Elevated levels of TRF2 induce telomeric ultrafine anaphase bridges and rapid telomere deletions

Bernadette Nera¹, Hui-Shun Huang¹, Thao Lai¹ & Lifeng Xu¹

The shelterin protein TRF2 is essential for chromosome-end protection. Depletion of TRF2 causes chromosome end-to-end fusions, initiating genomic instability that can be cancer promoting. Paradoxically, significant increased levels of TRF2 are observed in a subset of human cancers. Experimental overexpression of TRF2 has also been shown to induce telomere shortening, through an unknown mechanism. Here we report that TRF2 overexpression results in replication stalling in duplex telomeric repeat tracts and the subsequent formation of telomeric ultrafine anaphase bridges (UFBs), ultimately leading to stochastic loss of telomeric sequences. These TRF2 overexpression-induced telomere deletions generate chromosome fusions resembling those detected in human cancers and in mammalian cells containing critically shortened telomeres. Therefore, our findings have uncovered a second pathway by which altered TRF2 protein levels can induce end-to-end fusions. The observations also provide mechanistic insight into the molecular basis of genomic instability in tumour cells containing significantly increased TRF2 levels.

¹Department of Microbiology and Molecular Genetics, University of California, Davis, California 95616, USA. Correspondence and requests for materials should be addressed to L.X. (email: lfxu@ucdavis.edu).
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helterin—a six-protein complex bound to chromosome termini—is essential for protecting the integrity of natural chromosome ends1. Within the shelterin complex, POT1 binds single-stranded telomeric overhangs, while TRF1 and TRF2 bind duplex telomeric DNA3–5 and recruit TIN2, TPP1, POT1 and Rap1 to telomeres through protein–protein interactions6–11. When shelterin proteins are experimentally depleted, telomeres are sensed by cells as aberrant DNA. This triggers DNA damage signalling at telomeres, resulting in inappropriate repair, which can produce chromosome end-to-end fusions.

Shelterin is also involved in regulating the length of the telomeric tract in addition to chromosome end protection. The most recognized pathway is through its dual role in the regulation of telomerase: The shelterin component TPP1 promotes telomerase function by recruiting telomerase to telomeres via a direct interaction between its N-terminal OB-fold domain and the telomerase catalytic subunit12–16. Mutations that disrupt this interaction compromise telomerase-dependent telomere elongation. In contrast, the shelterin component TRF1 is thought to block telomerase access to telomeres through anchoring POT1 to telomeres17. Overexpression of TRF1 results in gradual telomere shortening18,19 and epistasis experiments have demonstrated that this effect is through inhibition of telomerase activity20.

However, telomere length homeostasis is dictated by more than simply telomerase action. In young primary human somatic cells, occasionally extremely shortened telomeres can be detected well before senescence21. Ultrashort telomeres (named ‘t-stumps’) of sizes significantly different from the bulk telomere size distribution also exist in cancer cells, which contain active telomerase22. It is speculated that such ultrashort telomeres in primary or cancer cells are generated through stochastic loss of long tracts of telomeric repeats21, a process that is different from the progressive telomere loss caused by replicative attrition due to lack of telomerase. Notably, the shelterin protein TRF2 has been reported to trigger telomere shortening by an unknown mechanism in a telomerase-independent manner19,20,23,24: overexpression of TRF2 can accelerate the rate of telomere erosion in human primary cells that do not have telomerase20,24, and even trigger a DNA damage response25. This suggests that TRF2 is involved in a telomere-processing function that is different from telomerase inhibition.

Purified shelterin components have also been reported to stall replication fork progression at telomeric sequences in an in vitro SV40 DNA-based replication system26, suggesting another mechanism by which telomere length might be modulated. Unresolved DNA structures during replication can persist through mitosis and cause the formation of ultrafine anaphase bridges (UFBs)27–31. Unlike canonical anaphase bridges that originate from covalent chromosome fusions, UFBs arise from interlinked sister chromatids. Two different types of UFBs have been described: one type of UFB forms at centromeres and likely derives from fully replicated DNA sequences held together by DNA catenation. They can be induced by topoisomerase II inhibitors27,30,31. The second type of UFB, which usually associates with common fragile sites (CFS), presumably derives from incompletely replicated DNA sequences and can be exacerbated by replication inhibitors28,29. Mammalian telomeres have been suggested to resemble CFS32, appearing as decondensed or multiple split signals in metaphase chromosomes under replication stress. Ultrafine anaphase bridges that are composed of telomeric sequences, however, are extremely rare, even when cells were challenged with replication inhibitors28.

In this study, we have asked whether elevated levels of TRF2 might promote the pathway that gives rise to ultrashort telomeres. We show here that TRF2 overexpression in human cells stalls replication at telomeric sequences and induces the formation of thin threads of telomeric bridges that arise during segregation of anaphase chromosomes. The induction of these telomeric UFBs precedes stochastic loss of large segments of telomeric sequences, with a subsequent increase in chromosome fusions. Since significantly elevated levels of TRF2 have been detected in many tumour samples and cancer cell lines23,33–35, as well as during the transformation of human primary mammary epithelial cells36, our findings provide mechanistic insight into a specific molecular mechanism driving genome instability in tumour cells.

Results
Elevated levels of TRF2 cause stochastic telomere shortening.
We carried out western blotting analysis to examine TRF2 protein expression in multiple human melanoma, breast cancer and primary cell lines. TRF2 was found to be expressed at significantly higher levels (approximately two- to eightfold) in several breast cancer (MDA-MB-453, MDA-MB-468, ZR-75-1 and MCF-7) and melanoma cell lines (LOX, WM115, WM278, WM983A and WM1158) compared with the primary cells (IMR90, BJ and WI38) (Fig. 1a, Supplementary Fig. 1a), consistent with observations reported by other groups23,33–35.

To understand how elevated TRF2 levels might affect telomere maintenance, we overexpressed full-length, untagged wild-type TRF2 (Fig. 1b) in HT1080 human fibrosarcoma cells to approximately sevenfold of endogenous level using a lentiviral expression system and analysed the effects on telomeres. HT1080 cells were chosen for this study because their endogenous TRF2 level is comparable to that in primary cells (Fig. 1a), and they have active telomerase (Supplementary Fig. 1b), which maintains telomeres at a stable intermediate-length range. As shown in Fig. 1c, within six population doublings (PDs), the distribution of bulk telomeres in cells overexpressing TRF2 changed from a tight cluster between 6 and 10 kb to a smear that extended from ~10 kb to below 2 kb; bulk telomeres in control cells overexpressing GFP maintained stable lengths, as expected.

To investigate this effect at a higher resolution, we used Single Telomere Length Analysis (STELA), which examines the length of individual telomeres21. In this assay (depicted in Fig. 1d), an anchor oligonucleotide comprising a unique sequence of 20 bases followed by 7 bases of telomeric repeat homology is ligated to the 5’-end of the C-rich strand of telomeric DNA. After ligation, the genomic DNA is diluted and different aliquots that each contain a small population of telomeres are analysed by PCR with the indicated primers. Southern hybridization to a probe containing the subtelomeric sequence of a specific chromosome (for example, a sequence at the end of the common subtelomeric sequence on the short arms of X and Y chromosomes, XpYp) is then used to detect the PCR products. Individual bands in the STELA analysis therefore represent the double-stranded region of a single telomere (also containing a short defined subtelomeric sequence). As shown in Fig. 1e, the majority of STELA products of XpYp telomeres in HT1080 cells overexpressing GFP ranged between 6 and 10 kb, consistent with the bulk telomere length results shown in Fig. 1c. In contrast, in cells overexpressing TRF2, the STELA products were very heterogeneous, with sizes ranging between 0.5 and 10 kb. This argues that TRF2 overexpression resulted in stochastic telomere shortening events that occurred within a very limited number of cell divisions.

Notably, we detected STELA products as short as ~0.5 to 0.6 kb. Considering that the STELA products of XpYp telomeres contain ~0.4 kb of subtelomeric region, these results indicate that TRF2 overexpression can infrequently lead to the loss of almost the entire telomeric tract of some chromosomes, potentially
causing chromosome end deprotection. To test whether this was indeed the case, we examined telomere morphology by performing fluorescence in situ hybridization (FISH) with a telomeric repeat probe on metaphase chromosomes. HeLa1.2.11 cells were used for this assay because their long telomeres (mean telomere lengths \( \sim 20 \text{ kb} \)) provide strong and easily detectable fluorescence signal for the FISH-based detection of telomeres (Fig. 2a). Cells were collected seven population doublings (PD7) after TRF2 overexpression for this analysis. We observed a statistically significant increase in signal-free chromosome ends (Fig. 2b) and chromosome end-to-end fusions (Fig. 2c) in cells overexpressing TRF2. Strikingly, the majority of TRF2-induced chromosome fusions lacked detectable telomeric signals at the fusion junction (Fig. 2a), suggesting that the loss of telomeric sequences precedes the chromosome fusion events.

We further examined these TRF2-induced chromosome fusions using a PCR-based assay \(^{37} \) (Fig. 2d). In this assay, individual chromosome fusions were first amplified by PCR utilizing telomere-proximal subtelomeric oligonucleotide primers of selected chromosomes, and then detected by Southern hybridization with a subtelomeric probe. Once again, we observed a significant increase of chromosome fusions in HeLa1.2.11 cells overexpressing TRF2 with this molecular assay (Fig. 2e), consistent with the FISH data. Sequencing of the amplified fusion products revealed that all fusions involved chromosome ends that completely lacked telomeric repeat DNA, with deletions often extending well into the telomere-adjacent subtelomeric tracts (Fig. 2f, Supplementary Fig. 2). All of the fusion molecules sequenced contained unique deletion points at the fusion junction, reflecting the stochastic nature of the deletion events. Most of the fusions also had one to six nucleotides of microhomology between the fused chromosomes at the fusion points (Fig. 2f, Supplementary Fig. 2). Similar large deletions and microhomologies have been observed in chromosome fusions detected in mammalian cells containing short dysfunctional telomeres and in early-stage colon carcinoma and chronological lymphocytic leukaemia cells \(^{37–41} \).

Collectively, the above observations indicate that in response to elevated levels of TRF2, infrequently a fraction of chromosome termini are critically shortened, which leads to a loss of end protection and subsequent end-to-end fusions.
Stalled telomere replication and telomeric UFB formation. Analysis of anaphase chromosomes provided insight into the molecular basis for TRF2-induced stochastic telomere shortening: we observed numerous telomeric bridges, identified by a telomeric repeat PNA FISH probe, between the segregating anaphase chromosomes in different cell lines overexpressing TRF2 (Fig. 3a, Supplementary Fig. 3a). These telomeric anaphase bridges were detected as early as 24 h after the cells were infected with lentivirus expressing TRF2, before a significant amount of TRF2-induced telomere shortening became detectable (Supplementary Fig. 3b). The fine thread-like telomeric bridges were visible through FISH with a telomeric repeat probe, but not through DAPI staining.

Quantification of both chromosome fusions and telomeric anaphase bridges in HeLa1.2.11 cells overexpressing TRF2 at PD3 and PD7 showed that the number of telomeric anaphase bridges increased over time. Figure 2 | Infrequent chromosome end-to-end fusions in HeLa1.2.11 cells overexpressing TRF2. (a) Representative metaphase spread image of HeLa1.2.11 cells infected with lentivirus expressing GFP or TRF2. Infected cells were passaged and collected at PD7 for metaphase spread followed by FISH analysis. Chromosomes (blue) were hybridized with PNA probes for telomeric sequences (green) or centromeric sequences (red). Regions in white boxes are enlarged to the bottom of the corresponding image for better visualization. Yellow arrows indicate signal-free telomeres; arrowhead indicates chromosome end-to-end fusions. For b and c, 50 metaphases (~3,360 chromosomes) each of GFP- or TRF2-overexpressing cells were examined for telomeric abnormality. All quantifications were carried out blindly. Each point on the scatter plot represents a single metaphase. Mean values are indicated in red. Two-tailed Student’s t-tests were performed to make pairwise comparison for statistical significance. (b) Quantification of signal-free telomeres in HeLa1.2.11 cells overexpressing GFP or TRF2. (c) Quantification of chromosome end-to-end fusions in HeLa1.2.11 cells overexpressing GFP or TRF2. (d) Schematic diagram of Fusion PCR analysis. (e) Individual chromosome end-to-end fusions assessed by Fusion PCR. HeLa1.2.11 cells overexpressing GFP or TRF2 were harvested at PD6. Multiple aliquots of 100 ng of genomic DNA were independently subjected to fusion PCR using a mix of XpYp, 17p and 21q subtelomeric primers. PCR products were resolved on 1% agarose-TBE gel and detected by Southern hybridization with an XpYp-specific subtelomeric probe. (f) Representative sequence of fusion molecules between XpYp, 17p and 21q. The fusion points, size of deletion, and microhomology (in red) are indicated.
and CldU in the presence of aphidicolin (see Supplementary Fig. 3c for representative images). We also observed that overexpression of TRF2 increased the frequency of fragile telomeres (Fig. 3c), which are aberrant decondensed and multiple split telomere signals whose formation closely correlates with replication stalling at telomeric regions\(^\text{32}\). This suggested that TRF2 overexpression was causing telomere replication stalling and the subsequent formation of telomeric UFBs. To assess this, we examined telomere replication by performing Chromatin Fibre-FISH analysis\(^\text{32,42–44}\). We used human LOX melanoma cells for this analysis because their very long telomeres (mean telomere length \(\sim 50 \text{ kb}\)) allow better linear resolution. Briefly, replicating DNA in LOX cells overexpressing a luciferase control or TRF2 protein were labelled consecutively with IdU and CldU before the cells were lysed and the chromatin fibres stretched onto a positively charged glass slide. Immunostaining was then carried out with antibodies decreased from 2.14 to 0.74 per cell, while that of chromosome fusions increased from 0.19 to 0.76 per cell (Fig. 3b). These data, together with the fact that TRF2-induced chromosome fusions lacked telomeric repeat tracts (Fig. 2a,f), support the conclusion that the TRF2-induced telomeric anaphase bridges were unlikely to originate from chromosome fusions. Instead, the TRF2-induced telomeric bridges were reminiscent of the UFBs, which derive from either catenated sister chromatids or incompletely replicated DNA during mitosis\(^\text{27–31}\). We also observed that overexpression of TRF2 increased the frequency of fragile telomeres (Fig. 3c), which are aberrant decondensed and multiple split telomere signals whose formation closely correlates

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\begin{array}{cccc}
\text{Subtelomere} & \text{Telomere} & \text{Luciferase control} & \text{Subtelomere} \\
\text{GFP} & \text{CldU} & \text{GFP} & \text{TRF2} \\
\text{TTAGGG} & \text{Merge} & \text{TTAGGG} & \text{Merge} \\
\text{CldU} & \text{Merge} & \text{CldU} & \text{Merge} \\
\text{IdU} & \text{Merge} & \text{IdU} & \text{Merge} \\
\text{Luciferase} & \text{control} & \text{TRF2} & \\
\end{array}
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Figure 3 | TRF2 overexpression induced telomeric ultrafine anaphase bridges. (a) Formation of thinly stretched telomere bridges between anaphase chromosomes in HeLa2.11 cells overexpressing TRF2. Telomeric DNAs were detected by in situ hybridization with a PNA telomeric probe (red). Chromosomes were stained with DAPI (blue). (b) Quantification of telomeric anaphase bridges and chromosome end-to-end fusions in HeLa2.11 cells overexpressing TRF2 at PD3 and PD7. HeLa2.11 cells were infected with lentiviruses expressing TRF2. Parallel cultures were collected at PD3 or PD7 for PNA telomere-FISH to examine fragile telomeres or for metaphase spreading followed by PNA telomere-FISH to examine chromosome end-to-end fusions. (c) Quantification of fragile telomeres in HeLa2.11 cells overexpressing TRF2 at PD3. HeLa1.2.11 cells were infected with lentiviruses expressing luciferase control or TRF2. At PD3, cells in logarithmic growth were labelled sequentially with IdU and CldU for 4 h before Chromatin fibre-FISH analysis was carried out. Telomeres were identified by FISH with a telomeric repeat probe. IdU and CldU were identified with replication stalling at telomeric regions\(^\text{32}\). This suggested that TRF2 overexpression was causing telomere replication stalling and the subsequent formation of telomeric UFBs. To assess this, we examined telomere replication by performing Chromatin Fibre-FISH analysis\(^\text{32,42–44}\). We used human LOX melanoma cells for this analysis because their very long telomeres (mean telomere length \(\sim 50 \text{ kb}\)) allow better linear resolution. Briefly, replicating DNA in LOX cells overexpressing a luciferase control or the TRF2 protein were labelled consecutively with halogenated nucleotides IdU and CldU before the cells were lysed and the chromatin fibres stretched onto a positively charged glass slide. Immunostaining was then carried out with antibodies decreased from 2.14 to 0.74 per cell, while that of chromosome fusions increased from 0.19 to 0.76 per cell (Fig. 3b). These data, together with the fact that TRF2-induced chromosome fusions lacked telomeric repeat tracts (Fig. 2a,f), support the conclusion that the TRF2-induced telomeric anaphase bridges were unlikely to originate from chromosome fusions. Instead, the TRF2-induced telomeric bridges were reminiscent of the UFBs, which derive from either catenated sister chromatids or incompletely replicated DNA during mitosis\(^\text{27–31}\). We also observed that overexpression of TRF2 increased the frequency of fragile telomeres (Fig. 3c), which are aberrant decondensed and multiple split telomere signals whose formation closely correlates
against IdU and CldU, followed by FISH analysis with a telomeric repeat probe. The replication status of telomeres was determined by analysing the incorporation of halogenated nucleotides within telomeres.

We adopted a previously established pulse-labelling procedure for human cells, which incubates proliferating cells sequentially in IdU and CldU for 4 h each. As the replication fork generally progresses at ≈2 kb min⁻¹ in mammalian cells, all of the halogenated nucleotide-incorporating telomeres in cells overexpressing luciferase control were completely labelled with either IdU or CldU (Fig. 3d). In cells overexpressing TRF2, many telomeres were only partially labelled or not labelled at all, even though their adjacent subtelomeric tracts were fully labelled with IdU or CldU, indicating that the replication forks stalled specifically at telomeric repeat tracts. We did not observe any telomeric tracts containing IdU segment flanked by a CldU labelling segment on either side (Fig. 3d), suggesting that the stalled telomere replication fork failed to restart during the 4 h of CldU labelling. Quantification showed that the overexpression of TRF2 resulted in an approximately threefold decrease of replicated telomeres (Fig. 3e). The extent of telomere replication fork stalling induced by TRF2 was comparable to that induced by 1 μg ml⁻¹ of DNA polymerase inhibitor aphidicolin (Fig. 3e, Supplementary Fig. 3c). Aphidicolin-treated cells, however, failed to replicate through both the subtelomeric region and the telomeric region. This suggests that aphidicolin does not specifically stall replication forks at telomeres (Supplementary Fig. 3c).

Immunostaining demonstrated that the characteristic markers of ultrafine anaphase bridges were associated with these TRF2-induced telomeric UFBs. It has been reported that the PICH protein and the BLM helicase colocalize with the centromere- and the CFS-originated UFBs. A subset of these UFBs, presumably those that have been unwound by BLM, was marked by the single-stranded DNA-binding protein RPA (Fig. 3f). BLM and RPA often exhibited a PICH protein (Supplementary Fig. 4a). BLM and RPA often exhibited an interspersed association pattern along UFBs, suggesting that BLM may dissociate from the unwound DNA strands bound by RPA.

Taken together, our data argue that the telomeric anaphase bridges induced by overexpression of TRF2 are ultrafine anaphase bridges, which arise from persistent replication stalling at telomeres.

**Reduced TRF1 induces fragility but not UFBs at telomeres.** Depletion of TRF1 in mouse embryonic fibroblasts has also been reported to cause replication fork stalling and the formation of fragile telomeres. We therefore examined the possibility that elevated levels of TRF2 may compete with TRF1 for telomere binding, resulting in decreased telomeric TRF1, which leads to telomere replication defects and telomeric UFB formation.

Overexpression of TRF2 indeed significantly decreased the levels of telomere-bound TRF1 (Supplementary Fig. 5a,b). To examine whether the TRF2-induced telomeric UFB formation was simply a secondary consequence of depletion of TRF1, we knocked down TRF1 in HT1080 cells by shRNA treatment (Supplementary Fig. 5c). Bulk telomere length analysis showed that the TRF1 depletion resulted in progressive telomere extension (Supplementary Fig. 5d), in contrast to the stochastic telomere shortening phenotype induced by TRF2 overexpression (Fig. 1c). Furthermore, TRF1 depletion in HT1080 cells led to increased sister telomere associations and fragile telomeres (Supplementary Fig. 5e,f), which is consistent with the TRF1 depletion phenotype previously observed in mouse cells. Notably, we did not detect any telomeric UFBs in TRF1-depleted HT1080 cells or HeLa.1.2.11 cells, among ~90 anaphases examined for each experiment. These data demonstrate that depletion of TRF1 by itself is not sufficient to induce telomeric UFBs, and also suggest that fragile telomeres may derive from telomere associations that are resolved before cells enter into anaphase.

**UFBs correlate with TRF2-induced telomere shortening.** It is possible that elevated levels of TRF2 lead to the formation of an excess of tight DNA–protein complexes, which impede replication fork progression at telomeres. This model predicts that longer telomeres containing more TRF2-binding sites would exacerbate TRF2-induced UFBs. To examine whether TRF2-induced telomeric UFB formation correlated with telomere lengths, we compared the induction of telomeric UFB in cells containing different mean telomere lengths: HeLa 1.2.11 ~20 kb; HT1080 A6 ~8 kb; and UM-UC-3 ~3 kb. As shown in Fig. 5a, comparable levels of TRF2 induced significantly more telomeric UFBs in cells with longer telomeres. Interestingly, in UM-UC-3 cells containing very short telomeres (mean length ~3 kb), TRF2 overexpression did not induce any telomeric UFBs. To determine whether short telomere length was the sole reason responsible for the failure to induce telomeric UFBs in UM-UC-3 cells, telomeres were elongated by overexpressing the telomerase RNA subunit (Fig. 5a), and examined for the induction of telomeric UFBs. Although TRF2 overexpression failed to induce telomeric UFBs in parental UM-UC-3 cells or in cells expressing an empty vector, comparable levels of TRF2 expression induced significant numbers of telomeric UFBs in UM-UC-3 cells containing pre-extended telomeres (Fig. 5a). Bulk telomere-length analysis conducted on seven population doublings after TRF2 overexpression showed drastic and rapid telomere shortening in UM-UC-3 cells containing pre-extended telomeres, but not in control cells whose telomeres are not pre-extended (Fig. 5b). These data demonstrate that the TRF2-induced telomere shortening closely correlated...
with the formation of telomeric UFBs, suggesting that the shortening of telomeres result from cells’ resolution of telomeric UFBs.

**Discussion**

In this study, we have elucidated the molecular series of events that occur at chromosome ends in response to elevated levels of TRF2. By examining the length of individual telomeres in cells overexpressing TRF2, we uncovered a subpopulation of termini that had undergone loss of almost the entire telomeric tract, which was often accompanied by end-to-end fusions. Our data also demonstrate that persistent replication stalling was induced by TRF2 overexpression, resulting in the formation of UFBs during the subsequent anaphase. Strikingly, telomeric UFBs between segregating anaphase chromosomes could be observed as early as the first cell division after TRF2 overexpression, before detection of significant telomere shortening (which required at least three to four cell divisions after TRF2 overexpression). These data support a model in which the primary defect caused by TRF2 overexpression is inhibition of duplex telomeric DNA replication, with resolution of the resulting UFBs leading to stochastic loss of large segments of telomeric sequences.

Our observations therefore provide a second mechanism by which perturbation of normal TRF2 levels can influence genomic instability. Experimental removal of TRF2 from telomeres causes chromosome end-to-end fusions, which often preserve long tracts of telomeric repeats on either side of the fusion junction. In contrast, we found that the majority of the TRF2 overexpression-induced chromosome fusions were accompanied by extensive deletions into the subtelomeric regions of involved chromosomes. Furthermore, these fusion junctions often contained one to six nucleotides of microhomology between the fused chromosomes. Fusions of similar features have been
detected in human and mouse cells containing critically shortened telomeres, as well as in early-stage colon carcinoma and chronic lymphocytic leukaemia cells. Critically shortened telomeres are known to be fused by the alternative non-homologous end-joining (A-NHEJ) repair process. Extensive deletions and limited microhomology at the fusion junctions are among the characteristic features of A-NHEJ. Future studies are needed to determine the involvement of A-NHEJ in TRF2 overexpression-induced fusions. Since chromosome fusions can inflict genomic instability, it will also be important to examine TRF2 levels in staged tumour samples and determine whether dysregulation of TRF2 correlates with tumorigenic transformation.

The specialized telomeric DNA structures at mammalian chromosome ends impose great challenges for replication: the single-stranded telomeric 5'-TTAGGG-3' repeats exposed during replication can form G-quadruplex structures, which hinder lagging-strand replication; the T-loop structures formed by invasion of the 3'-single-stranded telomeric overhang into the duplex region of telomeres present topological barriers for telomere replication. Shelterin protein TRF1 and multiple helicases (that is, BLM, RTEL1 and WRN) are implicated in the cellular regulatory system that remove them during replication under normal conditions, thus stall replication at telomeres. In fact, purified recombinant TRF1 and TRF2 proteins have been observed to stall replication fork progression at telomeric DNA in an in vitro SV40-based replication system. Curiously, the RTEL1 helicase interacts with TRF2 (ref. 60) and knockout of RTEL1 in mouse embryonic fibroblasts was found to cause telomere fragility and stochastic deletion of telomeric tracts, phenocopying the consequences of TRF2 overexpression. Although we failed to detect any telomeric phenotypes by knocking down RTEL1 to ~30% of the endogenous levels in HeLa1.2.11 cells, it is premature to exclude the involvement of RTEL1 in telomeric UFB formation/resolution since the residual RTEL1 in cells may be sufficient to carry out its telomeric functions. A recent live microscopy study of TRF1 overexpression in mouse embryonic stem cells demonstrated that very high TRF1 levels resulted in telomere associations that later became anaphase telomeric bridges and interphase telomere aggregates. It will be interesting to examine whether TRF1 overexpression causes the same type of telomeric UFBs as TRF2 overexpression.

It is noteworthy that the PICH protein often associates along a telomere repeat probe in telomeric FISH analysis. The genomic sequences from which these UFBs originate remain to be determined.

Elevated levels of TRF2 might lead to the formation of an excess of tight DNA–protein complexes, which exhaust the cellular regulatory system that remove them during replication under normal conditions, thus stall replication at telomeres. In fact, purified recombinant TRF1 and TRF2 proteins have been observed to stall replication fork progression at telomeric DNA in an in vitro SV40-based replication system. Curiously, the RTEL1 helicase interacts with TRF2 (ref. 60) and knockout of RTEL1 in mouse embryonic fibroblasts was found to cause telomere fragility and stochastic deletion of telomeric tracts, phenocopying the consequences of TRF2 overexpression. Although we failed to detect any telomeric phenotypes by knocking down RTEL1 to ~30% of the endogenous levels in HeLa1.2.11 cells, it is premature to exclude the involvement of RTEL1 in telomeric UFB formation/resolution since the residual RTEL1 in cells may be sufficient to carry out its telomeric functions. A recent live microscopy study of TRF1 overexpression in mouse embryonic stem cells demonstrated that very high TRF1 levels resulted in telomere associations that later became anaphase telomeric bridges and interphase telomere aggregates. It will be interesting to examine whether TRF1 overexpression causes the same type of telomeric UFBs as TRF2 overexpression.

It is noteworthy that the PICH protein often associates along a segment of the TRF2-induced telomeric UFBs, but along the entire length of the centromere- or CFS-originated UFBs (Fig. 4, Supplementary Fig. 4). Although we cannot exclude the possibility that annealing of the telomeric FISH probe interferes with detection of PICH at telomeric UFBs, this difference might be due to the unique nature of telomere replication: First, unlike CFS where opposing replication forks converge, at telomeric sequences the replication fork progresses largely unidirectionally from the subtelomeric region toward the end of the chromosome. The stalled unidirectional, non-converging replication fork could be processed differently from the converging replication forks. Second, the association and dissociation of PICH and BLM with telomeric tracts might be
influenced by the shelterin protein complexes: for example, both the duplex telomeric DNA-binding protein TRF2 and the single-stranded telomeric DNA-binding protein POT1 have been reported to interact with BLM and stimulate its helicase activity.\(^6\) Last, other helicases (that is, RET1L and WRN) in addition to BLM are known to facilitate telomere replication.\(^6\) Therefore, they may also be involved in resolving TRF2-induced telomeric UFOs.

**Methods**

**Cell lines.** HT1080 fibroblasts cells, HeLa cervical cancer cells, UM-UC-3 urinary bladder cancer cells, breast cancer cell lines MDA-MB-231, MDA-MB-453, MDA-468, ZR-75-1, MCF7, SK-BR-3, human primary fibroblast cell lines MDF, MDA-MB-468, ZR-75-1, MCF7, SK-BR-3, human primary fibroblast cell lines from ATCC and the Cell Line Repository at the Wistar Institute. All cell lines from ATCC and the Cell Line Repository at the Wistar Institute. All cell lines from ATCC and the Cell Line Repository at the Wistar Institute.

**Immunoblotting analysis.** Whole-cell extracts were resolved with 10% SDS–PAGE and transferred to PVDF nitrocellulose membranes. Immunoblots were incubated with a mouse monoclonal anti-TRF2 (BD Transduction Laboratories), a rabbit polyclonal to BLM (Santa Cruz Biotechnology, sc-7790, 1:500), an anti-hsTR antibody (Sigma, T6273, 1:1000), an anti-PICH antibody (Abnova, H54821-B01P, 1:500), or an anti-PICH antibody (Genex, FE435, 1:1000). A rabbit polyclonal anti-tubulin antibody (Sigma–Aldrich) was used as loading controls. Full scans of western blots are provided in Supplementary Fig. 6.

**Lentiviral plasmids.** The pHR'CMV lentiviral expression vector system used in this study was provided by Dr Didier Trono. TRF2 expression lentiviral vector containing TRF2 coding sequence. Full-length or subcloned, wild-type TRF2 cDNA driven by the CMV promoter, followed by an internal ribosome entry site and a hygromycin resistance gene. The GFP-TRF2 expression lentiviral vector used in Supplementary Fig. 5 contains an N-terminal GFP-tagged TRF2 cDNA. Telomerase RNA expression lentiviral vector contain the wild-type hTR cDNA driven by the IU1 promoter and a GFP gene driven by the CMV promoter.\(^69\) The shRNA expression lentiviral vector was constructed as described previously.\(^69\) The target sequence for TRF1 shRNA is 5'-GGGACATGTGACAACTCTACGTA-3'.

**Terminal restriction fragment analysis.** Five microgram of genomic DNA was digested with Hinfl and RsaI, fractionated by 0.6% agarose-TBE gel electrophoresis, and transferred to Hybond XL membrane. Southern blotting was carried out with a mouse monoclonal anti-tubulin antibody (Sigma–Aldrich). Blots were washed with the ImageQuant software. Mean telomere lengths were calculated according to the positions of molecular weight markers run on the same gel.

**Single telomere length analysis.** Briefly, 20 ng EcoRI-digested genomic DNA was incubated in a 10-μl ligation reaction containing 0.9 μM anchor oligo 1 and 1 U T4 DNA ligase (Roche) in 1× manufacturer’s ligation buffer at 37°C for 12 h. The ligated DNA was diluted to 50 pg/μl for subsequent multiple PCRs. Each PCR (94°C for 2 min, 25 cycles of 94°C for 15 s, 65°C for 30 s, and 68°C for 10 min followed by a final extension step at 68°C for 20 min) was carried out in a 15-μl reaction volume containing 100 pg of ligated DNA, 0.5 μM each primer, 0.3 mM each dNTP, 75 mM Tris-HCl (pH 8.8), 20 mM (NH4)2SO4, 0.01% Tween-20, 1.5 mM MgCl2, and 1.5 U Extensor A Human Telomeric Protein. A human telomeric protein.

**Real-time PCR.** Total RNA was extracted with the TRIzol reagent (Invitrogen). cDNA was prepared using the High Capacity RNA-to-cDNA kit (Invitrogen). Real-time PCR was performed using the StepOnePlus real-time PCR (Invitrogen). Telomerase RNA levels were normalized against GAPDH mRNA levels. Primer sets used: TRF1 forward 5'-CCACAGGGGCTAGGATTTTAC-3', TRF1 reverse 5'-ATCATCAGGGCTGAT-3'; and calibrator 5'-CTTGGGCTGAAAGTGGACC(A/T)ATCAG-3'. Signals were detected by phosphorimaging (Molecular Dynamics). Sequence of oligonucleotides used: anchor oligo, 5'-CCACAGGGGCTAGGATTTTAC-3'; TRF1 forward 5'-CCACAGGGGCTAGGATTTTAC-3', TRF1 reverse 5'-ATCATCAGGGCTGAT-3'; and calibrator 5'-CTTGGGCTGAAAGTGGACC(A/T)ATCAG-3'.

**Fusion PCR.** Briefly, genomic DNA was extracted using Gentra Puregene Cell kit (Qiagen) and diluted to 20 ng μl\(^{-1}\) in 10 mM Tris-HCl (pH 7.5). Each PCR (94°C for 2 min, 25 cycles of 94°C for 15 s, 95°C for 15 s, 92°C for 10 s, and 72°C for 10 s) followed by a final extension step at 72°C for 5 min was carried out in a 15-μl reaction volume containing 100 ng of genomic DNA, 0.5 μM each of telomere-adjacent primers (XpYpE2 and XpYpB2), 0.3 mM each dNTP, 75 mM Tris-HCl (pH 8.8), 20 mM (NH4)2SO4, 0.01% Tween-20, 1.5 mM MgCl2, and 1.5 U Extensor.

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**Author contributions**

B.N. and L.X. conceived the experiments. B.N., H-S.H., T.L. and L.X. performed the experiments and analysed the data. B.N. and L.X. wrote the manuscript.

**Additional information**

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