tails that highly variable in size (5), a telomeric sigmoidal arc is expected. As expectation, a sigmoidal arc of telomere-homologous species is detected in genomic DNA from non-synchronized HeLa cells (Fig. 1B).

To exclude the possibility that sigmoidal arc is the unique structure of HeLa, we detected the sigmoidal arcs using 2D gel method in variety of human cells, including primary cells (human T cell & fibroblast BJ), telomerase positive cells (HeLa S3 & A549) and ALT cells (VA13 & U2OS) (Fig. 1C). The sigmoidal arcs were observed in all of these cells, some of which have been observed by EM in previous study (5). It seems that the sigmoidal arc represents a special telomeric structure which predicted as t-loops.

Characterization of DNA structures in the sigmoidal arc

The DNA in the sigmoidal arc region of the 2D gel method was characterized by biochemical methods as follows. First, the sigmoidal arc gradually decreased as temperature rising up to 65°C, the temperature at which most t-loops dissociate (Fig. 2A), indicating low thermal stability of t-loops. Second, the sigmoidal arc is barely detected in undigested HeLa genomic DNA (Fig. S1A), indicating that the telomeric DNA in the sigmoidal arc is not extrachromosomal DNA. Trace amounts of sigmoidal arc signal may due to DNA breakage at subtelomere or telomere during purification. Third, after a second round of purification of HeLa genomic DNA (Fig. S1A), indicating that the telomeric DNA in the sigmoidal arc is not extrachromosomal DNA. Trace amounts of sigmoidal arc signal may due to DNA breakage at subtelomere or telomere during purification. Third, after a second round of purification of HeLa genomic DNA, the sigmoidal arc decreased, suggesting that this DNA fraction is not an artifact of genomic DNA causing by purification (Fig. S1B). Fourth, in the experiments described above, HeLa genomic DNA was purified without proteinase treatment at 55°C to preserve t-loops structure. To test the susceptibility of putative t-loop structures to protease digestion, cell lysate was digested with proteinase K at 55°C (10min) before purified by phenol/chloroform extraction or chromatography on Qiagen™ resin. Results showed that the sigmoidal arcs were almost the same as Fig. S1B (left panel) (Fig. S1C). This result suggests that structures in the sigmoidal arc are not mediated by obligate protein-protein or protein-DNA interactions.

In addition, HeLa genomic DNA was digested with Plasmid-safe DNase, an ATP-dependent exonuclease that degrades linear dsDNA to deoxymononucleotides, but not circular dsDNA. Based solely on its enzymatic properties, Plasmid-safe DNase should remove the linear tail from a t-loop, leaving loop (circular DNA) (Fig. 2B and C). Plasmid-safe DNase digested approximately 80% of linear telomeres, removing the sigmoidal arc entirely, while generating a new signal corresponding to circular telomeric DNA (Fig. 2C).

In t-loop structure, the telomeric ssDNA 3'-G-rich overhang is thought to invade into the dsDNA telomeric repeat tract, adapting a D-loop structure (5). Under this circumstance, the invading G-rich strand would be resistant to exonucleolytic digestion because of lacking a free 3′-end. To test this hypothesis, HeLa genomic DNA was digested with Exonuclease I (ExoI), analyzed by 2D gel method, and hybridized under native and denatured conditions to G-strand or C-strand specific probes. The exact same amount of undigested DNA was used as control. Results showed that DNA in the sigmoidal arc includes G-rich ssDNA (C-probe hybridization) but not C-rich ssDNA (G-probe hybridization) (Fig. 2B and C). In particular, most of the signal detected by the C-rich probe in the sigmoidal arc is resistant to ExoI, whereas little of the signal detected by the C-rich probe in the linear chromosome fraction is ExoI-resistant (Fig. 2D). These results support the conclusion that the DNA structures in the sigmoidal arc are t-loops, in which the G-rich 3′-ssDNA telemetric overhang invades into the dsDNA telomeric repeat tract to adapt a D-loop.

Moreover, we observed the t-loop structure in the sigmoidal arc by EM. The area of gel corresponding to sigmoidal arc
(equal to linear fragments ≥7.5 kb in length) was excised (Fig. S2A and B), and DNA was purified and analyzed by EM. In three independent experiments, we observed about 400 DNA molecules, of which 74 were scored as t-loops (Fig. S2C) with average size of 11.7 kb, consistent with excised telomeric DNA ranging from 7.5 to 15kb (Fig. S2D). The loop sizes were varied from 2 to 16kb with a mean size of 7.8kb, while the t-loop tails range from 0.3 to 11.3kb with the average length of 3.8kb (Fig. S2D). Tail lengths of t-loops were positively correlated with their loop sizes, indicating that bigger loop carries longer tail in general (Fig. S2E). Linear telomere-homologous molecules excised from the linear dsDNA region were used as a control (Fig. S2A). No t-loop shaped structure was observed (data not shown).

In conclusion, these evidences suggested that the sigmoidal arc detected by the 2D gel method represent t-loops.

Depletion of TRF2 induces the loss of t-loops

TRF2 is a component of shelterin that protects telomeres by repression of ATM signaling and NHEJ (17,18). Previous studies have revealed that TRF2 has the ability to generate t-loops in vitro (5,19). Recently, it is also demonstrated that TRF2 is required for the formation or maintenance of t-loops in vivo (12). To further verify this conclusion, we constructed TRF2 knock-out HeLa cells with two stably expressed sgRNAs (targeting TRFH domain of TRF2) and inducible Cas9 (Fig. 3A), and cells with empty vector was used as control. After 7 days of doxycycline (DOX) treatment, TRF2 is depleted in sgTRF2 cells (Fig. S3A), resulting in dramatically increase of chromosome end fusion (from 0 to 32.3%) and decreased free chromosome end compared with the control (Fig. 3D, E and Fig. S3B). Results of 2D gel method were quantified and the percentage of t-loops was calculated as described in the method. Compared with control, t-loops were dramatically decreased in TRF2 deficient cells (30.7% to 12.2%) (Fig. 3B and C). In addition, we found inhibition of ATM phosphorylation in TRF2 deficient cells could suppress the chromosome fusion but barely rescue the t-loop disruption (Fig. 3B-E and Fig. S3), which means TRF2 is directly responsible for t-loop formation or maintenance, consistent with previous study (12).

Immediate folding of t-loops after telomere replication during S phase

Our previous studies implied that telomeres must be unfolded (i.e., not in t-loops) at least twice during S phase in proliferating cells: once during S phase to permit telomere replication, and then again, at the end of S phase to permit C-strand fill-in DNA synthesis (20,21). In this instance, t-loops could either refold immediately after they are replicated, or they could remain unfolded in an open linear confirmation until C-strand fill-in synthesis completed at the end of S phase. To answer this question, HeLa S3 cells were synchronized at G1/S, released into S phase, and then harvested at different time points, corresponding to early S, middle S, late S/G2 and G1 phase (Fig. 4A). Genomic DNA was isolated and analyzed for the presence of t-loops by 2D gel method. The results demonstrate that the amount of t-loops is fairly constant throughout the cell cycle (Fig. 4B), which is consistent with the hypothesis that telomeres unfold and immediately refold after being replicated during S phase; therefore, they must unfold and refold a second time to allow for C-strand fill-in DNA synthesis at late S/G2 phase.

To further confirm that t-loop unfolding/refolding is tightly coupled to telomere replication, HeLa S3 cells were synchronized at G1/S phase, released into S phase for 3h, pulse-labeled for 1h with BrdU during mid-S phase, and harvested immediately (Fig. 4C). DNA was isolated from the pulse-labeled cells, and separated by CsCl density gradient centrifugation according to density. Telomere DNA that had replicated during the 1h pulse (labeled with BrdU) was divided from unreplicated and previously replicated DNA (unlabeled) (Fig. 4D), and analyzed by 2D gel method. It shows that both newly synthesized leading and lagging telomeres have t-loops (Fig. 4E). Because the density-labeled
telomeres represent the cohort of telomeres that were replicating during the 1h time window of exposure to BrdU, these data indicate that t-loops refold immediately following telomeres replication.

**Decondensation of telomeric chromatin leads to the disruption of t-loops**

The structure of t-loops was compared in primary cells (T cells and BJ fibroblasts), telomerase positive cells (HeLa S3 and A549) and ALT cells (VA13 and U2OS) (Fig. 1C). The results show that the percentage of t-loops were varied by cell type as follows: T cells from human blood, 24.5%; BJ fibroblasts, 16.9%; HeLa cells, 17.2%; A549, 17%; U2OS cells, 6.5%; VA13 cells, 7.3% (Fig. 1C). These results are consistent with a previous report that 6.9-9.2% of telomeres have t-loops in ALT cells and 15-40% in telomerase-positive HeLa cells (5,22). T-circles were observed in VA13 and U2OS cells, but they were barely detectable in other cell lines (23,24).

In addition, it has been reported that telomeric DNA is less compacted in ALT cells than in telomerase-positive cancer cells (25). To explore whether the decondensation of telomeric chromatin is correlated with the low abundance of t-loops, non-ALT HeLa cells were treated with trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor which regulate histone acetylation indirectly. After 48h treatment with TSA, the density of acetylated histones H3 and H4 on telomeres increased, suggesting the level of telomeric heterochromatin decreased (Fig. 5A and B). Consistently, micrococcal nuclease (MNase) assay showed that up to 60% of telomeric DNA in HeLa cells treated with TSA was digested to mono-nucleosomes by MNase (16min digestion), similar to U2OS cells (65% of telomeric DNA was converted to mono-nucleosomes by MNase digestion for 16min), while only 30% of telomeric DNA in untreated HeLa cells was converted to mono-nucleosomes under comparable digestion conditions (Fig. 5C). When HeLa cells were exposed to TSA, the intensity of the t-loops decreased in dose dependent manner (Fig. 5D and E). In addition, we also observed that t-circle is increased after TSA treatment, mechanism of which needs further research (Fig. 5D).

**The loss of t-loops is associated with activation of homologous recombination at telomeres**

Linear chromosomes lacking functional telomeres induce a DNA damage response (DDR), one marker of which is formation of 53BP1 foci at DNA damage sites (26). Here, the colocalization of antibody to 53BP1 and a hybridization probe for telomeric DNA sequences was used to assess the abundance of telomere dysfunction-induced foci (TIFs) (27). The results showed that ~58% of TSA-treated HeLa cells have at least one TIF, in comparison with ~20% in DMSO-treated HeLa cells (Fig. 6A and B), indicating the DNA damage level increasing at telomere in cells exposed to TSA.

Aggregates of nuclear protein, known as promyelocytic leukemia (PML) bodies begin to accumulate after HeLa cells are exposed to TSA (Fig. 6C). Some of these PML bodies are associated with telomeric chromatin, forming ALT-associated PML bodies (APBs). 48h after treatment with TSA, the number of PML bodies, as well as APBs, in HeLa cells increased dramatically, to include up to 50% of telomeres (data not shown). Quantitative analysis showed >9 APBs/cell in ~27% of TSA-treated cells and >4 APBs/cell in ~64% cells (Fig. 6D). In control cells treated with DMSO, 98% cells had < 3 APBs/cell and in 42% of the cells, no APBs were detected (Fig. 6D).

APBs are often associated with telomeric recombination in human ALT cells (28). To this end, chromosome orientation FISH (CO-FISH) was performed to determine the frequency of telomere sister chromatid exchanges (T-SCEs) (29). By scoring the signals on 1688 and 1633 chromosomes from control cells and TSA treated cells, respectively, we found that while T-SCEs were rare in control cells (2.4%), TSA treated cells exhibited a more than ten folds increase in the frequency of T-SCEs (30.7%) (Fig. 6E).
and F), indicating the occurrence of HR at telomeres.

In addition, it is possible that telomeric HR, which is induced by TSA mediated chromatin decondensation, subsequently results in loss of t-loops. If so, unchanged t-loops would be expected if HR is inhibited in TSA treated cells. To test this, HeLa cells were co-treated with TSA and B02, the latter is a specific inhibitor of Rad51 that plays an essential role in HR. We still observed decrease of t-loops (Fig. S4A and B) and generation of t-circle, demonstrating that the loss of t-loops is not caused by HR. We also showed that TSA and/or B02 treatment did not significantly alter telomere length (Fig. S4C). In addition, TERRA (Telomere Repeat-Containing RNA) level transcribed from 6p, 7p, XpYp, 7q and 17q telomeres was not changed by TSA treatment (Fig. S4D).

Discussion
Detection of t-loops by non-denatured 2D agarose gel electrophoresis

Neutral/neutral 2D gel method, which detects native DNA structure in a gentle condition, was widely used in studying DNA replication intermediate (RCIs) in different kinds of species (30-33). In recent 20 years, 2D gel method has been widely used in studying the dynamic of special telomeric structures, including G/C rich overhang, extrachromosomal telomeric circle and t-circle-tail (34-37). Based on the structural similarity between t-loops and RCIs, in this study, we developed a new method that employs 2D gel electrophoresis followed by Southern blotting to detect t-loops in cell-free extracts. In order to detect t-loops/sigmoidal signal using 2D gel electrophoresis, genomic DNA need to be isolated under moderate condition avoiding acute vortex and high temperature digestion of proteins. Moreover, electrophoresis was carried out at low-voltage/4°C to avoid the heat accumulation that may disrupt t-loop structure. The following evidences indicated that the sigmoidal arc observed in our 2D gel method represents t-loops. First, this structure is a part of chromosome and is sensitive to heating. Second, this structure can convert into circular DNA by plasmid-safe nuclease that specifically digests linear DNA but not circular DNA. Third, the structure consists of a single-stranded G-rich DNA that is resistant to Exo I digestion. Fourth, the result that ALT cells have much less t-loops compared to telomerase positive cells observed by our method is consistent with the observations by EM (5,22). Finally, we observed t-loops by EM with DNA extracted from the sigmoidal arc, and the t-loops showed similar loop and tail sizes as previous result of other groups(5,12). All of these results demonstrate that this method can document the t-loop structure.

In addition to the biochemical and EM validation of t-loop analysis using our 2D gel method, we also test the robustness of this method by reproduced necessity of TRF2 on stabilization of t-loop. TRF2 has been reported to be essential for t-loop formation and maintenance (12). Here we showed that t-loops decreased when TRF2 was depleted independent of chromosome fusion frequency. These results are consistent with previous report (12,38,39), further verified the authenticity of 2D gel method.

Advantage and disadvantage of 2D gel method to detect t-loops

The study of t-loops has been challenging previously, due to lack of a sensitive and handy method. EM and STORM are reliable methods to visualize the t-loops directly (12). However, the application of these two methods are limited for technical difficulty such as cross-linking level and sample preparation (5,12,22). The 2D gel method we presented here is a widely applicable method for it combined 2D gel electrophoresis and in-gel hybridization with telomeric probes. This method has several obvious advantages. First, it does not require special equipment and technology. Second, only a small amount of DNA (5μg ~ 10μg genomic DNA) is needed. Third, it is capable of analyzing
multi-samples simultaneously in the same condition. Finally, it can be finished in a couple of days. Therefore, this method provides a convenient way to study t-loop applicable to almost all researchers.

Although 2D gel method shows many advantages as described above, a few limitations still exist. Firstly, because of the 2D gel method is performed without DNA cross-linking, a part of t-loops would be disrupted during DNA purification, digestion and electrophoresis. That is why we detected less t-loop (17% in HeLa) than EM result (15-40% in HeLa) (5). Secondly, the sigmoidal arc represents a converged fraction of physically similar t-loops (Fig. 1A), while part of t-loops migrating to the region between the circle and sigmoidal arc (gray curves in Fig. 1A) are invisible for low abundance. Thirdly, the quantification of sigmoidal arc (t-loop) is based on intensity analysis of southern blot (relative ratio), which was not equal to the molecular number. However, even in this case, it still shows a similar relative abundance of t-loops between 2D gel method and EM (5,22), suggesting that t-loop quantification using 2D gel method is reliable.

**T-loops formation during cell cycle**

The dynamics of t-loops formation during cell cycle and heterochromatin-regulation of t-loop structure are fundamental issues remained poorly understood. Previously, it has been proposed that t-loops must unfold during S phase to allow replication processing (40) and C-rich strands fill in also requires an “open” telomere structure (20,21). Our results showed that t-loops are consistently present at chromosome ends throughout the cell cycle, and its formation is closely coupled with telomere replication, i.e. t-loops are immediately formed after the replication is finished (Fig. 4). These results implying that t-loop refolding follows closely with telomere synthesis, in other words, t-loop unfold during late S/G2 and immediately re-fold after C-rich strand synthesis is completed. The apparent need for consistent presence of t-loops at chromosome ends further suggests that they play a critical role in protecting linear chromosome ends at all stages of the cell cycle.

**Heterochromatin state of telomeres and the maintenance of t-loops**

Previous study showed that telomeres and subtelomeric regions exist as condensed heterochromatin (41). Mammalian telomeric repeats are characterized by low levels of acetylated H3 and H4, and high levels of trimethylated lysine 9 in histone H3 (H3K9me3) and lysine 20 in histone H4 (H4K20me3) (42). In yeast, the formation of back-folding loops is dependent on the activities of several histone deacetylases that play roles in chromatin silencing (43). In consistent with these findings, our data demonstrated that TSA-induced decondensation of telomeric chromatin triggers disruption of t-loops (Fig. 5). In general, heterochromatin regions are less accessible to DNA modifying proteins and nucleases (44,45). As such, the heterochromatic state might help to protect t-loops against DNase attack, helicase unwinding and other undesired processes.

As expected, in association with the loss of t-loops, significantly increased TIFs were observed in cells treated with TSA (Fig. 6A and B), indicating that the deprotection of chromosome ends activates DNA damage response (DDR). The frequency of T-SCEs also increased after TSA treatment (Fig. 6E and F), which may be causes by the disruption of t-loops. In support of this hypothesis, human ALT cancer cells display much less t-loops and higher frequency of telomeric HR, compared with non-ALT cells (Fig. 1C and 6E) (22,25).

**Experimental procedures**

**Cell culture, treatment and transfection**

HeLa S3, A549 and U2OS cells were grown in DMEM (Hyclone) containing 10% Calf Serum (PAA) and 100U/mL penicillin & 100μg/mL streptomycin at 37°C under 5% CO2. Human foreskin fibroblasts (BJ) and VA13 were grown in DMEM (Hyclone) containing 10% FBS (PAA). For drug treatment, HeLa cells...
treated with 0.5µM Trichostatin A (unless indicated otherwise), or 27.4µM B02 (Millipore) for 48h. For TRF2 knockout, we used inducible CRISPR/Cas9 system. First, HeLa cells were transfected with lentivirus carrying inducible-Cas9 (pHAGE-TRE-Cas9) and selected with neomycin for 10 days. Second, two lentivirus with individual sgRNAs targeting to TRF2 were sequentially transfected into cells, and the empty vector was used as control. Two rounds of selection with puromycin (2µg/ml) and blasticidin (10µg/ml) were used to select cells express sgRNAs stably. The sequences of sgRNAs are as following:

- **sgTRF2-1-F**
  GCCTTTCGGGGTAGCCGGTA
- **sgTRF2-1-R**
  TACCGGCTACCCCGAAAGGC
- **sgTRF2-2-F**
  GAACCCGCAGCAATCGGGACA
- **sgTRF2-2-R**
  TGTCCCGATTGCTGCGGGTTC

Third, cells with sgTRF2 were treated with doxycycline (1µg/ml) for 7 days to induce Cas9 expression and deficient TRF2, and KU60019 (10μg/ml, inhibitor of phosphorylation of ATM) was added at the last 4 days during doxycycline treatment to inhibit ATM phosphorylation. Cells with sgCtrl were treated in same condition as control.

**Cell cycle synchronization**

HeLa S3 cells were synchronized at G1/S phase by double thymidine block as described previously(20). Cells were then released into S phase and harvested 3, 6, 8, 10 or 15 h after release. Genomic DNA was purified from recovered cell pellets immediately after harvest. For FACS analysis, Cells were fixed with 75% ethanol and stained with PI.

**Genomic DNA isolation**

Unless indicated otherwise, genomic DNA was extracted using AxyPrepTM Blood Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, USA) following the manufacturer’s instruction. All steps were performed at room temperature omitting vortexing. Genomic DNA was also isolated using the DNAeasy kit (QIAGEN, Valencia CA) and using phenol-chloroform extraction as the primary deproteinization step (5), after which DNA samples prepared by different methods were used for side-by-side comparison.

**Neutral-neutral 2D agarose gel electrophoresis and southern blotting (2D gel method)**

2D agarose gel electrophoresis was performed as described (46,47). Briefly, 10µg (5µg at least) genomic DNA was digested with Rsal and Hinfl (NEB) and loaded onto a 0.4% agarose gel, the electrophoresis was carried out in 1×TBE at 1V/cm for 12h at room temperature. The lane containing DNA was excised from gel and the gel buffer was exchanged with 1×TBE with 0.3µg/ml ethidium bromide (EB) (Sigma). The gel slice was placed and cast with 1% agarose gel in 1×TBE containing 0.3µg/ml EB. The gel was run at 4°C for 6 h at 3V/cm. In-gel hybridization analysis of telomeric DNA was performed as described (35) with minor modification: the gel was dried for 1 h at 50°C and denatured for 30 min in 0.5M NaOH & 1.5M NaCl. The gel was rinsed several times in distilled water, neutralized with 0.5M Tris-HCl & 1.5M NaCl (pH=8.0) and hybridized with a telomere probe in 1×hybridization buffer (2µg/ml sonicated E.coli DNA, 10×Denhardt’s buffer, 0.5% SDS and 5×SSC) at 42°C overnight. The gel was washed four times in washing buffer (2×SSC, 0.5% SDS) and exposed to a PhosphorImager screen. Telomere probes (G-rich and C-rich) were prepared as described previously (20).

The percentage of t-loops was calculated as following: (the intensity of signal in the sigmoidal arc) / ( intensity of total signal) × 100%".

**Electron microscopy**

DNA from sigmoidal arc was purified with Qiagen MinElute Gel-extraction kits (Qiagen, Cat NO.28604). The enriched DNA in TE buffer (10mM Tris-Cl, pH 8.0 and 1mM EDTA) was applied to a layer of benzalkonium chloride (BAC) (6µl deionized formamide, 0.6µl 40% glyoxal, 1µl enriched DNA and 1µl 0.02%
BAC in 12 μl) and transferred to pretreated thin carbon membrane-coated EM grids. After rinsing with distilled water, DNA was stained with 0.75% formic acid uranium at near neutral pH and dehydrated with 5μl 100% ethanol. The EM grids were examined under a JEM-100CXII transmission electron microscope. Images were captured using an OSIS, MEGAVIEW G2 CCD camera. The dimension of t-loops was measured by iTEM software (TEM IMAGING PLATFORM, Olympus).

**IF-fluorescent in situ hybridization (IF-FISH)**

IF-FISH for telomere dysfunction-induced foci (TIFs) and APBs detection was essentially done as previously described (48). The following antibodies and telomeric probe were used in this study: PML (Mouse mAb, Santa Clus), 53BP1 (Rabbit, Novus Biologicals), Cy3-(TTAGGG); (Peptide Nucleic Acid, Bio-Synthesis). Fluorescence microscopy was performed on a ZEISS AxioImager Z1 microscope.

**Chromosome orientation fluorescence in situ hybridization (CO-FISH)**

CO-FISH were performed as described previously (29). Control cells and TSA-treated HeLa cells were cultured in 100μM BrdU for 16h, followed by treatment with 1μg/ml nocodazole for 2h. Telomeric daughters were probed by Alexa Fluor-488-(CCCTAA)3; (Peptide Nucleic Acid, Bio-Synthesis) and Cy3-(TTAGGG); (Peptide Nucleic Acid, Bio-Synthesis), respectively. Photographs were taken on a Zeiss AxioImager Z1 microscope.

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) was essentially done as previously described (49). The following antibodies were used in this study: anti-H3K9ac (Rabbit, Sigma), anti-H4ac (Rabbit, Millipore), Human IgG (Sigma). Telomeric DNA was detected by hybridization with telomere specific probe.

**Micrococcal nuclease assay**

Micrococcal nuclease assay was performed as previously described (25).

**Immunoblot**

Antibody to TRF2 (Mouse, Millipore) was used in western blot to determine the expression level of TRF2 in control and CRISPR/Cas9 knockout cells.

**Telomere restricted fragment (TRF) assay**

The telomere length assay was performed as previously described (35).

**Determination of TERRA Level**

RNA was isolated by RNAiso Plus (Takara) according to the instructions of manufacturer. Quantitative PCR was performed by cDNA synthesis (PrimeScript II 1st Strand cDNA Synthesis Kit (Takara)) followed by amplification with RealStar Power SYBR Mixture (GenStar, China). GAPDH was used as internal control. PCR primers detecting TERRA transcribed from different telomeres were listed: GAPDH-forward 5’-CCCATGTTCGTCATGGG TGT-3’;  GAPDH-reverse 5’-TGGTCATGAGTCTTCCACGATA-3’; 6p-forward 5’-TCTGTTCCTTCTCGT GTGACC-3’;  6p-reverse 5’-TGTGTTCACGTCCACAG-3’;  7p-forward 5’-GTTGTTCCACGCC TGTAAT-3’;  XpYp-forward 5’-AAGAACGAAGCTTCCACAG-3’; XpYp-reverse 5’-GGTCAGAGCACCTTGGC 3’;  12q-forward 5’-CCCTAACCCTAAGCTT-3’;  12q-reverse 5’-CCTCAACCCTAAGCTT-3’; YpXp-forward 5’-CTTCGTCGGGAGCAGATT AGAGAATAA-3’; YpXp-reverse 5’-CCCTAACCCTAAGCTT-3’;  7q-forward 5’-CTTCGTCGGG AGAGAATAA-3’;  7q-reverse 5’-GGTCAGACACGATGAGA-3’.

**Statistical analysis**

Two-tailed unpaired student’s t-test was used for analysis between two samples (Graphpad Prism). Error bars represent the mean±SD of no less than three biological repeats/independent experiments, * P<0.05, ** P<0.005, *** P<0.001. For sample treated with more than two samples, analysis was performed by one or two-way ANOVA (Graphpad Prism) and P values were indicated in Figure legends.
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Conflict of interest
The authors declare that they have no conflict of interest.

Author contributions
H.L, Y.Z, Z.Z, T.Z, and Y.G designed the experiments, Z.Z, T.Z, H.L, M.T performed the experiments, Y.Z, W.M, Q.Z, S.G, J.S, W.E.W analyzed the data, Y.Z, H.L oversaw the project and wrote the paper.

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FOOTNOTES
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Figure 1. A sigmoidal arc was putative as t-loop structure and observed in different cell lines by 2D gel method. (A) Electrophoretic mobility of typical rolling circle intermediates (RCIs) (13). Insert indicates the eyebrow formed by RCIs with the same loop size but different tail lengths. (B) HeLa genomic DNA was purified, digested with RsaI and HinfI and analyzed by 2D gel method. Sigmoidal arc, t-circle-tail (35) and linear telomere were indicated. (C) Telomere homologous DNA was analyzed in human primary cells (T-cells & BJ fibroblast), telomerase positive cells (HeLa S3 & A549) and ALT cells (VA13 & U2OS). Sigmoidal arc (black arrow) and t-circle (red allow) (24) were indicated.
Figure 2. Biochemical Characterization of t-loops. (A) DNA samples were heated at the indicated temperatures overnight and analyzed by 2D gel method. (B) Schematic of the migration of linear dsDNA, ssDNA and open circles in 2D gel method. (C) Plasmid-safe DNase converted the molecules in the sigmoidal arc region into t-circles (right). DNA is analysis by 2D gel method. Untreated DNA was used as a control (left). (D) Samples were treated with or without Exonuclease I as indicated and analysis by 2D gel method, which probed with C-rich (left) or G-rich telomeric probe (right) under native or denaturing conditions.
**Figure 3.** The deletion of TRF2 results in decreased t-loops. (A) Schematic of CRISPR/Cas9 for editing TRF2 gene. Two sgRNAs were designed to target the sites in the vicinity of 73 and 103aa of TRF2 protein. (B) TRF2 knocked-out samples with/without inhibition of ATM phosphorylation (p-ATM) by K60019 were analyzed by 2D gel method, cells with empty vector were used as control. Fusion telomere was indicated by red arrow. (C) Quantification of t-loop percentage in (B) (mean±SD, n=3). P values were calculated using the student’s t-test, *** p<0.001. (D) Metaphase spreads of samples same as (B). Chromosome fusions were indicated by white arrows. Scale bar: 10μm. (E) Quantification of fusion chromosomes (%) in (D), (mean±SD, n=3 with more than 3357 chromosomes). Two-way ANOVA was performed: sgTRF2 p<0.0001; iATM p<0.001; interaction p<0.001. The student’s t-test was also performed: ** p<0.01, *** p<0.001.
Figure 4. Cycles of T-loops folding/unfolding during S phase. (A) HeLa cells were released from G1/S arrest, harvested at the indicated time points and analyzed by FACS. (B) Genomic DNA corresponding to cells in (A) was assessed for t-loops frequency/persistence by 2D gel method. Phases of cell cycle are as follows: 3 and 6 h samples: S phase, 8 and 10 h samples, late S/G2, and 15 h sample, G1. (C) Schematic showing experimental design for 1 h pulse-labeling of cells with BrdU. (D) Samples prepared as described in (C) were applied to a CsCl density gradient. Differential density separates sample into three peaks, corresponding to unlabeled telomere, leading and lagging telomere fragments, as indicated. (E) T-loops formation in DNA fragments, corresponding to leading and lagging telomeres, were analyzed by 2D gel method in the same gel side by side.
Figure 5. Decondensation of telomeric chromatin induces disruption of t-loops. (A) ChIP assay with DNA from HeLa cells or TSA-treated HeLa cells. Slot blots were probed with telomeric probe. (B) Quantification of (A), values are mean±SD of three independent experiments. Bar graph shows level of enrichment for epigenetic marks in TSA-treated HeLa cell DNA. P values were calculated using the student’s t-test, *** p<0.001. (C) U2OS, HeLa, or TSA-treated HeLa cells were treated with MNase for the indicated length of time. Density scanning of gel lane corresponding to sample digested for 16 min is shown to the right of each panel. The fraction of sample detected as mono-nucleosomes (1N) was calculated and is showed at bottom right of each panel. (D) Genomic DNA from TSA-treated (48h with indicated doses) and untreated HeLa cells were analyzed by 2D gel method. Putative t-loops and t-circles are indicated. Results from the same gel were spliced and rearranged. (E) Quantification of t-loop percentage in (D) (mean±SD, n≥3). One-way ANOVA was performed: p<0.0001.
Figure 6. Loss of t-loops is associated with telomere homologous recombination in TSA-treated HeLa cells. (A) Telomere dysfunction induced foci (TIFs) were detected by IF-FISH using antibody against 53BP1 and telomere specific probe. TIFs are indicated by arrows. Scale bar: 10μm. (B) Quantification of data in (A), values are mean±SD of three independent experiments. P values were calculated using the student’s t-test, ** p<0.01. (C) Telomeres were detected by FISH using telomere probe and APBs were detected using antibody to PML protein. DMSO-treated cells were used as a control (Ctrl). APBs are indicated by arrows. Scale bar: 10μm. (D) APBs per cell were counted and percentage of cells with 0, 1-3, 4-9, or >9 APB foci per cell were calculated (mean±SD, n=3). (E) Typical images of CO-FISH showing that high frequency of T-SCE occurs in TSA treated cells but not in control cells (Ctrl). Recombinated telomeres are indicated by arrows. Enlarged images showed sister chromatids after T-SCE. Scale bar: 10μm in overview figures (white line), 2μm in magnification (gray line). (F) Quantification of E (mean±SD, n=3 with more than 1633 chromosomes). P values were calculated using the student’s t-test, *** p<0.001.
2D gel electrophoresis reveals dynamics of t-loop formation during the cell cycle and t-loop in maintenance regulated by heterochromatin state

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