CDK phosphorylation of TRF2 controls t-loop dynamics during the cell cycle

The protection of telomere ends by the shelterin complex prevents DNA damage signalling and promiscuous repair at chromosome ends. Evidence suggests that the 3′ single-stranded telomere end can assemble into a lasso-like t-loop configuration1,2, which has been proposed to safeguard chromosome ends from being recognized as DNA double-strand breaks3. Mechanisms must also exist to transiently disassemble t-loops to allow accurate telomere replication and to permit telomerase access to the 3′ end to solve the end-replication problem. However, the regulation and physiological importance of t-loops in the protection of telomere ends remains unknown. Here we identify a CDK phosphorylation site in the shelterin subunit at Ser365 of TRF2, whose dephosphorylation in S phase by the PP6R3 phosphatase provides a narrow window during which the RTEL1 helicase can transiently access and unwind t-loops to facilitate telomere replication. Re-phosphorylation of TRF2 at Ser365 outside of S phase is required to release RTEL1 from telomeres, which not only protects t-loops from promiscuous unwinding and inappropriate activation of ATM, but also counteracts replication conflicts at DNA secondary structures that arise within telomeres and across the genome. Hence, a phospho-switch in TRF2 coordinates the assembly and disassembly of t-loops during the cell cycle, which protects telomeres from replication stress and an unscheduled DNA damage response.

Telomere homeostasis is crucially dependent on the function of the shelterin complex but how this is regulated during the cell cycle remains uncertain. Using phospho-proteomic analysis of the shelterin complex, we identified a putative CDK2 phosphorylation site in human TRF2 at Ser365 (Ser367 in mouse; Extended Data Fig. 1a), which is abolished by treatment with λ-protein phosphatase (Fig. 1a, left and middle) or mutation of the phospho-site to alanine (Myc-tagged TRF2(S367A); Fig. 1a, right). Analysis of the cell cycle revealed that this modification is abundant in G1, G2 and M phases but is markedly reduced in S phase (Fig. 1b, Extended Data Fig. 1b).

Deletion of Trf2 (also known as Terf2) results in telomere deprotection and chromosome end-to-end fusions1 (Fig. 1c, right). By contrast, Trf2WT1 mouse embryonic fibroblasts (MEFs) complemented with wild-type TRF2 or phospho-dead (Myc–TRF2(S367A)) or phospho-mimetic (Myc–TRF2(S367D) and Myc–TRF2(S367E)) mutants lacked telomere fusions (Fig. 1c, left, Extended Data Fig. 1c, d). The TRF2 Ser367 mutants also retained interactions with other shelterin proteins, including TRF1 and RAP1, and depletion of RAP1 did not result in telomere fusions in cells expressing Myc–TRF2(S367A)4 (Extended Data Fig. 2a–c). Hence, TRF2 Ser367 mutants retain the ability to engage with other shelterin components and to protect telomeres against fusions.

Further analysis of TRF2 null cells expressing the TRF2 Ser367 mutants showed that the phospho-dead Ser367Ala mutant (Myc–TRF2(S367A)) resulted in high levels of telomere fragility, which indicates problems in telomere replication5, whereas the phospho-mimetic mutants (Myc–TRF2(S367D/E)) resulted in frequent loss of telomeres, signal-free ends and high levels of extra-chromosomal telomere circles6 (Fig. 1d–g). Because the distinct phenotypes of the phospho-dead and phospho-mimetic TRF2 Ser367 mutants resemble cells that fail to recruit the helicase RTEL1 to replication forks and telomeres, respectively7, we reasoned that Ser365 or Ser367 of TRF2 might serve as a phospho-dependent protein-interaction surface, which could cooperate with the TRFH domain that was previously shown to interact with RTEL18.

Pull-down experiments using biotinylated human TRF2 peptides encompassing amino acids 354–383 revealed a prominent RTEL1 band with the unphosphorylated peptide (S365) but not with the phosphorylated peptide (pS365) or an unrelated TRF2 control peptide (384–413) (Fig. 2a, Extended Data Fig. 3a). These results raised the possibility that the phosphorylation of Ser365 or Ser367 of TRF2 negatively regulates the interaction between TRF2 and RTEL1. The addition of λ-protein phosphatase was found to enhance this association in cell extracts (Fig. 2c), whereas addition of the phosphatase inhibitor PhosSTOP prevented a robust TRF2–RTEL1 interaction (Fig. 2c). Treatment of cells with the CDK inhibitor R-roscovitine, but not with a PLK1 inhibitor (BI-2536), also enhanced levels of Myc–TRF2 co-immunoprecipitated with RTEL1.
The interaction of RTEL1 with wild-type Myc–TRF2, but not with the Myc–TRF2(S365A) mutant, was inhibited after incubation with recombinant cyclinA–CDK2 (Fig. 2f), which supports previous findings that Ser365 of TRF2 is a cyclinA–CDK substrate. Inhibition of ERK1/2 by UBLCP1, PP1R10, PP4R1, PP4R2, PP6R2, PP2R5C and PP6R3 showed increased association with TRF2 and RTEL1 complexes purified from S phase and asynchronous cells including UBLCP1, PP1R10, PP4R1, PP4R2, PP6R2, PP2R5C and PP6R3. Of these phosphatases, knockdown by short interfering RNA (siRNA) of PP4R2 or PP6R3 or their respective catalytic subunits (PP4C or PP6C, respectively), greatly reduced the TRF2–RTEL1 interaction in two different cell lines (Extended Data Figs. 4b, c, 5a, b). Co-immunoprecipitation studies confirmed that PP4R2 and PP6R3 regulatory subunits interact with TRF2 and RTEL1 in vivo (Extended Data Fig. 4d). Notably, phosphorylation of human TRF2 at Ser365 and mouse TRF2 at Ser367 were greatly enhanced after silencing of PP6R3 but not in cells subjected to PP4R2 knockdown (Extended Data Fig. 5c, d). Cells depleted of the PP4R2 or PP6R3 regulatory subunits also exhibited telomere loss (Extended Data Fig. 5e) and a greater than threefold increase in telomere fragility, arrowheads denote loss of telomere signal. Red, telomere peptide nucleic acid (PNA) FISH; blue, DAPI. g, Phi29-dependent telomere circles (TCs; top) and quantification (bottom) in DNA isolated from Trf2F/− MEFs stably expressing empty vector control, wild-type or mutant TRF2, 96 h after infection with control or Cre-expressing adenovirus (Ad-GFP or Ad-Cre-GFP, respectively). Data are mean ± s.d. from three independent experiments. All P values determined by one-way analysis of variance (ANOVA).
induction in telomere circles when compared with controls (Extended Data Fig. 5f). These data indicate that PP6R3 dephosphorylates TRF2 Ser365 or Ser367 to permit the transient recruitment of RTEL1 to telomeres in S phase.

Because RTEL1 facilitates global and telomere replication through its ability to interact with PCNA, we considered the possibility that the phospho-dead TRF2(S367A) mutant might sequester RTEL1 and limit its ability to bind PCNA. Indeed, cells expressing the phospho-dead TRF2(S367A) mutant, but not wild-type TRF2 or the phospho-mimetic mutants, were compromised for the RTEL1–PCNA interaction in co-immunoprecipitation and proximity ligation assay (PLA) experiments (Fig. 2h, i). Furthermore, analysis of global replication dynamics revealed that TRF2-null cells expressing the phospho-dead TRF2(S367A) mutant, but not wild-type TRF2 or the phospho-mimetic mutants, exhibited reduced replication fork extension rates and increased asymmetric forks across the genome (Fig. 3a, Extended Data Fig. 6a, b). Cells expressing the Myc–TRF2(S367A) phospho-dead mutant also exhibited increased levels of replication stress, which manifested as increased asymmetric forks across the genome (Fig. 3a, Extended Data Fig. 6a, b).
as micronuclei, mitotic catastrophe and increased S3BP1 nuclear foci (Extended Data Fig. 6c–f).

We reasoned that if the phospho-dead TRF2(S367A) mutant sequesters RTEL1 at telomeres, then expressing RTEL1 with a mutation in the C4C4 motif that is defective for TRF2 binding (RTEL1(R1237H)) should mitigate this effect. Indeed, co-expression of the phospho-dead Flag-tagged TRF2(S367A) with the V5-tagged RTEL1(R1237H) mutant, but not with wild-type V5–RTEL1, restored the interaction between RTEL1 and PCNA in mouse ear fibroblasts (Fig. 3b, c). This co-expression also suppressed the levels of fragile telomeres (Fig. 3d), rescued the DNA replication defects (Fig. 3e, f, Extended Data Fig. 7a, b), and suppressed the formation of micronuclei, mitotic catastrophe and S3BP1 foci in mouse ear fibroblasts expressing the phospho-dead Flag–TRF2(S367A) mutant (Extended Data Fig. 7c–f). These data suggest that the TRF2(S367A) mutant sequesters the endogenous pool of RTEL1, potentially at both telomeres and pericentromeric regions, which restricts its ability to engage with PCNA leading to replication stress at telomeres and across the genome.

RTEL1 has been shown to unwind D-loops based on genetic studies and its ability to resolve such structures in vitro. However, evidence demonstrating a direct role in unwinding t-loops in vivo, which contain a D-loop at the point of strand invasion, remains lacking. Because the TRF2(S367A) phospho-dead mutant sequesters RTEL1 at telomeres, we asked what would happen to t-loops in this context. Visualization of telomere secondary structures by Airyscan super-resolution microscopy revealed no measurable reduction in t-loop abundance in TRF2-null cells expressing wild-type TRF2 (Fig. 4a, Extended Data Fig. 8a). However, the frequency of t-loops was significantly diminished in TRF2-null cells expressing the Myc–TRF2(S367A) phospho-dead mutant (Fig. 4b, c). Hence, sequestration of RTEL1 at telomeres leads to promiscuous t-loop unwinding, decreasing the overall levels of t-loops.

The spurious t-loop unwinding observed in the TRF2(S367A) mutant presented an opportunity to directly test whether t-loops are important for suppressing the DNA damage response (DDR) at telomeres. Analysis of TRF2-null cells expressing the phospho-dead Myc–TRF2(S367A) mutant revealed a largely ATM-dependent DDR induction at telomeres, albeit with a modest accumulation of DNA damage-induced RPA foci and activation of ATR due to telomere fragility (Fig. 4d–g, Extended Data Fig. 8b–d). Measuring the lengths of telomere contours in super-resolution micrographs revealed that linear telomeres from cells expressing Myc–TRF2(S367A) overlapped in length distribution with looped telomeres from the wild-type Myc–TRF2 control with protected chromosome ends (Extended Data Fig. 8e, f). These data suggest that promiscuous t-loop unwinding results in linear telomeres that activate an ATM-dependent DDR (Fig. 1c). Collectively, these data reveal that the t-loop structure is important for suppressing the activation of ATM at telomere ends.

In conclusion, our study identifies a phospho-switch in TRF2 that regulates the transient recruitment and release of RTEL1 from telomeres, which is required to temporarily disassemble t-loops during S phase to avert telomere catastrophe, while also preventing promiscuous t-loop unwinding during other cell cycle stages. We suggest that such
Fig. 4 | Expression of the TRF2(S367A) mutant promotes telomere-dysfunction-induced foci (TIF) and impairs the formation of t-loops. a, Western blotting analysis of cells of the indicated genotype. Short and long indicate short and long exposure times. b, Quantification of t-loops observed in TRF2(S367A) MEFs expressing wild-type Myc–TRF2 or the Ser367Ala mutant with or without treatment with 4-hydroxytamoxifen (4-OHT) for 120 h. Data are exclusive of ambiguous molecules (n = 3 biological replicates scoring ≥1,192 molecules per replicate). Data are mean ± s.e.m. c, Representative images of t-loops and linear telomeres identified by Airyscan super-resolution imaging. Scale bar, 1 µm. d, Quantification of telomere-dysfunction-induced foci (TIF) per metaphase (n = 120 metaphases). Data are mean ± s.e.m. e, Representative images of TIF metaphase assays. TIF examples (white arrows) and mitotic catastrophe (red arrow) are shown. f, Representative images of TIF interphase assay with or without ATR or ATM inhibitors. Co-localization of 53BP1 and telomeres is shown in the merged panels. g, Quantification of TIF interphase assay in cells of the indicated genotype with or without ATR or ATM inhibitors (ATRi or ATMi, respectively) (n > 300 nuclei). Data are mean ± s.d. In a and c–g, the experiments were independently repeated at least three times with similar results. P values determined by one-way ANOVA (b, d, g). Box plots are as in Fig. 3e.

exquisite control of TRF2 to regulate t-loop opening and the need to ‘protect’ t-loops from promiscuous unwinding by RTEL1 outside of S phase, further demonstrate that t-loops are essential for physiological telomere homeostasis and chromosome end protection.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41586-019-1744-8.

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Cell culture procedures
SV40-LT-immortalized Ret1fl/fl MEFs\(^2\), Trf2\(^{-}\) MEFs (a gift from T. de Lange)\(^3\), Trf2\(^{-}\)/Rosa26\(^{cre}\)MEFs (from E. Lazzerini-Denchi)\(^1\) and Trf2\(^{-}\)/Ret1fl\(^{-}\) mouse ear fibroblasts were transfected in DMEM supplemented with 15% fetal bovine serum (FBS; Invitrogen), L-glutamine and penicillin–streptomycin. HEK 293 and Phoenix Amphi 293 cells were kept in DMEM with 10% FBS. Trf2\(^{-}\)/Ret1fl\(^{-}\) conditional double-knockout mouse ear fibroblasts were isolated from adult mice obtained by crossing the individual targeted Trf2\(^{-}\) and Ret1fl\(^{-}\) mice. Genotypes were determined by Transnetyx using quantitative PCR with allele-specific probes. Production of retroviral supernatants and transductions of Ret1fl\(^{-}\) and Trf2\(^{-}\) MEFs were done essentially as previously described.\(^4\) Trf2\(^{-}\) MEFs were infected with pLPC-puromycin retrovirus expressing control vector, Myc-tagged wild-type TRF2 or S367 mutants. Trf2\(^{-}\)/Rosa26\(^{cre}\)MEFs were infected with pLPC-puromycin retroviruses expressing Myc-tagged wild-type TRF2 or mutant TRF2 (S367A). Human HEK 293 cells were transduced with pLPC-puromycin retroviruses carrying Myc-tagged wild-type or mutant (S365A) TRF2. Trf2\(^{-}\)/Reta1fl\(^{-}\) mouse ear fibroblasts were complemented with pLPC-hygro in retroviruses carrying Flag-tagged TRF2 (S367A) and pBABE-puromycin retroviruses expressing mouse V5-tagged wild-type RTEL1 or RTEL1(R1237H) mutant. Transduced cells were selected with puromycin (2 µg/ml) for 2–6 days. Trf2\(^{-}\)/Reta1fl\(^{-}\) mouse ear fibroblasts were kept under puromycin (2 µg/ml) and hygromycin (150 µg/ml) selection for 5 days. Deletion of floxed alleles in Ret1fl\(^{-}\) and Trf2\(^{-}\) MEFs was carried out with Ad-Cre-GFP adenovirus (Vector Biolabs) and cells were genotyped by PCR at 96 h after infection as previously described.\(^5\) Trf2\(^{-}\) was deleted in Trf2\(^{-}\)/Rosa26\(^{Cre}\)MEFs by adding 1 µM 4-hydroxytamoxifen (Sigma-Aldrich) to the culture medium. Cell lines were routinely tested for mycoplasma contamination with negative results.

Cell lysis, western blotting, immunoprecipitation and drug treatments
Cells were rinsed twice with PBS, transferred to an ice-cold NET lysis buffer (50 mM Tris (pH 7.2), 150 mM NaCl, 0.5% NP-40, 1× EDTA-free Complete protease inhibitor cocktail (Roche), 1× PhosSTOP phosphatase inhibitor cocktail (Roche)) and lysed for 10 min on ice. The cell lysates were then briefly vortexed and passed through a 23G syringe five times. The soluble protein fractions were collected after centrifugation at 16,000g for 10 min at 4 °C. Western blotting analysis was performed as previously described. Immonoblot of whole-cell extracts from Trf2\(^{-}\)/Rosa26\(^{Cre}\) cells with or without exogenous expression of the Myc–TRF2 allele was performed as previously described.\(^1\) For protein immunoprecipitation, whole-cell extracts were precleared with protein G Sepharose (Sigma-Aldrich) and 1–2 µg of precleared extract was incubated with the indicated antibodies. Immunocomplexes were subjected to SDS–PAGE followed by immunoblotting using nitrocellulose membrane (GE Healthcare). See Supplementary Table 1 for a list of antibodies used. For inhibition of CDKs, R-roscovitine (Sigma-Aldrich), was used at a final concentration of 10 µM for 24 h. PLK-1 was inhibited by BI-2536 (Axon Medchem) at a final concentration of 100 nM for 24 h. The MEK–ERK signalling pathway was inhibited by the MEK1 and MEK2 inhibitor U0126 (Selleckchem), at a concentration of 30 µM for 24 h. An equal amount of DMSO was used as a vehicle control.

siRNA treatment and siRNA oligonucleotides
Transfections with siRNA oligonucleotides were performed using the Lipofectamine RNAiMax (Thermo Fisher Scientific). In brief, human cells at density of 2.0 × 10⁵ cells per well were transfected in a 6-well plate with 40 pmol siRNA. Mouse cells at density of 3.0 × 10⁶ cells per well were transfected with 150 pmol siRNA. The medium was exchanged 24 h after transfection. Then, 72 h after transfection, the cells were collected and the levels of proteins of interest were assessed by immunoblot analyses as described. For silencing experiments in human and mouse cells, pre-designed SMARTpool ON-TARGETplus and Accell siRNA oligonucleotides (Dharmacon; GE Healthcare) were used, respectively. For siRNA oligonucleotide details, see Supplementary Table 2.

A-Phosphatase treatment
Whole-cell extracts were prepared as described above except for the absence of phosphatase inhibitors. Lysates were incubated with 800 U of λ-phosphatase (New England Biolabs) in NET lysis buffer supplemented with 1 mM of MnCl\(_2\) along with protease inhibitors for 30 min at 30 °C. Next, the lysates were incubated on ice for 15 min and subjected to immunoprecipitation as detailed in the main text.

In vitro kinase assay
Whole-cell extracts from HEK 293 cells were incubated for 1 h at 4 °C with the rabbit polyclonal anti-RTEL1 antibody. Immunocomplexes were coupled to protein G Sepharose beads for an additional 1 h at 4 °C and washed three times with the NET lysis buffer followed by two washes with kinase buffer containing cold adenine triphosphate (ATP) and 1 µg recombinant CDK2–cyclinA protein complex for 20 min at 37 °C. Reactions were washed twice with kinase buffer and terminated by the addition of 5× SDS–PAGE sample buffer, and resolved by SDS–PAGE.

Generation of pTRF2(Ser365/367) phospho-specific antibodies
Rabbit polyclonal antibodies against human TRF2 phosphorylated at Ser365 and mouse TRF2 phosphorylated at Ser367 were generated by Kaneka Eurogentec S.A. Biologics Division. The antibodies were raised against the phosphorylated human C–(PTQALP)pS(pALKNKR)-N and mouse C–(ANLSPS)p5p(ALKHKKR)-N TRF2 sequences conjugated through the added C-terminal cysteine to keyhole limpet hemocyanin (KLH). Phosphoerine 365- and 367-specific antibodies were purified with the use of the corresponding sulfolinked phospho- and unphosphorylated peptides. The specificity of each antibody was confirmed by ELISA and immunoblot assays.

Peptide synthesis and peptide pull-down experiments
The peptide pull-down was carried out using the biotinylated peptides. In brief, 36 µg of each of the peptides was coupled to 40 µl of streptavidin–coated magnetic beads (Invitrogen) and added to 1 µg of nuclear extract of HEK 293 cells expressing PHAG-HA-Flag-RTEL. Nuclear extracts were precleared by incubation for 30 min at room temperature with uncoupled beads before pull-down incubation. The coupled beads and the lysates were incubated for 2 h at 4 °C. The beads were washed four times with TBST (Tris-buffered saline, 0.1% Tween-20), resuspended in 2× SDS loading sample buffer, and boiled for 5 min.

Slot-blot assay
TRF2 peptides diluted into a final volume of 200 µl in SSC 2× were applied under gentle vacuum to Trans-Blot nitrocellulose membrane (Bio-Rad) using a Minifold 48 slots, Whatman apparatus (GE Healthcare). Each well was washed with 200 µl aliquots of SSC 2×. After removing SSC 2× with gentle suction, the membrane was removed from the apparatus and washed once with SSC 2×. The membrane was blocked at room temperature in a blocking buffer (5% BSA in TBST) for 1 h and probed with horseradish peroxidase (HRP)-conjugated anti-biotin antibody. Incubation was allowed to proceed for 1 h at 4 °C with rocking. After incubation, the antibody solution was removed and the membrane rinsed twice with TBST followed by detection by the ECL method.

Site-directed mutagenesis
Amino acid substitutions were performed with the primers as indicated in key resources table. Primers were designed with the QuikChange
Molecules were classified as linear when we observed an individual molecule consisting of a closed loop structure with a single attached tail. Molecules were classified as t-loops when we could discern an individual contour of ≥1 µm, and contained no gaps in telomere staining ≥500 nm. Telomere molecules were scored if they had a traceable telomere manually quantified with researchers blinded to the experimental conditions. Specifically, after capture and processing, images were obtained from super-resolution microscopy were scored as previously described.

**PNA FISH and immunofluorescence**

Telomeric PNA FISH on cytogenetic chromosome spreads was performed as previously described. In brief, cells were treated with 0.2 µg/ml of colcemid for 90 min to arrest cells in metaphase. Trypsinized cells were incubated in 75 mM KCl, fixed with methanol:acetic acid (3:1), and spread on a glass slide. To preserve the chromosome architecture better, the slides were rehydrated in PBS for 5 min, fixed in 4% formaldehyde for 5 min, treated with 1 mg/ml pepsin for 10 min at 37°C, and fixed in 4% formaldehyde for 5 min. Next, slides were dehydrated in 70%, 85% and 100% (v/v) ethanol for 15 min each and air-dried. Metaphase chromosome spreads were hybridized with telomeric TAMRA-TelG 5′- (TTAGGG)3-3′ PNA probe (Panagene) and slides were mounted using ProLong Gold antifade with DAPI (Life Technologies). Chromosome images and telomere signals were captured using Zeiss Axio Imager M1 microscope equipped with an ORCA-ER camera (Hamamatsu) and using the Velocity 6.3 software (Perkin Elmer).

**Cell cycle synchronization**

HEK 293 cells were synchronized by the double-thymidine-block method with minor modifications. In brief, cells were treated with 2 mM thymidine (Sigma-Aldrich) for 18 h, thymidine-free medium for 9 h to release the cells, and 2 mM thymidine was added to medium for an additional 16 h to arrest the cells at the G1-to-S transition. Cells were washed twice with PBS and then released in fresh complete DMEM. Cells were analysed at 70-min time intervals by immunoblotting and in situ PLA assay. For synchronization in mitosis, a thymidine—nocodazole block was used. In brief, cells at a confluence of 60% were treated with 2 mM thymidine for 24 h, washed twice in PBS, and released into complete DMEM for 3 h. Next, cells were treated with 50 µg ml⁻¹ of nocodazole (Sigma-Aldrich) for 15 h, and the cells were washed twice with PBS and a fresh complete medium was added to the cell culture. Synchronized cells were analysed at 90-min time intervals by western blotting with antibodies as indicated.

**Indirect immunofluorescence**

Cells were washed with PBS and fixed with 4% formaldehyde for 10 min at room temperature, permeabilized with 0.3% Triton X-100 in PBS for 5 min at room temperature and then blocked with 3% BSA, 10% FBS in PBS for 1 h at room temperature. Samples were then incubated with rabbit anti-53BP1 antibody overnight at 4°C, washed with 0.05% Tween-20 in PBS and incubated with anti-rabbit IgG Alexa Fluor 594 (Molecular Probes). DNA was counterstained with DAPI and images were acquired using a Zeiss Axiosmager MI, using a Hamamatsu digital camera and the Velocity 4.3.2 software (Perkin Elmer).

**Airyscan super-resolution imaging**

Sample preparation for super-resolution microscopy, cross-linking efficiency determination and Airyscan imaging were performed as described previously. Images obtained from super-resolution microscopy were scored as previously described. Specifically, after capture and processing, images were exported to ImageJ as.tif images with maintained scales. Images were manually quantified with researchers blinded to the experimental conditions. Telomere molecules were scored if they had a traceable telomere contour of ≥1 µm, and contained no gaps in telomere staining ≥500 nm. Molecules were classified as t-loops when we could discern an individual molecule consisting of a closed loop structure with a single attached tail. Molecules were classified as linear when we observed an individual molecule with two visible ends, containing no loops or branched structures. All molecules that did not conform to the looped or linear definition were classified as ambiguous. Densely packed areas of coverslips with overlapping telomere molecules were not scored. Each loop and linear molecule were measured for contour length using the ImageJ trace function.

**Telomere circle assay**

Cells grown at a confluence between 70% and 80% were collected from two 10-cm dishes and extraction of genomic DNA for T-circle assay was performed as previously described. Total gDNA was digested by Alul/Hinf restriction enzymes and the TCA assay was performed with two essential modifications as described: (1) Phi29 DNA (Thermo Scientific) polymerization used a mammalian telomere primer; and (2) Southern blotting membrane was hybridized to a γ[32P]-labelled (TTAGGG)₃ telomeric probe. Southern blot images were captured with Storm 840 scanner and the extent of [32P] incorporation was quantified from the autoradiographs using ImageQuant TL Software Analyzer (Amersham Biosciences). The level of γ[H2AX primary antibody and subsequently with anti-mouse Alexa Fluor 568 secondary antibody (Molecular Probes) and a TAMRA-TelG 5′- (TTAGGG)₃-3′ PNA probe (Panagene). Metaphase TIF assays were done as previously described. In brief, cells were treated with 20 ng/ml colcemid for 1 h before collecting and resuspending in a hypotonic buffer of 0.2% trisodium citrate in 0.2% KCl. The cells were swollen for 5 min then cytocentrifuged onto glass slides using a Tharmac Cellspin 1, before fixation and processed for immunolabelling with an anti-γ-H2AX primary antibody and appropriate filter cubes, and a CoolCube1 camera (MetaSystems). After acquisition, images were imported into ImageJ (NIH) and Adobe Photoshop CS5 for manual quantification and processing.

**DNA combing**

Cells were sequentially pulse-labelled with 25 µM CldU (Sigma) and 250 µM IdU (Sigma) for 20 min and, after collection of the cells, low-melting agarose (Sigma) plugs each containing 200,000 cells were prepared. DNA fibres were extracted from the plugs and combed on silanized.
coverslips using the FiberPrep DNA extraction kit and the molecular combing system (Genomic Vision), according to the manufacturer’s instructions. Combed fibres were fixed at 60 °C for 2 h and DNA was denaturated in 0.5 M NaOH for 25 min. Fibres were then blocked in 1% BSA in 0.1% Tween-20 in PBS for 1 h, incubated with rat anti-BrdU (detects CldU, BU1/75, AbD Serotec, 1:200) and mouse anti-BrdU (detects IdU, B44, BD Biosciences, 1:200) for 1 h followed by anti-rat IgG AlexaFluor 594 and anti-mouse IgG AlexaFluor 488 (Molecular Probes, 1:500) for 1.5 h.

For DNA combing with single-stranded DNA (ssDNA) staining, cells were sequentially pulse-labelled with 25 µM CldU (Sigma) and 250 µM IdU (Sigma) for 15 min, and, after collection of the cells, low-melting agarose (Sigma) plugs each containing 250,000 cells were prepared. DNA fibres were extracted from the plugs and combed on silanized coverslips using the FiberPrep DNA extraction kit and the molecular combing system (Genomic Vision), according to the manufacturer’s instructions. Combed fibres were fixed at 60 °C for 2 h and DNA was denaturated in 0.5 M NaOH for 25 min. Fibres were then blocked in 1% BSA in 0.1% Tween-20 in PBS for 1 h, incubated with rat anti-BrdU (detects CldU, abcam, ab6326, 1:500) and mouse anti-BrdU (detects IdU, B44, BD Biosciences, 1:250) for 1 h followed by anti-rat IgG AlexaFluor 594 and anti-mouse IgG AlexaFluor 488 (Molecular Probes, 1:500) for 1.5 h. Fibres were then incubated with mouse anti-ssDNA antibody (Millipore, MAB3034, 1:200) for 45 min followed by anti-mouse IgG AlexaFluor 647 (Molecular Probes, 1:200) for 45 min.

Images were acquired using a Zeiss Axiomager M1, equipped with Hamamatsu digital camera and the Volocity software (Perkin Elmer). Fibre length was analysed using ImageJ (http://rsbweb.nih.gov/ij/).

Mass spectrometric analyses and protein identification
Coomassie-stained polyacrylamide gel slices were excised from SDS–PAGE gels using a scalpel and processed for mass spectrometry using the Janus liquid handling system (PerkinElmer). In brief, the excised protein gel pieces were placed in individual wells of a 96-well microtitre plate and destained with 50% (v/v) acetonitrile and 50 mM ammonium bicarbonate, reduced with 10 mM DTT, and alkylated with 55 mM iodoacetamide. After alkylation, the samples were digested with trypsin (Promega), overnight at 37 °C. The resulting peptides were extracted in 1% (v/v) formic acid, 2% (v/v) acetonitrile. Digests were subsequently analysed by nano-scale capillary LC–MS/MS. Peptide mixtures were separated on a 50 cm, 75 µm i.d. EasySpray C18 LC-MS column over a 30-min gradient and eluted directly into the LTQ Orbitrap Velos (Thermo Scientific) mass spectrometer. The mass spectrometer was operated in data dependent mode with the top-10 most-intense multiply charged precursor ions fragmented in the linear ion trap using collision-induced dissociation. Raw mass spectrometric data were processed in MaxQuant18 (v.1.3.0.5) for peptide and protein identification, the database search was performed using the Andromeda search engine against the Homo sapiens canonical sequences downloaded from UniProtKB (release 2012_08).

Statistical analysis
Statistical analyses were performed using GraphPad PRISM version 7.0 software (GraphPad). Statistical significance of data was assessed by two-tailed Student t-test or one-way ANOVA unless noted otherwise. Data represent mean ± s.e.m. or mean ± s.d. as indicated. P > 0.05 was considered not significant. No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment unless stated otherwise.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

Data availability
The mass spectrometry proteomics dataset is publicly available through ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014843. Source Data for Figs. 1–4 and Extended Data Figs. 1–8 are available with the online version of the paper. All other data are available from the corresponding author upon reasonable request.

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Acknowledgements We thank members of the Boulton and Cesare laboratories for suggestions, discussions and critical reading of the manuscript. We thank N. O’Reilly and D. Joshi for peptide synthesis, the Australian Cancer Research Foundation Telomere Analysis Centre at the Children’s Medical Research Institute (Sydney) for imaging support and A. Colomba for providing reagents. G.S. is supported by an EMBO advanced fellowship (ALTF 1656-2014). P.K. and P.R. are supported by the Crick Institute core funding. The work in the Chowdhury laboratory is supported by the National Institutes of Health (NIH) ROI CA208044. The work in the Boulton laboratory is supported by the Francis Crick Institute, which receives its core funding from Cancer Research UK (FC0010048), the UK Medical Research Council (FC0010048), and the Wellcome Trust (FC0010048); a European Research Council (ERC) Advanced Investigator Grant (TelMetab), and a Wellcome Trust Senior Investigator Grant. The Cesare laboratory is supported by National Health and Medical Research Council of Australia (1106241) and the Cancer Institute NSW (11/FRL/5-02).

Author contributions S.J.B. and G.S. conceived the study; G.S., P.K., P.R., D.V.L., A.J.C. and S.J.B. designed experiments; G.S., P.K., P.R., D.V.L., P.M., V.B., X.-F.Z. and H.R.F. conducted experiments; G.S., P.K., P.R., D.V.L., P.M., X.-F.Z., H.R.F., A.P.S., D.C., A.J.C. and S.J.B. analysed data; S.J.B., G.S. and A.J.C. wrote the manuscript with editorial help from P.K., P.R., P.M. and D.V.L.

Competing interests The authors declare no competing interests.

Additional information Supplementary information is available for this paper at https://doi.org/10.1038/s41586-019-1744-8.

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Peer review information Nature thanks Eric Gilson, Joachim Lingner and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Extended Data Fig. 1 | TRF2 is phosphorylated at Ser365. a, Annotated spectrum for the TRF2 phosphorylated peptide. The data were acquired on the LTQ Orbitrap Velos and processed in MaxQuant v.1.3.0.5 with the database search performed against the canonical sequences Homo sapiens from UniProt. For the spectrum shown, the posterior error probability value was 0.018258 and the localization score for the site was DLVLPTQALPAS(1)PALK.
b, HEK 293 cells were released from a double-thymidine block (left) or a thymidine plus nocodazole block (right). Cells were subjected to SDS–PAGE analysis and progression through the cell cycle was monitored by immunoblotting with cell cycle markers as indicated. Asterisks indicate time points after synchronization. c, PCR analysis of genomic DNA isolated from Trf2F/− MEFs stably expressing empty vector, wild-type or mutant TRF2, 96 h after infection with control or Cre-expressing adenovirus. d, Western blotting analysis of the cells described in c to monitor loss of endogenous TRF2 after Cre expression and to determine complementation efficiency with ectopic wild-type and mutant TRF2. The asterisk indicates endogenous TRF2. In a–d, the experiments were independently repeated at least twice with similar results.
Extended Data Fig. 2 | Mutations of TRF2 at the Ser365 or Ser367 phosphosite do not affect interaction with shelterin components. a, Whole-cell extracts from HEK 293 cells stably expressing empty vector, wild-type or mutant Myc-tagged TRF2 as indicated were immunoprecipitated with anti-Myc antibody or normal mouse IgG. Protein complexes were analysed with antibodies against RAP1, TRF1 and Myc. b, Trf2F/F MEFs expressing wild-type or phospho-dead (Ser367Ala mutant) TRF2 were transfected with either control siRNA (non-targeting control, NTC) or siRNA against RAP1 (siRAP1) and treated with 4-OHT for 96 h. Whole-cell extracts were analysed 72 h later as indicated. c, Quantification (left) and representative images (right) of chromosome fusions in the Trf2F/F MEFs depicted in b performed 96 h after 4-OHT treatment (n = 30 metaphases analysed). Data are mean ± s.e.m. P values were determined by one-way ANOVA. In a–d, the experiments were independently repeated at least twice with similar results.
Extended Data Fig. 3 | Inhibition of MEK–ERK signalling pathway does not affect TRF2 phosphorylation at Ser365 or Ser367. a, Quantity screen for TRF2-biotinylated peptides. Slot-blot assay in which biotin-tagged TRF2 peptides were incubated with streptavidin-coated beads to ensure that the correct amounts were used in the peptide pull-down assay. b, HEK 293 cells (left) or Rtel1 F/F MEFs (right) were pre-treated with vehicle control (DMSO) or with 25 µM of MEK1/2 kinase inhibitor (U0126) for 48 h. Whole-cell extracts were subjected to SDS–PAGE analysis followed by immunoblotting with antibodies as indicated. In a and b, the experiments were independently repeated at least twice with similar results.
Extended Data Fig. 4 | Identification of TRF2- and RTEL1-interacting phosphatases and protein phosphatase regulatory subunits. a, Intensity-based absolute quantification (iBAQ) scatter plots comparing protein abundance in cells synchronized during S phase versus asynchronous control cells. Immunoprecipitates from asynchronous or S-phase-synchronized HEK 293 cells stably expressing Flag–haemagglutinin (HA)-tagged RTEL1 (top), N-terminal FLAP (Flag–GFP)-tagged RTEL1 (middle) or Myc–TRF2 (bottom) were separated by SDS–PAGE and stained with Coomassie blue to visualize proteins. Immunoprecipitations with haemagglutinin (top), GFP (middle) and Myc (bottom) antibodies were performed. The proteins along the entire length of the gel were extracted and analysed by liquid chromatography–tandem mass spectrometry (LC/MS–MS). b, HEK 293 cells stably expressing wild-type Myc–TRF2 were transfected with either non-target control or siRNA against protein phosphatase regulatory subunits, as specified. Three days later, protein levels were analysed with the indicated antibodies. c, FLAP-tagged RTEL1 HEK 293 cells expressing Myc-tagged wild-type TRF2 were transfected with either control siRNA or siRNA against PP4R2 or PP6R3. Whole-cell extracts were immunoprecipitated with anti-Flag antibody and immuno complexes were analysed for Myc (TRF2) and Flag (RTEL1). Inputs (5%) are shown on the right. d, HEK 293 cells expressing wild-type Myc–TRF2 (left) or Flag–HA-tagged RTEL1 (right) were subjected to immunoprecipitation with normal rabbit IgG or antibodies against PP4R2 and PP6R3. Immune complexes were analysed by western blotting with the indicated antibodies. In b–d, the experiments were independently repeated at least twice with similar results.
Extended Data Fig. 5 | PP6R3 controls phosphorylation of TRF2 at Ser365 or Ser367. HEK 293 cells expressing wild-type Myc–TRF2 were transfected with a non-targeting control siRNA or siRNAs against protein phosphatase regulatory subunits (a) or catalytic subunits (b). Cells were collected, and whole-cell extracts were immunoprecipitated with anti-RTEL1 antibody. Immunocomplexes were resolved by SDS–PAGE and analysed by western blotting as indicated. c, HEK 293 cells (c) and Trf2−/− MEFs (d) expressing Myc-tagged wild-type TRF2 were transfected with control siRNA or siRNA targeting PP4R2 or PP6R3 (Pp4r2 or Pp6r3 for MEFs). Whole-cell extracts were immunoprecipitated with anti-TRF2 antibody, and immunocomplexes were resolved by SDS–PAGE and analysed for human phospho-TRF2 (pS365 TRF2; left panel in c) or mouse phospho-TRF2 (pS367 TRF2; left panel in d). e, Top, frequency of telomere loss and telomere fragility per metaphase in Rtel1 F/F MEFs transfected with control siRNA or with Pp4r2 or Pp6r3 siRNA (n = 58 (NTC), n = 57 (Pp4r2), and n = 55 (Pp6r3) of analysed metaphases). Efficiency of siRNA knockdown was determined by western blotting with PP6R3 and PP4R2 antibodies as indicated. Data are mean ± s.e.m. P values determined by one-way ANOVA. Bottom, representative images of the telomere FISH experiments. The arrowheads show loss of telomere signal. Red, telomere PNA FISH; blue, DAPI. f, Phi29-dependent telomere circles (top) detected in cells as indicated in e. The extent of [32P] incorporation was quantified (bottom) from the autoradiographs, and the level of [32P] incorporation by cells transfected with control siRNA was arbitrarily assigned a value of 100%. Data are mean ± s.d. and from two independent experiments. P values determined by one-way ANOVA.

In a–f, the experiments were independently repeated at least twice with similar results.
Extended Data Fig. 6 | Replication defects in Trf2−/− MEFs in the absence of TRF2 phosphorylation at Ser365 or Ser367. a, Quantification of global replication fork dynamics (left) and rates of replication fork progression (right) of the IdU/CldU double pulse-labelling experiment in Trf2−/− MEFs complemented with empty vector, wild-type or mutant TRF2, performed 96 h after infection with control- or Cre-expressing adenovirus (n denotes number of analysed forks). Data are mean ± s.e.m. of triplicate experiments. Box plots are as in Fig. 3e. b, Representative images of the experiment from a. c–e, Quantification of micronuclei (c; 500 nuclei per replicate), mitotic catastrophe (d; 500 nuclei per replicate), and 53BP1 foci frequency (e; 150 nuclei per replicate) in Trf2−/− MEFs complemented as in a. Data are mean ± s.e.m. of three (c, d) or two (e) independent experiments. f, DNA damage in Trf2−/− MEFs complemented as in a was estimated by counting the frequency of cells with five or more 53BP1 foci. For each independent experiment (n = 2), a minimum of 150 nuclei of each condition were analysed. All P values were determined by one-way ANOVA.
Extended Data Fig. 7 | Suppression of constitutive binding of RTEL1 to the TRF2(S367A) phospho-dead mutant rescues replication defects in MEFs. 

a, Quantification of rates of replication fork progression (left) and representative images (right) of the IdU/CldU double pulse-labelling experiment in double-knockout Trf2F/F;Rtel1F/F mouse ear fibroblasts stably expressing Myc–TRF2(S367A), together with wild-type V5–RTEL1 (WT) or C4C4 mutant V5–RTEL1(R1237H) (R/H), performed 96 h after infection with Cre-expressing adenovirus. Data are mean ± s.e.m. of triplicate experiments.

b, Quantification of replication fork dynamics (top) and fork asymmetry (bottom) from cells as in a. Staining with anti-ssDNA antibody (right) was used to exclude broken DNA tracks (n denotes number of analysed forks). Box plots are as in Fig. 3e. Data are mean ± s.e.m.

c–e, Quantification of the frequency of micronuclei (c; 500 nuclei per replicate), mitotic catastrophe (d; 500 nuclei per replicate), and 53BP1 foci (e; 150 nuclei per replicate) in Trf2F/F;Rtel1F/F mouse ear fibroblasts complemented as indicated in a. Data are mean ± s.e.m. of three (c, d) or two (e) independent experiments.

f, DNA damage in Trf2F/F;Rtel1F/F mouse ear fibroblasts complemented as in a was estimated by counting the frequency of cells with five or more 53BP1 foci. For each independent experiment (n = 2), a minimum of 150 nuclei of each condition were analysed. All P values were determined by one-way ANOVA.
Extended Data Fig. 8 | TRF2(S367A) mutation induces TIFs and impairs formation of t-loops. **a**, Quantification (top) of the cross-linking efficiency test (bottom) in the Trf2<sup>F/F</sup> MEFs stably expressing wild-type or S367A mutant Myc–TRF2 120 h after treatment with 4-OHT (n = 3 independent biological replicates). Data are mean ± s.e.m. **b**, Left, quantification of TIFs per interphase in Trf2<sup>F/F</sup> MEFs complemented with empty vector, wild-type Myc–TRF2, phospho-dead mutant TRF2(S367A), or phospho-mimetic Myc–TRF2(S367D) and Myc–TRF2(S367E) mutants 96 h after infection with Cre-expressing adenovirus. Right, representative interphase TIF images. **c**, Quantification of TIFs per interphase in Trf2<sup>F/F;Rtel1<sup>F/F</sup></sup> mouse ear fibroblasts stably expressing Myc–TRF2(S367A) together with wild-type V5–RTEL1 or mutant V5–RTEL1(R1237H) 96 h after infection with GFP- or Cre-expressing adenovirus. Analysis was carried out 96 h after infection with Cre-expressing adenovirus. Data in **b**–**d** are mean ± s.d. from three independent experiments (n = 100 cells in each treatment group analysed per independent experiment). **e**, Quantification of linear and t-loop molecules shown in Fig. 4c (n = 3 biological replicates scoring ≥1,192 molecules per replicate). T-loop measurements are a sum of the loop and tail portions of the molecule. Data are mean ± s.e.m. **f**, Measurement of the loop portion of t-loops from the experiments depicted in Fig. 4c (n = 3 biological replicates scoring ≥1,192 molecules per replicate). All P values were determined by one-way ANOVA.
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