The TEL patch of telomere protein TPP1 mediates telomerase recruitment and processivity

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Human chromosome ends are capped by shelterin, a protein complex that protects the natural ends from being recognized as sites of DNA damage and also regulates the telomere-replicating enzyme, telomerase1–3. Shelterin includes the heterodimeric POT1–TPP1 protein, which binds the telomeric single-stranded DNA tail4–9. TPP1 has been implicated both in recruiting telomerase to telomeres and in stimulating telomerase processivity (the addition of multiple DNA repeats after a single primer-binding event)10–14. Determining the mechanisms of these activities has been difficult, especially because genetic perturbations also tend to affect the essential chromosome end-protection function of TPP1 (refs 15–17). Here we identify separation-of-function mutants of human TPP1 that retain full telomere-capping function in vitro and in vivo, yet are defective in binding human shelterin. The seven separation-of-function mutations map to a patch of amino acids on the surface of TPP1, the TEL patch, that both recruits telomerase to telomeres and promotes high-processivity DNA synthesis, indicating that these two activities are manifestations of the same molecular interaction. Given that the interaction between telomerase and TPP1 is required for telomerase function in vivo, the TEL patch of TPP1 provides a new target for anticancer drug development.

Genetic analysis of TPP1 regulation of telomerase is complicated by the potential of TPP1 mutations to uncap telomeres, which can give unnatural telomerase hyperextension or induce a DNA-damage response. Our search for separation-of-function mutants of TPP1 that would affect only its telomerase interactions was conducted on the TPP1 OB domain (TPP1(OB)), which is separate from its POT1 single-stranded DNA (ssDNA)-interaction domain (Fig. 1a) and previously implicated in telomerase interaction13,14. We engineered 12 single amino-acid mutants and two double mutants of TPP1(OB), choosing residues that are both conserved among mammalian TPP1 proteins and reside on the protein surface9 (Supplementary Fig. 1a, b). All mutant and wild-type proteins were expressed recombinantly in *Escherichia coli* and purified (Supplementary Fig. 1c; see Methods).

The POT1–TPP1 heterodimer has a higher affinity for telomeric ssDNA than does POT1 alone4, which is consistent with the role of this complex in protecting single-stranded telomeric DNA from the DNA-damage response machinery15. Quantitative DNA-binding experiments demonstrated that all the mutant proteins, like wild-type TPP1, were able to enhance POT1–DNA affinity (Fig. 1b and Supplementary Fig. 2a). Furthermore, all TPP1 mutants formed stable POT1–TPP1–ssDNA ternary complexes in electrophoretic mobility shift assays (Supplementary Fig. 2b). These results suggest that the TPP1(OB) surface mutations do not disrupt the overall structure or the DNA-end protection function of TPP1.

Addition of purified POT1–TPP1 to telomerase enzyme activity assays enhances telomerase processivity 2–3-fold in vitro9. We developed a new direct telomerase assay in which TERT, TR, TPP1 and POT1 were reconstitutively co-expressed in human cells. Lysates from these cells were used in direct telomerase activity assays involving extension of primer a5 (TTAGGTTAGCGTGTTAGG; the underlined G-to-C mutation ensured positioning of the POT1–TPP1 complex at the 5′ portion of the DNA primer, thereby providing a homogenous substrate for telomerase extension9). The 14 TPP1 mutants varied substantially in their stimulation of processivity; some such as S106A, S111A and R175V, resulted in long extension products indicative of high processivity, similar to wild-type TPP1, but others, such as E169A/E171A and L212A, resulted in low-processivity patterns similar to that with no TPP1 added (Fig. 1c).

Quantification of data (Fig. 1d) showed that several TPP1 mutants gave significantly decreased processivity (*P < 0.005). Western blot analysis showed that TERT, POT1 and TPP1 proteins were expressed at similar levels across all transfections (Fig. 1e). Note that processivity, unlike activity, is not influenced by enzyme concentration.

As an independent test of processivity, lysates from HEK 293T cells transiently overexpressing telomerase were supplemented with purified POT1 and TPP1 proteins before the telomerase assay, as described previously10,11,18 (Supplementary Fig. 3; see also alternative quantification of processivity in Supplementary Fig. 4). This traditional assay gave results in agreement with the new co-transfection method. We also analysed the 14 TPP1 mutants using a primer with a purely telomeric sequence and a different sequence permutation (GGT TAGGGTTAG GTTAGG); we obtained essentially the same results as with primer a5 (Supplementary Fig. 5), verifying that the TPP1 mutant phenotype was not primer specific. All three assays identified the same separation-of-function mutants of TPP1 (L104A, D166A/E168A, E169A/E171A, R180A, L183A and L212A), with E215A and R208V being intermediate and the other mutants having wild-type activity. Further dissection of the two double mutants into four single mutants revealed that E168, E169 and E171, but not D166, were important for telomerase stimulation (Supplementary Fig. 3). Most of the TPP1 mutants that were defective in processivity also gave a statistically significant decrease in activity (Supplementary Fig. 3b). In agreement with previous results9, the stimulation of activity (and its dependence on specific TPP1 residues) was observed even in the absence of added POT1 protein (Supplementary Fig. 6).

The effects of TPP1 mutations might be explained by disruption of a TPP1–telomerase interaction. To test this directly, we developed a co-immunoprecipitation assay involving co-transfection of Flag–TPP1 and untagged telomerase (TERT plus TR) in HeLa-EM2-11ht cells. Inspection and quantification by immunoblot of proteins co-immunoprecipitated with anti-Flag beads shows that TERT, but not actin, was associated with Flag–TPP1 (Fig. 2a, compare lanes 1 and 2). More TERT protein was co-precipitated with Flag–TPP1 when POT1, the binding partner of TPP1, was also present (compare lanes 1 and 2). The addition of primer a5 (a ligand for POT1–TPP1 and a substrate for telomerase extension9) also resulted in a statistically significant decrease in activity (Supplementary Fig. 4).

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TEN domain of TERT, which is consistent with the implication of telomerase with TPP1 involves contacts both within and outside the ref. 21). These data indicate that the observed immunoprecipitation did not abolish telomerase binding (Supplementary Fig. 7c and 7d). However, deletion of the TEN domain or mutating G100V (ref. 19) did not pull down telomerase even in the presence of a5 DNA (lane 5). The enhanced interaction between TPP1 and telomerase was reduced when either TR or TERT was omitted from co-transfections (Supplementary Fig. 2a for dissociation constant ($K_d$) values. WT, wild type. c. Direct telomerase activity assay with primer a5 of lysates from cells co-transfected with a TR plasmid and Flag-tagged POT1, TPP1 and TERT plasmids. 'No POT1–TPP1' indicates transfection without POT1 and TPP1. The number of telomeric repeats added to primer are indicated along the left. d. Processive extension (>15 repeats/total) with TPP1 mutants relative to that with wild-type TPP1 (green line) obtained from three independent sets of experiments (as in panel c); error bars indicate s.d. Stimulation of processivity is assessed relative to the 'no POT1–TPP1' negative control (red line). Two-tailed student’s t-test with respect to R175V: *P < 0.02, **P < 0.005. Red labels indicate significantly defective; green labels indicate not significantly defective. e. Immunoblot of lysates used in panel c probed with anti-Flag antibody–HRP conjugate shows uniform expression.

$\sim$2.5-fold compared to the basal interaction in the absence of POT1 and a5 DNA (compare lanes 2 and 6). The enhanced interaction between telomerase and POT1–TPP1 is unlikely to be occurring indirectly via bridging of TPP1 and telomerase by POT1–DNA, because Flag–POT1 did not pull down telomerase even in the presence of a5 DNA (lane 5).

The interaction between TPP1 and telomerase was reduced when either TR or TERT was omitted from co-transfections (Supplementary Fig. 7a, b). These results indicate that TPP1 interacts with telomerase optimally in the context of the fully assembled RNP. The TEN (telomerase essential N-terminal) domain of TERT has been implicated in a functional interaction with POT1–TPP1 and in binding to TPP1(OB). However, deletion of the TEN domain or mutating G100V (ref. 19) did not abolish telomerase binding (Supplementary Fig. 7c and ref. 21). These data indicate that the observed immunoprecipitation of telomerase with TPP1 involves contacts both within and outside the TEN domain of TERT, which is consistent with the implication of C-terminal TERT residues in TPP1(OB)-mediated recruitment of telomerase to telomeres.

When the TPP1 mutants were tested for binding telomerase (Fig. 2b, c), there was strong correlation with the telomerase stimulation results. Five of the six mutants that showed a defect in telomerase stimulation in vitro also showed defects in telomerase association in the presence of POT1 and DNA primer, and the intermediate mutant E215A was also defective in binding. An exception was mutant L104A, which showed wild-type levels of associated telomerase.

When the separation-of-function mutations that fail to stimulate processivity and also interfere with telomerase binding are mapped on the surface of the TPP1(OB) structure, the seven critical amino acids (E168, E169, E171, R180, L183, L212 and E215) cluster to reveal a surface the TEL patch (TPP1 glutamate (E) and leucine (L)-rich patch). The fact that the TEL patch promotes both processivity and
telomerase binding fulfills a prediction of a previous model for telomerase processivity\textsuperscript{31}. The ‘back face’ of TPP1(OB) (bottom view of Fig. 2d) contains amino acids not involved in binding telomerase.

Previously, knockdown of TPP1 in human HTC75 cells resulted in telomere lengthening\textsuperscript{13}, whereas skin keratinocytes from TIFs\textsuperscript{13} displayed telomere shortening\textsuperscript{12}. However, loss of both telomere lengthening\textsuperscript{7, whereas skin keratinocytes from TIFs\textsuperscript{13}. However, expression of the TPP1 mutants resulted in the genous TPP1 (Supplementary Figs 9a, b and 10a, b). Proteins were ectopically expressed without knocking down the endo-

Figure 2 | TPP1 mutations that disrupt telomerase stimulation also disrupt telomerase binding. a, Pull down of transiently expressed Flag–TPP1, Flag–POT1 and associated untagged TERT from HeLa-EM2-11ht lysates on anti-Flag conjugated beads. ‘Input’ indicates immunoblot of soluble cellular lysates before incubation with beads; ‘beads’ indicates immunoblot of proteins retained on antibody beads after 2 h incubation at 4 °C and washing. The TERT signal was plotted after correction for loading differences (using the actin signal). b, Comparison of TERT pull down by Flag–TPP1 mutants in the presence of Flag–POT1 and primer a5. Control 1, Flag–POT1/TPP1 and primer a5 omitted; control 2, Flag–TPP1 (WT) present, Flag–POT1 and primer omitted; control 3, Flag–POT1 and primer present, Flag–TPP1 omitted. c, The mean TERT signal on beads obtained from quantification of three independent sets of experiments of which panel b is representative; error bars indicate s.d. Two-tailed Student’s t-test with respect to R175V: *P < 0.05, **P < 0.01. The red label indicates significantly defective; green label indicates not significantly defective. d, Structure showing the TEL patch (amino acids in orange), the surface of the OB domain of TPP1 that mediates telomerase association and stimulation. Other amino acids mutated in this study are shown in cyan.
Figure 3 | TPP1 TEL-patch mutants fail to stimulate telomere lengthening in human cells. a, Engineering HeLa-EM2-11ht stable cell lines containing single-copy integration of bidirectional Tet-inducible shTPP1 and shRNA-resistant Flag–TPP1* (wild type or mutants) genes. b, A stable cell line encoding shTPP1 (no exogenous Flag–TPP1) shows doxycycline-dependent knockdown of endogenous TPP1 protein. Nonspecific bands serve as loading controls. c, Quantitative RT–PCR showing knockdown of endogenous TPP1 mRNA in the indicated cell lines ± s.d. (n = 3). d, Western blot showing similar protein levels of shRNA-resistant Flag–TPP1 in the indicated cell lines also expressing shTPP1. e, Telomeric restriction fragment (TRF) Southern blot of DNA from HeLa-EM2-11ht (untransfected HeLa) and stable cell lines expressing shTPP1 and the indicated TPP1 constructs at the indicated population doublings (PD). DNA length standards are indicated along the left. f, Change in mean telomere length for data shown in panel e was plotted against population doublings. The vertical bars at the right indicate the distinct ranges of telomere length attained by wild-type* and R175V* versus E169A/E171A* and L212A* cells after 81 population doublings.

Figure 4 | Failure to stimulate telomere lengthening correlates with inability to recruit telomerase to telomeres. a, Fluorescence in situ hybridization (FISH) detects TR (green) and immunofluorescence (IF) detects the indicated Flag–TPP1 proteins (red). In the merge panels, yellow spots indicate recruitment of telomerase to telomeres. b, Quantification of telomerase recruitment data of which panel a is representative. The average percentage of Flag–TPP1 foci containing TR (y axis) and standard deviations (error bars) of 15 fields of view (40–120 cells total) were plotted for the indicated stable cell lines. c, IF–FISH experiment showing that telomerase (TR in green) in E169A/E171A* and L212A* cells fails to be recruited to telomeres (Flag–TPP1 in red) and instead remains localized in Cajal bodies (collin in blue).
In the simplest model, the TEL patch binds telomerase directly. If there were a bridging molecule, it would probably be an abundant cellular component, because TPP1–telomerase binding is robust under conditions of TPP1 and telomerase overexpression, where any telomerase-specific factors would be substoichiometric. There is precedence for such a bridging molecule in Schizosaccharomyces pombe, where Ccql physically connects Tp1 (the S. pombe TPP1) to telomeraser22–24. However, there is no identifiable homologue of Ccql in humans (see Supplementary Discussion for comparison with telomerase recruitment in budding yeast).

The substantial reduction of telomerase recruitment by subtle mutations in the TEL patch of TPP1 indicates the potential for novel strategies to inhibit telomerase for cancer therapy. By targeting the TEL patch, instead of telomerase itself, a compound could potentially inhibit telomerase action only at telomeres without interfering with other hTERT functions25,26. Furthermore, such an inhibitor should not perturb genomic stability in normal cells lacking telomerase, because the TEL patch is physically and functionally separate from the portion of TPP1 engaged in chromosome end-protection.

METHODS SUMMARY
All experiments involving recombinant TPP1 expressed in bacteria9 used TPP1(N) (amino acids 87–334), which recapitulates all in vitro functions of the full-length human protein. Experiments involving TPP1 expressed in human cells used full-length TPP1 that we define as starting from amino acid Met 87 (refs 9, 14) (Fig. 1a). Telomerase was obtained from HEK 293T cells transfected with human TERT and TR plasmids or from HeLa-EM2-11ht cells transfected with Flag-TERT, TR, Flag-POT1 and Flag-TPP1 plasmids. Cell lines were engineered by integrating gene cassettes containing Tet-inducible TPP1 genes into a unique targeting site in the genome of the HeLa-EM2-11ht cell line (Tet Systems Holdings GmbH & Co. KG) using Flp recombinase-mediated cassette exchange. Where indicated, the integration cassette contained a bi-directional Tet-inducible promoter driving both shTPP1 and shRNA-resistant TPP1 genes in opposite directions. FISH and IF experiments were performed by modifying existing protocols27 (http://delanegalab.rockefeller.edu/protocols.html).

Full Methods and any associated references are available in the online version of the paper.
METHODS

All experiments involving recombinant TPP1 expressed in bacteria^9,10 used TPP1(N) (amino acids 87–334), which recapitulates all in vitro functions of the full-length human protein^9. Experiments involving TPP1 expressed in human cells used full-length TPPI constructs starting from amino acid Met87 (refs 9, 14) (Fig. 1a).

Oligonucleotides. Synthetic oligonucleotides used in gel-shift, filter-binding and telomerase activity assays were purchased from Integrated DNA Technologies and resuspended in 10 mM Tris–Cl (pH 8.0) to obtain 50 μM stocks that were stored at −20 °C and diluted to required concentrations immediately before use.

Plasmid constructs for cell-based assays. Mammalian cell expression plasmids encoding human TERT (pTERT-cDNA6/Myc-His C^9) and human TR (pHTR-miR1) vectors (replacing the existing d1GFP gene in the vector) that encoded human TERT (pTERT-cDNA6/Myc-His C^9) and human TR (pHTR-miR1) vectors (replacing the existing d1GFP gene in the vector) were used as the template in the PCR, whereas for expression in the HeLa-EM2-11ht cell line, p3×-Flag-TPP1-B14 was used as the template in the PCR. The TPP1 genes in the mutant plasmids were sequenced completely to verify the presence of the intended mutation(s) and exclude the acquisition of unwanted mutations during PCR amplification and cloning.

Protein purification. Purified mutant and wild-type Smt3–TPP1(N) fusion proteins were lysed from soluble lysates of bacterial Smt3–TPP1(N) expressing cells and purified by nickel-agarose chromatography, treatment with Ulp1 protease to cleave the Smt3 tag^9 and size-exclusion chromatography^2. Purified recombinant human POT1 protein was obtained from baculovirus-infected insect cells as described previously^2.

Gel-shift and filter-binding assays. Electrophoretic mobility shift assays and filter-binding assays of POT1–oligonucleotide complexes were performed exactly as described previously^2.

Cell culture. All human cell cultures were cultured at 37 °C with 5% CO2. HEK 293T cells were cultured in growth medium containing Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamax (Life Technologies), 100 units ml^−1 penicillin and 100 μg ml^−1 streptomycin. Doxycycline was added to a final concentration of 200 ng ml^−1 for induction of pTet-driven genes.

Telomerase preparations. Telomerase preparations from HEK 293T cell extracts (super-telomerase) were obtained from transient transfection of TERT and TR using a published protocol^11. For telomerase preparations from HeLa-EM2-11ht cells, 300,000 cells per well of a 6-well plate were seeded and transfected 42 h later using Lipofectamine 2000 (Invitrogen) and indicated plasmid DNA using the manufacturer’s recommendation. 1 μg p3×-Flag-TERT-cDNA6/Myc-His C and 3 μg pHTR-Bluescript II SK(+)^10 were added per transfection. For transfections involving POT1 or TPP1, 1 μg of p3×-Flag-POT1-B14 or p3×-Flag-TPP1-B14 was added per transfection. In control transfections where POT1 or TPP1 were omitted, pTet-B14^9 (empty vector) was included. Medium was removed after 5 h and exchanged with fresh medium containing 200 ng ml^−1 doxycycline to induce TPP1 and/or POT1 expression from the tetracycline-driven promoter. After 48 h of transfection, the cells were trypsinized, washed with phosphate buffered saline (PBS), re-suspended in 100 μl CHAPS lysing buffer^11 containing 1 μl of RNasin plus (Promega), mixed with rocking on a nutator at 4 °C for 20 min, and centrifuged (13,600 r.p.m., 10 min) to remove cell debris. Aliquots of the soluble cell lysates were flash frozen in liquid nitrogen and stored at −80 °C.

Telomerase activity assays. Telomerase reactions were carried out as 20 μl reactions containing: 50 mM Tris–Cl (pH 8.0), 30 mM KCl, 1 mM MgCl2, 1 mM spermidine, 5 mM β-mercaptoethanol, the indicated concentration of primer a5 or b^9, 500 μM dATP, 500 μM dTTP, 2.92 μM unlabeled dGTP, 0.33 μM radiolabeled dGTP (3,000 Ci mmol^−1), and 3 μl of super-telomerase cell extract (or telomerase from HeLa-EM2-11ht cell extracts) at 30 °C for 30 min. Reactions were stopped with buffers containing 100 μM of 3.6 mM ammonium acetate, 20 μg of glycerol and a 5° C end-label signal (7.5 pmol μl^−1) 18mer oligonucleotide loading control, and precipitated with ethanol. The pellets were re-suspended in 10 μl H2O and 10 μl of loading buffer (95% formamide, 5% H2O, loading dye), heated at 95 °C for 10 min, and loaded onto a 10% acrylamide, 7 M urea, 1× TBE sequencing gel. Gels were run at 90 W for 1.75 h, dried, exposed to an image plate and imaged on a phosphorimager (Typhoon Trio). The data were analysed using Imagequant TL software and telomerase activities quantified as described previously^11. Processivity calculations were performed as described previously^11 (Supplementary Fig. 4) although it was not possible to include the higher molecular weight products on the gel in these calculations due to severe band overlap. To include the contributions of these higher molecular weight products which are the result of the highly processive action of telomerase (data not shown) in processivity measurements, we defined a ‘15+’ relative processivity term as the fraction of the total activity present in bands resulting from 15 (number chosen arbitrarily to define the lower limit of high processivity) or more telomeric repeats added by telomerase (Fig. 1d and Supplementary Fig. 3c), noting that the ‘15+’ method does not correct for the greater number of G nucleotides (three per repeat) in the upper bands compared to lower bands.

Co-immunoprecipitation experiments. The protocol was adapted from a previously published protocol^12. HeLa-EM2-11ht cells were seeded and transfected as stated above for telomerase preparations. After 24 h of transfection, cells were washed twice with PBS, dislodged with trypsin and harvested using 400 μl ice-cold lysis buffer (50 mM Tris–Cl (pH 7.4), 20% glycerol, 1 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 0.02% SDS, 1 mM dithiothreitol, 2 mM phenylmethylsulphonyl fluoride, complete protease inhibitor cocktail (Roche)) and kept on ice. After 5 min, 20 μl of 5 M NaCl was added and mixed. After another 5 min on ice, 420 μl of ice-cold
water was added and mixed before immediate centrifugation (13,600 × g, 10 min). Supernatants were collected and used directly for immunoprecipitation. Lysate (40 μl) added to 40 μl of 4 × LDS sample loading buffer (Invitrogen) was kept aside for analysis of ‘input’ samples. Anti-Flag (M2 affinity gel beads (50 μl); Sigma) pre-incubated with 100 mg ml⁻¹ of bovine serum albumin in PBS were added to lysate prepared from each well of a 6-well plate. Mixtures were mixed with rocking on a rotator for 2 h at 4 °C. Beads were washed three times with 1:1 diluted lysis buffer and proteins were eluted with 180 μl 1:1 diluted 4 × LDS sample loading buffer (Invitrogen) for analysis of ‘beads’ samples. All ‘input’ (10 μl) and ‘beads’ (15 μl) samples were heated at 90 °C for 12–14 min and analysed by SDS-PAGE and immunoblotting.

**Stable cell line generation using HeLa-EM2-11ht cells and p3X-Flag-TPP1-F3 plasmids.** Detailed protocols for stable transfection of HeLa-EM2-11ht cells are reported elsewhere. HeLa-EM2-11ht cells were co-transfected in a 6-well format using either Lipofectamine 2000 (Life Technologies) or TransIT (Mirus) using the manufacturer’s protocol with 1 μg each of the p3X-Flag-TPP1-F3 (containing wild type, E169A/E171A, R175V, or L212A mutants for experiments in Supplementary Fig. 8), and containing shTPP1 and shTPP1-resistant wild type, E169A/E171A, R175V, or L212A mutants for experiments in Fig. 3), and 1 μg of plasmid expressing Fpl recombinase and conferring puromycin resistance. Twelve hours post-transfection, transfected cells were selected for 36 h using 5 μg ml⁻¹ puromycin. Subsequently, fresh medium lacking puromycin but including 50–100 μM ganciclovir (Sigma-Aldrich) was added and negative selection was conducted for 10 days. Next, 12 individual clones were picked from each transfection and expanded. To verify the identity of the clone and distinguish it from false-positive clones that survived selection, a small aliquot of cells was induced by doxycycline and expanded. To verify the identity of the clone and distinguish it from false-positive clones that survived selection, a small aliquot of cells was induced with 200 ng ml⁻¹ doxycycline and observed under a fluorescence microscope (Supplementary Fig. 10a). Positive clones were selected based on fluorescence arising from their IRES-GFP locus downstream of the ORF (absent in Flag-tagged constructs) and continuing into the ORF was amplified, using the High Capacity cDNA reverse transcription kit (Applied Biosystems). A TRIzol reagent (Life Technologies). Total cDNA was prepared from total RNA and qPCR was performed with the iQ SYBR green Supermix (Biorad) using the UTR (absent in Flag-tagged constructs) and continuing into the ORF was amplified, using the High Capacity cDNA reverse transcription kit (Applied Biosystems). A TRIzol reagent (Life Technologies). Total cDNA was prepared from total RNA.

The DNA digests were run on a 0.8% Agarose-1% formaldehyde gel (10,000 c.p.m.) was run as a marker on a separate lane on the gel. Next, the gel was shaken in solution containing 0.5 M Tris-Cl and 1.5 M NaCl (15 min each) of shaking in solution containing 0.5 M NaOH and 1.5 M NaCl. The DNA samples were heated at 90 °C for 10 min. Supernatants were collected and used directly for immunoprecipitation. For TIF analysis using co-immunofluorescence (co-IF), 10,000 cells of HeLa-EM2-11ht-derived stable cell lines were seeded on coverslips in a 12-well culture plate containing growth medium adjusted to 200 ng ml⁻¹ doxycycline. After 96 h, medium was removed and all subsequent steps were performed at room temperature. Cells were washed once with PBS and fixed with 3% or 4% formaldehyde in PBS for 8 min. The fixative was removed and the cells washed three times with PBS. The cells were permeabilized with PBS containing 0.1% Triton X-100 (PBS-T) for 5 min and blocked in PBS-T containing nuclease-free 3% BSA for 30 min. Cells were incubated with mouse monoclonal anti-TRF2 (Imgenex; IMG-124A; 1:500) and rabbit polyclonal anti-53BP1 (Novus Biologicals; NB100-304; 1:1,000 dilution) in PBS-T containing nuclease-free 3% BSA for 1 h. The cells were then washed three times in PBS (5 min each), and incubated with Alexa Fluor 568-conjugated anti-mouse IgG (Life Technologies) and Alexa Fluor 647-conjugated anti-rabbit IgG (Life Technologies) diluted 1:500 in PBS-T containing nuclease-free 3% BSA for 30 min in the dark. The cells were then washed three times in PBS and the excess PBS was removed by blotting. The coverslips were mounted on microscope slides using Vectashield mounting medium with DAPI (Vector Laboratories), sealed using transparent nail polish and stored at 4 °C or −20 °C until the time of imaging. Detection of Flag–TPP1 and RAP1 by co-IF was done essentially as above, but with mouse monoclonal anti-Flag M2 (Sigma; F1804; 1:500) and rabbit polyclonal anti-RAP1 (Novus Biologicals; NB100-292; 1:500) primary antibodies.

For combined immunofluorescence–fluorescence in situ hybridization (IF–FISH) experiments, the IF was performed before FISH. Briefly, IF was performed as described above using the appropriate primary and secondary antibodies. After the final PBS wash, the cells were fixed again in 4% formaldehyde in PBS for 10 min at room temperature. The cells were washed twice with PBS and dehydrated by successive 5-min incubations in 70%, 95% and 100% ethanol. The ethanol was removed and the coverslips allowed to dry for 2 min. The cells were rehydrated in 50% formamide in 2 × SSC for 5 min. The coverslips were placed (with cells facing down) on a drop (~40 μl) of pre-hybridization solution containing 100 mg ml⁻¹ dextran sulphate, 0.125 mg ml⁻¹ E. coli tRNA, 1 mg ml⁻¹ nuclease-free BSA, 0.5 mg ml⁻¹ salmon sperm DNA, 1 mM vanadyl ribonucleoside complexes, and 50% formamide in 2 × SSC for 1 h at 37 °C in a humidified chamber. Hybridization solution was made by adding a mixture of three Cy5-conjugated TR probes (30 ng of each probe per coverslip) to the pre-hybridization solution and the cells were hybridized in this solution overnight at 37 °C in a dark humidified chamber. Next morning, the cells were washed twice in 50% formamide in 2 × SSC and twice in PBS, and the coverslips were then mounted on microscope slides using ProLong Gold Antifade Reagent (Life Technologies). After 24 h at room temperature in the dark, the coverslips were sealed using transparent nail polish and stored at 4 °C or −20 °C until the time of imaging. In addition to antibodies mentioned above, rabbit polyclonal anti-collin (Santa Cruz; sc-32860; 1:100) and Alexa Fluor 405-conjugated anti-mouse (and anti-rabbit) IgG antibodies (Life Technologies; 1:500) were used to stain and image Flag–TPP1 and Cajal bodies.

**Microscopy.** Imaging was performed using a Nikon TE2000-U inverted fluorescence microscope equipped with Photometrics Cascade II EM-CCD camera and Yokogawa Spinning disc Confocal (CSU-Xm2) (Nikon Instruments, Inc.) fluorescence microscope equipped with Photometrics Cascade II EM-CCD camera and Yokogawa Spinning disc Confocal (CSU-Xm2) (Nikon Instruments, Inc.) equipped with a 60× objective. Images were acquired using Metamorph software. Linear image adjustments were made when necessary using ImageJ and Adobe Photoshop and applied to all images in a given channel. The colours depicted in the figures do not necessarily correspond to the emission of the fluorescent labels, but are used to facilitate visualization/analysis of ‘merged’ signals from two or more channels. Representative cells are shown in all microscopy figure panels. Quantification of co-localizations was done manually and plots of numbers of co-localizations per cell (one focal plane) show data obtained from 15 or more fields of cells (40–120 cells in total) for each sample set processed in parallel on the same day.

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