## Experimental design

1. **Sample size**
   - Describe how sample size was determined.

   Sample sizes were chosen according to commonly used and accepted standards in the field. No statistical method was used to pre-determine sizes. The numbers of independent experiments (n) are indicated in the Methods section and in figure legends.

2. **Data exclusions**
   - Describe any data exclusions.

   No data were excluded from any analysis.

3. **Replication**
   - Describe whether the experimental findings were reliably reproduced.

   All experiments were reliably reproduced.

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.

   No randomization was used.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   No blinding was used for data analysis.

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**
   - For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

<table>
<thead>
<tr>
<th>n/a</th>
<th>Confirmed</th>
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</thead>
<tbody>
<tr>
<td>☒</td>
<td>✓ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)</td>
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<tr>
<td>✓</td>
<td>✓ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly</td>
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<tr>
<td>✓</td>
<td>✓ A statement indicating how many times each experiment was replicated</td>
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<tr>
<td>✓</td>
<td>✓ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)</td>
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<tr>
<td>✓</td>
<td>✓ A description of any assumptions or corrections, such as an adjustment for multiple comparisons</td>
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<tr>
<td>✓</td>
<td>✓ The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted</td>
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<tr>
<td>✓</td>
<td>✓ A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)</td>
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<tr>
<td>✓</td>
<td>✓ Clearly defined error bars</td>
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</table>

See the web collection on statistics for biologists for further resources and guidance.
7. Software

Describe the software used to analyze the data in this study.

The following software were used in this study: MetaMorph, DeltaVision SoftWoRx, Zen (Zeiss) for microscopy; ImageJ and Adobe Photoshop for image processing; Microsoft Excel and Graph Pad Prism for graph plotting and statistical analysis; Typhoon FLA 7000 Control Software and AlphaView (FluorChem HD2) for acquirement of radioactive and chemiluminescent signals, respectively.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All the materials are available upon request. Materials purchased from companies are reported in the Methods section.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Western blots were performed using a mouse monoclonal anti-TRF2 (Millipore, 05-521), a rabbit polyclonal anti-TRF1 raised against full-length recombinant TRF1 (kind gift from Jan Karlseder), a rabbit polyclonal anti-Tankyrase 1 (kind gift from Susan Smith), a rabbit polyclonal anti-RNaseH1 (GeneTex, GTX117624), a mouse monoclonal anti-hbetaActin (Abcam, ab8224), and a rabbit polyclonal anti-LaminA (GeneTex, GTX11677S). Secondary antibodies were HRP-conjugated goat anti-mouse and anti-rabbit IgGs (Bethyl Laboratories). The specificity of anti-TRF2, anti-TRF1, anti-Tankyrase 1, and anti-RNaseH1 antibodies was confirmed by western blot analysis of siRNA-depleted cells.

Indirect immunofluorescence experiments were performed using a mouse monoclonal anti-Flag (Sigma-Aldrich, F1804), a mouse monoclonal anti-Myc (Sigma-Aldrich M4439), a rabbit polyclonal anti-pSer33, (Bethyl Laboratories A300-244A), a mouse monoclonal anti-TRF2 (Millipore, 05-521), and a mouse monoclonal mix raised against a recombinant peptide spanning the A domain of hTRF1 (raised at the monoclonal antibody facility of Max F. Perutz Laboratories in Vienna, 1:100). Secondary antibodies were donkey anti-rabbit and donkey anti-mouse IgGs conjugated with Alexa Fluor 568 or Alexa Fluor 488 (Invitrogen). The specificity of anti-Flag, anti-Myc, anti-TRF2 and anti-TRF1 A domain antibodies was confirmed by indirect immunofluorescence staining combined with FISH for telomeric DNA of cells over-expressing Flag- and Myc-tagged TRF proteins. The specificity of the anti-pSer33 antibody was confirmed by indirect immunofluorescence staining of cells treated with camptothecin or hydroxyurea. DRIP experiments were performed using the mouse monoclonal S9.6 antibody (kind gift from Stephen Leppla). The specificity of the S9.6 antibody was confirmed by dot-blot analysis of total nucleic acids from different cell lines treated or not with RNaseH.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

We used HeLa telomerase positive cervical cancer cells (ATCC) and U2OS osteosarcoma cells (kind gift from Joachim Lingner).

b. Describe the method of cell line authentication used.

Telomere length and ALT-associated promyelocytic leukaemia body (APB) analyses.

c. Report whether the cell lines were tested for mycoplasma contamination.

Cells were tested for mycoplasma contamination and they were negative.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

The two cell lines are not listed as commonly misidentified (NCBI Biosample).
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Not applicable

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

Not applicable