Local enrichment of HP1alpha at telomeres alters their structure and regulation of telomere protection

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Enhanced telomere maintenance is evident in malignant cancers. While telomeres are thought to be inherently heterochromatic, detailed mechanisms of how epigenetic modifications impact telomere protection and structures are largely unknown in human cancers. Here we develop a molecular tethering approach to experimentally enrich heterochromatin protein HP1α specifically at telomeres. This results in increased deposition of H3K9me3 at cancer cell telomeres. Telomere extension by telomerase is attenuated, and damage-induced foci at telomeres are reduced, indicating augmentation of telomere stability. Super-resolution STORM imaging shows an unexpected increase in irregularity of telomeric structure. Telomere-tethered chromo shadow domain (CSD) mutant I165A of HP1α abrogates both the inhibition of telomere extension and the irregularity of telomeric structure, suggesting the involvement of at least one HP1α-ligand in mediating these effects. This work presents an approach to specifically manipulate the epigenetic status locally at telomeres to uncover insights into molecular mechanisms underlying telomere structural dynamics.
Telomere maintenance is indispensable for indefinite proliferation of cancer cells. Mammalian telomeres consist of tracts of hexameric DNA repeats (5'-TTAGGG-3') bound by protective nonhistone proteins in a complex called shelterin. Paradoxically, in spite of the nucleosome-disfavoring properties of telomeric repeats, mammalian telomeric DNA is also organized into closely packed nucleosomes. It is unknown how the resulting telomeric chromatin domain, consisting of the telomere nucleosomal chromatin plus shelterin complex, establishes a capping structure to maintain genome integrity. While functions associated with shelterin itself have been widely studied, molecular details of how this peculiar telomere chromatin impacts mammalian telomere maintenance remain largely unexplored.

Telomere chromatin is thought to be inherently condensed heterochromatin primarily based on findings in yeast, Drosophila, and mouse. In these organisms, establishment of telomeric and subtelomeric heterochromatin is crucial for chromosomal end protection. However, recent studies suggest that human and Arabidopsis telomere chromatin are relatively dynamically characterized by a mix of heterochromatic and euchromatic marks, as well as enrichments of histone modifications associated with active transcription. Besides canonical telomere capping, telomeric chromatin also regulates telomere position effect, telomere transcription, homologous recombination at telomeres, cellular differentiation, and nuclear reprogramming.

Roles for epigenetic regulation of telomere maintenance have been sought in many studies. Knockout of various histone modifying enzymes such as histone methyltransferases SUV39H1/2, SUV4-2OH11/2,10,17,21 result in defective telomere function, aberrantly increased telomere length, and chromosomal instability. Depletion of yeast histone methyltransferase Dot1 and its homolog in mouse (Dot1L), mammalian histone modifier ATRX and its chaperon DAXX, yeast histone deacetylases Sir2 and its orthologs in mouse (Sirt1) and human (Sirt6), result in a range of altered or defective telomere maintenance phenotypes. These include alterations in telomere length, recombination which characterizes alternative telomere lengthening, TPE, telomere transcription, DNA damage at the telomeres, or increased telomere fusion and premature senescence. However, in such knockout or knockdown studies, it is very difficult to interpret the molecular mechanisms underlying the dynamics of telomeric chromatin because they take place in settings of global genomic changes in chromatin and histone modifying enzymes. Therefore, we desired to set up an alternative approach to engineer localized manipulations of telomere chromatin.

A common feature of heterochromatin-mediated telomere protection in Drosophila and yeast is that their telomeric and subtelomeric chromatin marks are enriched in heterochromatin marks such as trimethylation of lysine 9 of histone H3 (H3K9me3) and H3K9me3. H3K9me3 provides a high affinity binding site for HP1 (heterochromatin protein 1) and recruits histone methyltransferase SUV39H1 to catalyze the propagation of this mark to establish heterochromatin. Extensive studies of heterochromatin marks, using chromatin immunoprecipitation (ChIP) and genome-wide chromatin state mapping, have reported enrichment of H3K9me3 and other heterochromatin marks in mouse subtelomere and telomeres. In striking contrast to this reported high H3K9me3 at mouse telomeres, unexpectedly low density of telomere H3K9me3 and rather infrequent HP1 are naturally localized at human telomeres. This provides an opportunity to enhance the presence of this naturally occurring component of telomeric chromatin to study its role in telomere biology.

In this report, we present an approach to study the consequences of locally altering telomere chromatin properties on the key functions of telomeres. We enrich heterochromatinization at telomeres by fusing HP1alpha (HP1a) to the telomere binding shelterin protein TRF1. We find that deposition of heterochromatin marks at telomeres is increased and telomerase-mediated telomere extension is attenuated. Mutational studies of such telomere-tethered HP1a show the chromo shadow domain (CSD) of the telomere-tethered HP1a is involved in attenuating telomere extension. Additionally, DNA-damage responses at telomeres, triggered by either expressing mutant-template telomerase RNA (hTR) or depletion of shelterin TRF2, are reduced, suggesting enhanced telomere stability. Direct super-resolution visualization of this HP1a-tethered telomere chromatin in cells by stochastic optical reconstruction microscopy (STORM) imaging shows previously unsuspected less globular, more irregularly shaped telomere structures. These findings provide a platform for understanding the crosstalk between altered chromatin environment, epigenetic regulation and telomere maintenance.

Results

A model system to study HP1a function at telomeres. To study how altered telomere chromatin regulates its maintenance, we set up a controlled system to enhance heterochromatin in a locus-specific manner. We fused shelterin TRF1, which confers telomeric locus-specificity, to HP1a, a protein involved in heterochromatin establishment and maintenance. HP1a contains a conserved N-terminal chromo domain (CD) that binds to dimethylated and trimethylated H3K9 (H3K9me2/3) and a C-terminal CSD for dimerization and ligand binding. These two domains are joined by a flexible hinge domain.

To validate our system, EGFP-tagged TRF1 fused with HP1a (Fig. 1a) was transiently cotransfected with mCherry-tagged TRF2, a core shelterin component, and tested for colocalization at telomeres (Fig. 1b) in human bladder cancer UM-UC3 cells. As expected, EGFP-HP1a is capable of localizing to nontelomeric genomic regions, resulting in a significantly higher total average HP1a occupancy (~16.7% area per nucleus) compared to EGFP-TRF1 (~5.0%) that localized exclusively to telomeres (Fig. 1c), as measured by percent EGFP per nucleus. Meanwhile, TRF1HP1a also localized to genomic regions other than telomeres with no significant difference of average nucleus occupancy (~17.3%) compared to control HP1a (~16.7%). Thus, TRF1HP1a also retained the functional abilities of HP1a for targeting and chromatin spreading (Fig. 1c). A point mutation in the CD domain of the TRF1HP1a-fusion construct (V22M), which abrogates recognition of H3K9me3 by HP1a, maintained its ability to localize at telomeres, as will be discussed further below, and reverted average EGFP occupancy in the nucleus to ~6.4%. Average colocalization with TRF2 was significantly higher for both EGFP-TRF1HP1a (~74.2%) and EGFP-TRF1 (~62.1%) compared to EGFP-HP1a alone (~46.9%) (Fig. 1d). Thus, TRF1HP1a is expressed and specifically enriched at telomeres.

TRF1HP1a expression increases H3K9me3 per H3 at telomeres. In addition to microscopy, we also used ChIP to follow the genomic localization of stably expressed TRF1HP1a cells (Fig. 1e–j). Immunoprecipitated chromatin was hybridized with either telomeric or control centromeric (CENPB) probe (Fig. 1f). After normalizing to intensity of 10% total chromatin input, TRF1HP1a showed ~28-fold increased average HP1a at telomeres compared to controls (Fig. 1f, g). While TRF1 overexpression resulted in a slight decrease of H3 at telomeres compared to vector only (Vonly) or HP1a, each of the three
control groups showed higher H3 compared to TRF1HP1α (Fig. 1f, h). Combining all three control groups, TRF1HP1α showed less H3 (~0.7 fold) per telomere (Fig. 1f, h). We then asked if H3K9me3 heterochromatin marks at telomeres were partly caused by random, coincidental overlaps with telomeres due to widespread HP1 colocalization of EGFP and TRF2 (mCherry) **p < 0.0001; n.s. (no significance). The high apparent colocalization of HP1 with TRF2 (within the HP1α group) is partly caused by random, coincidental overlaps with telomeres due to widespread HP1 spots; X–Y planes are projections of z-stacks. Significance is assessed by one-way ANOVA and Dunnett’s multiple comparison test with 95% confidence level. Error bars represent standard error of the mean (s.e.m.). Experimental set-up for ChIP to follow the localization of stably expressed TRF1HP1α in UM-UC3 after blasticidin selection (Bsd) at ~PD25. Experimental groups are immunoprecipitated with the indicated antibodies, and hybridized on a dot blot with either telomere or control centromere CENPB probe (CENPB) probe (assigned as a chromo domain (CD), a hinge, and a chromo shadow domain (CSD); AA (amino acid). Fluorescence imaging of UM-UC3 cells cotransfected with mCherry-tagged TRF2 and EGFP-TRF1, EGFP-HP1α, or EGFP-TRF1HP1α-fusion 48 h after transfection (n = 15–21 nuclei). Representative images. mCherry shown as magenta in merged image. Scale bar: 10 μm. Quantification of % EGFP area per nucleus **p < 0.0001; n.s. (no significance). Quantification of % telomere per nucleus with colocalization of EGFP and TRF2 (mCherry) **p = 0.0065; ***p < 0.0001. The high apparent colocalization of HP1α with TRF2 (within the HP1α group) is partly caused by random, coincidental overlaps with telomeres due to widespread HP1 spots; X–Y planes are projections of z-stacks. Significance is assessed by one-way ANOVA and Dunnett’s multiple comparison test with 95% confidence level. Error bars represent standard error of the mean (s.e.m.). Experimental set-up for ChIP to follow the localization of stably expressed TRF1HP1α in UM-UC3 after blasticidin selection (Bsd) at ~PD25. Experimental groups are immunoprecipitated with the indicated antibodies, and hybridized on a dot blot with either telomere or control centromere (CENPB) probe (n = 3 independent replicates). Upon signal normalization to 10% input, TRF1HP1α shows increased HP1α at telomeres compared to controls Vector only (Vonly), TRF1 and HP1α ***p < 0.0001; TRF1HP1α shows decreased H3 at telomeres *p = 0.0133. Upon normalization to H3 signal, TRF1HP1α shows increased H3K9me3 at telomeres per H3 *p = 0.0101 while j there is no significant change of TRF2 occupancy at telomeres. n.s. (no significance). The values for three independent experiments (Supplementary Fig. 1) are used to calculate the s.e.m. for each group. p values are calculated by two-tailed unpaired t test with 95% confidence level.

TRF1HP1α attenuates telomere extension. To investigate if tethered HP1α-induced heterochromatin regulates telomere extension by telomerase, EGFP-tagged TRF1HP1α or corresponding control groups (Vonly, TRF1, HP1α) were introduced into UM-UC3 cells via lentiviral construct infection. Blasticidin-selected cells were FACS sorted for medium EGFP expression (assigned as Population Doubling PD0). Protein expression was

Fig. 1 Tethered HP1α at telomeres locally increases H3K9me3. a Schematic of HP1α fused to TRF1. HP1α consists of a chromo domain (CD), a hinge, and a chromo shadow domain (CSD); AA (amino acid). b–d Fluorescence imaging of UM-UC3 cells cotransfected with mCherry-tagged TRF2 and EGFP-TRF1, EGFP-HP1α, or EGFP-TRF1HP1α-fusion 48 h after transfection (n = 15–21 nuclei). b Representative images. mCherry shown as magenta in merged image. Scale bar: 10 μm. c Quantification of % EGFP area per nucleus **p < 0.0001; n.s. (no significance). d Quantification of % telomere per nucleus with colocalization of EGFP and TRF2 (mCherry) **p = 0.0065; ***p < 0.0001. The high apparent colocalization of HP1α with TRF2 (within the HP1α group) is partly caused by random, coincidental overlaps with telomeres due to widespread HP1 spots; X–Y planes are projections of z-stacks. c, d Significance is assessed by one-way ANOVA and Dunnett’s multiple comparison test with 95% confidence level. Error bars represent standard error of the mean (s.e.m.). e Experimental set-up for ChIP to follow the localization of stably expressed TRF1HP1α in UM-UC3 after blasticidin selection (Bsd) at ~PD25. Experimental groups are immunoprecipitated with the indicated antibodies, and hybridized on a dot blot with either telomere or control centromere (CENPB) probe (n = 3 independent replicates). Upon signal normalization to 10% input, TRF1HP1α shows increased HP1α at telomeres compared to controls Vector only (Vonly), TRF1 and HP1α ***p < 0.0001; TRF1HP1α shows decreased H3 at telomeres *p = 0.0133. Upon normalization to H3 signal, TRF1HP1α shows increased H3K9me3 at telomeres per H3 *p = 0.0101 while j there is no significant change of TRF2 occupancy at telomeres. n.s. (no significance). g, h Experimental groups are immunoprecipitated with the indicated antibodies, and hybridized on a dot blot with either telomere or control centromere (CENPB) probe (n = 3 independent replicates). Upon signal normalization to 10% input, TRF1HP1α shows increased HP1α at telomeres compared to controls Vector only (Vonly), TRF1 and HP1α ***p < 0.0001; TRF1HP1α shows decreased H3 at telomeres *p = 0.0133. Upon normalization to H3 signal, TRF1HP1α shows increased H3K9me3 at telomeres per H3 *p = 0.0101 while j there is no significant change of TRF2 occupancy at telomeres. n.s. (no significance). The values for three independent experiments (Supplementary Fig. 1) are used to calculate the s.e.m. for each group. p values are calculated by two-tailed unpaired t test with 95% confidence level.
compensation by other proteins or selection of cell subpopulations. Fibroblasts and during that period (~5 to Vonly, TRF1-alone, or HP1
gramed in Fig. 2a. Southern blotting (Telomere Restriction
second round of infection with the experimental set-up dia-
TRF1HP1
 Fragment Length) analysis showed that the telomere-tethered
in telomere shortening37. To better resolve changes in length,
culturing of TRF1 overexpression in certain cancer cells resulted
vation is consistent with a previous report that only long-term
omere length up to ~PD80 (Supplementary Fig. 3). This obser-
overexpression cell lines showed only minimal alteration in tel-
validated by western blot analysis (Supplementary Fig. 2). All
overexpression cell lines showed only minimal alteration in tel-
omere length analysis of TRF1HP1α, various controls, and untreated parental cells (Prn) with WT hTR overexpression from PD0 to -PD30. e Quantification (average telomere length) shows TRF1HP1α attenuates the WT hTR overexpression-induced telomere extension. Similar findings are observed in two independent replicates. d Qualitative β-gal staining of BJ fibroblasts with earlier versus later PD. Bar: 100 µm. e Quantifications of relative β-gal fluorescence units are normalized to µg of protein. BJ PD68 shows significantly more β-gal fluorescence than BJ PD34 ***p < 0.0001. Two independent experiments; each contains triplicates. Error bars represent s.e.m. p values are calculated by two-tailed unpaired t test with 95% confidence level.
f Experimental set-up to determine if TRF1HP1α accelerates replicative senescence. These analyses were performed only 10–12 days after infection, and during that period (~5–6 PDs) telomere shortening was minimal. Thus, it is unlikely that the lack of any effect on β-gal was due to adaptive compensation by other proteins or selection of cell subpopulations. Fibroblasts g BJ (PD67-70) or h WI-38 (PD44) show no significant difference in β-gal signal. BJ, three independent experiments each contain triplicates. WI-38, single experiment with triple replicates. Error bars represent s.e.m.

Fig. 2 Telomere-tethered HP1α attenuates telomere extension by telomerase but does not accelerate replicative senescence. a Experimental set-up to study the impact of HP1α on telomerase-based telomere extension in UM-UC3. First infection: EGFP-tagged Vonly, TRF1, HP1α, or TRF1HP1α. b Telomere length analysis of TRF1HP1α, various controls, and untreated parental cells (Prn) with WT hTR overexpression from PD0 to -PD30. e Quantification (average telomere length) shows TRF1HP1α attenuates the WT hTR overexpression-induced telomere extension. Similar findings are observed in two independent replicates. d Qualitative β-gal staining of BJ fibroblasts with earlier versus later PD. Bar: 100 µm. e Quantifications of relative β-gal fluorescence units are normalized to µg of protein. BJ PD68 shows significantly more β-gal fluorescence than BJ PD34 ***p < 0.0001. Two independent experiments; each contains triplicates. Error bars represent s.e.m. p values are calculated by two-tailed unpaired t test with 95% confidence level.
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Tethering TRF1HP1α containing mutations within HP1α. To rule out potential indirect effects due to tethering of TRF1HP1α to nontelomeric HP1α genomic loci and to understand mechanistically how HP1α inhibited telomere elongation, HP1α constructs carrying various characterized separation-of-function mutations fused with TRF1, as above, were introduced into UM-UC3 cells (Fig. 3a): (i) CD mutant V22M39, defective in recognizing H3K9me3 marks; CSD mutants (ii) I165A39, deficient in dimerization and ligand binding and (iii) W174A39, which can dimerize but is deficient in ligand binding; (iv) N-terminal phosphorylation mutant NS2A40, to perturb oligomerization; and
driven by its TRF1 fusion and did not require HP1 tethering system, telomere anchorage of V22M was efficient (~6.3%), but not I165A (~27.1%), compared to WT TRF1HP1 (~22.5%) (Fig. 3b, c). Consistent patterns were observed by quantifying total numbers of fusion protein spots per nucleus (Supplementary Fig. 4). Thus, loss of H3K9me3 binding by V22M mutants V22M I165A or V22MI165A resulted in de-repression (v) hinge mutant KRKAAA, deficient in HP1α DNA/RNA interaction (Fig. 3a).

Validation of the ability of these mutant proteins to localize to telomeres or other genomic regions was performed as described for Fig. 1b–d. WT TRF1HP1α and all mutants tested had considerable amounts of tethering to other genomic regions except for V22M or V22MI165A (which do not recognize H3K9me3) (Fig. 3b). Average HP1α nucleus occupancy was reduced in V22M (~6.4%) and the double mutant V22MI165A (~6.3%), but not I165A (~27.1%), compared to WT TRF1HP1α (~22.5%) (Fig. 3b, c). Consistent patterns were observed by quantifying total numbers of fusion protein spots per nucleus (Supplementary Fig. 4). Thus, loss of H3K9me3 binding by V22M or V22MI165A resulted in deficient anchorage to nontelomeric chromatin.

However, all mutants, including V22M and V22MI165A, were efficiently tethered at the telomeres via their fused TRF1 (~67.1–83.4% colocalization; Fig. 3b, d). Thus, in this controlled tethering system, telomere anchorage of V22M was efficiently driven by its TRF1 fusion and did not require HP1α recognition of H3K9me2/3, that might potentially have contributed to nontelomeric localization. Therefore, we deliberately used V22M to control for possible indirect effects due to tethering of TRF1 to nontelomeric HP1α genomic sites. Meanwhile, there was no significant change in number of TRF2 foci per nucleus (Fig. 3e).

Chromo shadow domain of HP1α attenuates telomere extension. To determine which domain functions of HP1α control telomere extension by telomerase, we generated cells stably overexpressing TRF1HP1α-con structs harboring various mutations within HP1α (Fig. 3a–d), using the experimental set-up shown (Fig. 2a). Interestingly, WT TRF1HP1α and V22M limited telomere extension to similar extents (Fig. 3f, h). Hence, because TRF1 tethering of HP1α to telomeres bypassed the need for H3K9me2/3 recognition for HP1α recruitment to telomeres, HP1α recognition of H3K9me2/3 per se was not required for this inhibition of telomere extension. In contrast, I165A abolished the inhibition of telomere lengthening, as did V22MI165A (Fig. 3f, h). Since I165A abrogates both dimerization and ligand

![Fig. 3](image-url)
binding, we sought to separate which function was primary in this regulation of telomerase action. An additional CSD mutant W174A, which is deficient in ligand binding but can still dimerize, only partially restored the inhibition of lengthening rate (Fig. 3g, i). Thus, because dimerization was not sufficient to fully inhibit telomerase action down to the WT TRF1HP1α level, the ligand binding (and possibly also dimerization) function of CSD is required to inhibit telomerase extension. Finally, N-terminal phosphorylation and the hinge DNA-binding domain were not required to inhibit telomere extension (mutants NS2A and KRKAAA in Fig. 3g, i).

**TRF1HP1α reduces telomere damage induced by mutant hTR.** Knowing that TRF1HP1α inhibited telomere extension (Figs. 2, 3), using an independent readout for telomerase function, we determined whether TRF1HP1α-induced inhibition of telomerase would lead to less incorporation of mutant hTR-specified telomeric DNA, and hence lead to a diminished DNA-damage response at telomeres. Incorporated mutant telomere repeats cannot bind shelterin proteins, and lead to rapid uncapping and localized telomere damage foci. Cells were infected on day 0 with WT hTR or mutant hTRs, either 47A (5’-TTTGGG-3’)38 or TSQ1 (5’-GTTGGG-3’)43, and selected for stable expression after 48 h. On day 5, 53BP1 DNA-damage foci present at telomeres, also referred to as telomere dysfunction-induced foci (TIFs), were increased (Fig. 4a–c) compared to WT hTR (Fig. 4d, e). We tested TIF induction early, when cell growth was only mildly affected (Supplementary Fig. 5). Introduction of TRF1HP1α yielded fewer average 47A-induced TIFs (~13.4%) compared to controls Vonly (~23.8%), TRF1 (~28.6%) and HP1α (~23.8%) (Fig. 4a, b). Similar findings were also observed with TSQ1 treatment (Fig. 4c). Moreover, WT TRF1HP1α (~13.4%) and V22M (~16.7%) showed similar TIFs (Fig. 4a, b). However, elevated TIFs were observed in CSD mutants I165A (~27.7%), W174A (~22.6%), and V22MI165A (~26.8%). In cells overexpressing WT hTR, minimal baseline DNA damage at telomeres was observed in corresponding controls (ranging from 4.1–7.5%; Fig. 4d, e) or Vonly (5.8–8.9%; Fig. 4f).

In these experiments, the DNA damage caused by incorporated mutant repeats depends on telomerase action at telomeres. We showed that WT TRF1HP1α inhibited telomere extension to similar extents as mutants V22M, NS2A, and KRKAAA (Fig. 3). If reduced TIF levels were solely due to telomerase inhibition, we would expect that TIF induction upon 47A expression would be similar with all four fusion proteins. However, notably, upon 47A expression, NS2A and KRKAAA showed more TIFs compared to WT and V22M TRF1HP1α (Fig. 4a, b). These results indicate a separation of HP1α functions: on the one hand, in regulating telomere extension via its C-terminal CSD (ligand binding and dimerization); and on the other hand, in DNA-damage reduction (via its N-terminal CD and hinge domains).
Tethered HP1α reduces telomere damage induced by si-TRF2.
To further study the direct telomere-protective effect of HP1α, we used two additional, independent approaches. First, we induced telomere damage by efficiently knocking down TRF2 with si-TRF2 (Fig. 5a). Baseline TIFs were quantified using control non-targeting siRNA (Fig. 5b). TRF1HP1α mildly protected from si-TRF2-induced telomere damage (Fig. 5c). Furthermore, comparing across all of the TRF1HP1α mutants, the pattern of allele-specific effects on TRF2-depletion-induced TIFs closely paralleled their corresponding pattern on 47A-hTR-induced TIFs (compare Fig. 4b with Fig. 5c). This similarity of protective effects, against both telomerase-independent (TRF2 knockdown) and telomerase-dependent (47A hTR-induced) damage, indicates that in addition to its inhibitory effect on telomerase action, telomere-tethered WT TRF1HP1α can also protect telomeres.

Independently, we also developed a CRISPR/Cas9-based telomeric DNA-cutting strategy to induce telomere-specific damage in cells (Supplementary Fig. 6). Interestingly, expressing either TRF1 alone or telomere-tethered WT TRF1HP1α reduced CRISPR-induced telomere DNA cutting to similar extent in UM-UC3 cells. In summary, employing different approaches to induce telomeric damage has uncovered different aspects of how tethered HP1α affects telomere protection.

TRF1HP1α increases irregularly shaped telomere structures.
Telomere structures are smaller than the diffraction-limited resolution (~250 nm) of conventional light microscopy. Under stochastic optical reconstruction microscopy (STORM), the great majority of WT telomeres appear as spherical, globular structures. Using STORM, we examined whether HP1α tethering altered the size or globular shape of telomeres. Under our conditions, three-dimensional (3D) STORM provided XY precision of ~30 nm and Z resolution of ~70 nm. Cells stably expressing TRF1HP1α, or corresponding control groups (TRFI, HP1α), were collected for telomere length analysis or fixed for STORM analysis. We first verified that all experimental groups, collected at earliest passage after blasticidin selection (days 8–9 post lentiviral infection), showed similar population telomere lengths (Fig. 6a). Therefore, any observed telomere shape changes at the population level should not be a result of average telomere length alteration.

3D STORM showed significantly better resolution compared to conventional widefield imaging (Fig. 6b, top and middle panels). The overlay image also allowed us to exclude any nontelomeric background, ensuring the identified clusters correspond to telomeres (Fig. 6b, bottom panel). To quantify structural changes of individual telomeres, we measured the radius of gyration (Rg) of each cluster. Rg represented the root-mean-square distance of the localization points from the center of mass of a cluster according to $R_g^2 = (1/N) \sum_{k=1}^{N} (\vec{r}_k - \vec{r}_{center} - \vec{r}_{bias})^2$, where $\vec{r}$ denotes position, $k$ denotes the localization point index, and $N$ is the number of localization points. The average number of localization points of such filtered individual telomeres for TRF1, HP1α, WT TRF1HP1α, TRF1HP1α165A were 664, 420, 544, and 639, respectively (Supplementary Fig. 7). As an imaging quality control, we only analyzed telomere clusters with centers of mass near the focal plane, and consisting of more than 200 localization points (Fig. 6c, bottom panel). Telomeric localization points were clustered using Insight3 software to reconstruct structures of individual telomeric foci (Fig. 6c, top panel). Across all experimental groups, individual Rg values showed only weak correlations with number of localization points (Supplementary Fig. 7). Average Rg was similar in parental cells and Vonly, suggesting any observable changes in Rg were not caused by the vector itself (Supplementary Fig. 8).

Some generalities emerged from these analyses. As expected, most telomeres appeared spherical, but heterogeneous shapes...
were also observed\textsuperscript{45}. Figure 6c showed examples of individual telomere structures across a gradient of Rg in TRF1HP1α. Analyses showed telomeres with larger Rg displayed more variable and irregular shapes; specifically, while more spread out in three dimensions, they were compact (dense) in one dimension (Fig. 6c). The distributions of Rg heterogeneity among individual telomeres were consistently observed in multiple nuclei for each experimental group (Fig. 6d–g). This indicated that the observed structural differences among groups, as described below, were unlikely to have been simply skewed by specific nuclei that harbored Rg outliers.

To compare among the groups, we quantified the differences in telomeric structures. Rg distribution frequency of individual telomeres were represented by violin plots (Fig. 6h). Surprisingly, the Rg mean of WT TRF1HP1α (90.7 nm) was significantly higher than the mean RgS of controls TRF1 (84 nm) and HP1α (73.8 nm). The phenotype of the point mutant TRF1HP1α I165A (Rg mean 83.6 nm) resembled that of the TRF1 control (84 nm). We also noted that the Rg mean of TRF1 alone versus HP1α alone differed. Further studies are underway to better understand this phenomenon. We focused our analyses on the finding that the Rg mean of WT TRF1HP1α was significantly higher than both controls (TRF1 or HP1α) or point mutant TRF1HP1α I165A. To quantify the proportions of irregular telomere structures, mean Rg of TRF1 (84 nm) was applied as a reference cut-off (dashed line) and fractions of Rg equal or greater than the 84 nm cut-off in experimental groups

**Discussion**

The establishment of a dynamic telomeric chromatin is important for the structural and functional integrity of telomeres. However, how structural determinants impact telomere maintenance is largely unknown. We experimentally enhanced
heterochromatinization at chromosomal ends by enriching HP1α specifically at telomeres. The results reported here, summarized in Fig. 7, provide insights into how heterochromatin alters telomere maintenance and structure. Using TRF1 for telomere-tethering of HP1α, which is detected naturally at telomeres but at low occupancies, we report that an intact dimerization domain of HP1α, with its ligand binding function, is required to regulate telomere extension. Thus, HP1α-induced chromatin alteration can function as a gatekeeper of telomerase action. The requirement for ligand binding by HP1α suggests that this function requires interaction with other factors. Moreover, employing independent modes of inducing telomere damage (mutant DNA repeat incorporation or shelterin TRF2 depletion), we find that the tethered HP1α increases telomere protection. Future studies will be of interest to determine if the telomere-localized chromatin changes induced by HP1α may also play an active role in the DNA-damage responses themselves at the telomeres. Structurally, we find that enhancing heterochromatin by tethering HP1α increases the irregularity of telomere shapes, dependent on an intact HP1α dimerization domain. This correlation suggests the possibility that certain telomeric structural conformations facilitate ligand binding efficiency to result in inhibition of telomere extension by telomerase.

Previous reports, using in vitro nucleosome reconstitution assays, suggested TRF1 and TRF2 may play roles in both the formation and dynamics of telomeric nucleosomal arrays. Telomeric DNA, like other chromosomal DNA, wraps around histone protein cores, forming nucleosomes. We observed a slight decrease of the core histone protein H3 occupancy at telomeres by overexpressing just TRF1, and a further reduction upon decrease of the core histone protein H3 occupancy at telomeres, as expected some HP1α-occupied nucleosomes by the tethered TRF1HP1α. This is consistent with the in vitro finding that telomere sequence disfavors nucleosome assembly.

TRF2, like TRF1, also directly binds double-stranded telomeric DNA. Interestingly however, our ChIP analysis found that TRF1HP1α expression neither altered TRF2 occupancy (Fig. 1) nor elevated TIFs (Fig. 4d–f), suggesting TRF1HP1α cohabited with shelterin. We speculate that TRF1HP1α may directly interact with nucleosome-bound telomeric DNA in addition to nucleosomal-free telomeric DNA without interfering with TRF2 binding. This is consistent with previous reports, using micrococcal nuclease I mapping in mouse embryonic fibroblasts, showing no evident alteration of telomeric nucleosomal organization upon depletion of TRF2 or even the whole shelterin. If a significant amount of bulk TRF2 had been out-competed by TRF1HP1α for telomere binding, we would have expected a phenotype resembling that of overexpression of a dominant-negative mutant (TRF2ΔΔM), which was not observed. We cannot exclude that the balance of other shelterin components could be altered. These other components, including POT1, TIN2, RAP1, and TPP1, bind to single-stranded telomeric DNA and/or function as scaffold bridging proteins. Exactly how shelterins interplay with histones to regulate telomere dynamics are important topics for future studies.

Through these studies, we uncovered and dissected some specific functions of HP1α at telomeres. Telomerase plays a crucial role in maintaining unlimited cellular proliferation in the majority of cancer cells. Telomerase activity is regulated at multiple levels including transcriptional regulation, holoenzyme biogenesis, trafficking and recruitment of telomerase to telomeres. However, how local telomere chromatin dynamics regulate telomerase action and telomere length has been unclear. Our HP1α mutational analyses suggest that the CSD region functions as a negative regulator of telomerase action. The CSD is required for HP1α dimerization and interaction with proteins containing a conserved motif, PXVXL. Candidates for such ligands include shelterin component TIN2, and the telomere-associated chromatin remodeler ATRX, which both contain PXVXL motifs. We speculate that their recruitment by HP1α (directly or via another bridging complex) may impact telomerase action, potentially through regulating telomerase recruitment to the telomere, polymerization initiation and/or processivity.

A main function of the CD region for HP1α is to recognize H3K9me2/3. While WT TRF1HP1α enriched HP1α at telomeres, as expected some HP1α also localized to various other genome regions, presumably harboring the recognition heterochromatin marks (Fig. 3a–c). V22M mutant lacks the ability to bind to heterochromatin marks at nontelomeric genomic regions, and was exclusively tethered by TRF1 at the telomeres, and not to other regions in the genome (Fig. 3b–d). Therefore, to exclude potential confounding effects mediated via augmented binding to such regions, we exploited mutant V22M intentionally as a control, both to eliminate any tethering by TRF1HP1α of TRF1 at nontelomeric sites, and to prevent indirect effects caused by TRF1HP1α bound to genomic regions. Telomere-tethered HP1α-directed inhibition of telomere extension was independent of H3K9me2/3 recognition by the CD. Hence, H3K9m2/3 anchoring is separable from inhibition of telomere extension.
Here we have reported new connections between telomere structure, protection and telomerase action (Table 1 and Fig. 7). Overexpression of TRF1HP1α increased heterochromatin mark H3K9me3 on telomeres, increased telomere protection, reduced telomerase action and surprisingly induced irregular, often visually extended, telomeric structures. Previous reports have also suggested that silent chromatin was less condensed than euchromatin since subtelomeric and pericentromeric heterochromatin regions had lower protection in micrococcal nuclease assays compared to the rest of the genome.61 Despite the prevailing assumption that highly condensed chromatin configuration is transcriptionally inert, transcription factors were found to bind to heterochromatic repeat sequences across diverse species.62,63. Telomeres, while thought to be more heterochromatic than other genomic regions, are transcribed into telomere repeat-containing RNA (TERRA)16 which interacts with TRF1 and TRF2 to regulate telomere length.64 Although molecular component changes at telomeres can trigger a switch from a protected to a deprotected state,65 our observed increased irregularity of telomere shapes occur in the absence of DNA-damage responses. We propose that these changes in telomere structures can influence protection and telomerase action. It is also possible that the reduced H3 at telomeres (Fig. 1) may influence nucleosome arrangements to result in a more irregular telomere structure.

Telomere maintenance is crucial for cancer cell proliferation. Telomere homeostasis is regulated at many different levels. Telomere chromatin encompasses highly dynamic structures interconverting between different conformations. Thus, telomere chromatin states may add another layer of protection to play an important role in regulating chromosome end maintenance and protection. Chromatin states are often altered during tumorigenesis. It has become clear that, along with genomic instability, epigenetic abnormalities promote carcinogenesis. Heterochromatin-dependent, noncanonical telomere protection strategies, resembling those found in flies or yeasts, may have been selected for some human cancers. The possibility that some cancers can adapt heterochromatin changes to stabilize their telomeres will be interesting topics for future studies. Manipulating the epigenetic status at telomeres should provide new insights for the development of innovative telomere-directed, epigenetic cancer therapeutics.

**Methods**

**Cell culture.** UM-UC3 (ATCC), U2OS (ATCC), BJ (ATCC), WI-38 (ATCC), and lenti-X-293T (Clontech) cells were cultured at 37 °C in 5% CO₂ in high glucose DMEM medium (Hyclone, Logan, UT) containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% (vol/vol) penicillin-streptomycin (Gibco). Cotransfection was performed using PolyJet reagent (SignaGen Laboratories).

**Plasmids and lentivirus.** The pHR α-driven expression lentiviral vector contained a hygromycin resistance gene. pHR α expression lentiviral vector was a gift from Feng Zhang (Addgene Plasmid #52961). Lentivirus was packaged in lenti-X-293T (Clontech) using PolyJet reagent (SignaGen Laboratories). Drug selection was initiated 48 h post infection with 50 μg/ml blasticidin for 5 days (ThermoFisher Scientific). For introduction of a second round of infection with either WT or mutant hTRs, cells were selected using 8 µg/ml puromycin for 1 day (ThermoFisher Scientific).

**Western blotting.** Cells were lysed [10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% IGEPAL CA-630, 10% glycerol, 1 mM EDTA, 1× Halt protease inhibitor cocktail (ThermoFisher Scientific)] and 1 mM DTT, Benzonase nuclease 50 U/ml (Novagen)]. Lysates were spun at 13,000 rpm (15 min at 4 °C). Supernatants were then blocked for 30 min at room temperature with 5% milk in TBST (20 mM Tris pH 7.4, 150 mM NaCl, 0.05% IGEPAL CA-630, 10% glycerol, 1 mM EDTA, 1× Halt protease inhibitor cocktail) for 30 min. Protein concentration was measured using Precision Red protein assay dye (ThermoFisher Scientific) and exposed to the membranes. Primary antibodies used include 1:5000 rabbit anti-GAPDH (A-8347; Invitrogen), 1:10000 mouse anti-β-actin, 1:10000 mouse anti-TRF2 (NB110-57130), 1:1000 goat anti-Cas9 (A-9000; Epigenetek), 1:200 mouse anti-p53 (sc-126; Santa Cruz), and 1:1000 mouse anti-GAPDH (MA515738; ThermoFisher). Secondary antibodies used include 1:5000 Goat Anti-Mouse IgG-HRP (611-055-046; Jackson ImmunoResearch), 1:5000 Goat Anti-Rabbit IgG-HRP (611-055-046; Jackson ImmunoResearch), 1:5000 Donkey Anti-Goat IgG-HRP (sc2020; Santa Cruz Biotechnology). Uncropped blots are shown in Supplementary Fig. 9.

**Chromatin immunoprecipitation and dot blot assays.** 20×10⁶ cells were trypanosized and crosslinked with 1% paraformaldehyde (w/v) (ThermoFisher Scientific) at room temperature for 5 min, followed by 125 mM glycine (Sigma) for 5 min to quench the crosslinking and washed (cold 1× PBS, 1 mM PMSF). All subsequent steps were performed at 4 °C, unless noted otherwise. Cells were resuspended into ChIP lysis buffer (0.5% NP-40, 85 mM KCI, 20 µM Tris-HCl pH 8.0 with 1x Halt protease inhibitor cocktail (ThermoFisher Scientific)) for 15 min, homogenized with a pellet pestle (ThermoFisher Scientific), and spun at 450 x g for 5 min. Nuclei pellets were incubated in nuclear lysis buffer (1% SDS, 50 mM Tris-HCl pH 8.0, 10 mM EDTA with 1× Halt protease inhibitor cocktail) for 30 min, further lysed with a syringe, and sonicated with Covaris S2 to obtain fragments between 400 and 1000 base pairs. Fragment sizes were checked by running an aliquot of the sheared, purified chromatin on an agarose gel. Sheared chromatin was spun at 13,000 rpm for 10 min, and supernatant (2×10⁶ cells/reaction) was incubated overnight with 10 μg of ChIP-grade antibodies respectively: anti-H3 (ab1791; Abcam); anti-H3K14Ac (ab60028; Abcam); anti-Trf1 (ab77526; Abcam); anti-H3K9me3 (ab88898; Abcam); anti-Trf1 (NB110-57130; Novus Biologicals); anti-Trf1 (ab1423; Abcam.) and anti-rabbit IgG (#2729; Cell Signaling). Samples were then immunoprecipitated with Dynabeads Protein G (Life Technologies) for >6 h to overnight, washed and eluted (1× TE, 1% SDS, 250 mM NaCl, 40 mM Tris-HCl, pH 8.0, 5 mM EDTA) for 1 h at 65 °C for >6 h to overnight. DNA was purified using NucleaseSpin Gel and PCR cleanup kit (Macherey-Nagel), denatured (0.1 M NaOH) at 37 °C for 30 min, neutralized (6x SSC), and transferred to a Hybond-N membrane (GE Healthcare) for >6 h to overnight. DNA was then hybridized with 32P-dCTP labeled probes

| Table 1 Summary of experimental data describing impact of WT versus mutants TRF1HP1α on telomere lengthening and TIF (via 47A or TRF2 depletion) |
|------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Baseline        | TRF1HP1α         |                 |                 |                 |                 |                 |
| Telomere lengthening | + + +           | ++ +            | + + +           | ++ +            | ++ +            | ++ +            | ++ +            |
| TIF via 47A      | + + +           | ++ +            | + + +           | ++ +            | ++ +            | ++ +            | ++ +            |
| TIF via si-TRF2  | + + +           | ++ +            | + + +           | ++ +            | ++ +            | ++ +            | ++ +            |

+++ (strong telomere lengthening, high number of TIF), ++ (intermediate phenotype), + (weak telomere lengthening, low number of TIF)
stock, final 30 μM), 7 μM 32P-dCTP (3000 Ci/mmol), 4 μl 32P-dATP (3000 Ci/ mmol), 7.9 μl Millipore H₂O, and 1 μl Klonev (5 U/μl) were combined in a final volume of 25 μl. Room temperature extension was carried out for 30 min, and 95 °C for 5 min [to inactivate Klonev to prevent probe degradation upon uracil deglycosylase (UDG) treatment]. The reaction was cooled to room temperature, 0.5 μl UDG (1 U/μl) was added to degrade the UDG-resistant, incubated at 37 °C for 15 min, and then UDG was inactivated at 95 °C for 10 min. Free isotopes were removed using an ultima micropin G-25 column (GE Healthcare, Piscataway, NJ). CENPB (5'-CCCGGTCCGAAACGCGGA-3') probes were end-labeled with [γ-32P] ATP. Half of the blot was hybridized with C-strand telomeric probes, and the other half with CENPB probe at 42 °C overnight. The gel was then washed and exposed to a phosphorimager screen (GE Healthcare). Uncropped dot blots are shown in Supplementary Fig. 1.

Telomere restriction fragment length analysis. Genomic DNA was purified using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA). Telomere Restriction Fragment (TRF) length analysis was performed99. 1 μg purified genomic DNA was digested in 20 μl reaction with Alu, MspI, HaeIII, HinfI, RsaI and Ruv for 4 h at 37 °C. DNA was separated on a 0.8% agarose gel in 0.5× TBE. The gel was dried, denatured (0.5 M NaOH and 1.5 M NaCl for 1 h), rinsed with distilled water (3×), neutralized (0.5 M Tris-Cl pH 8.5 and 1.5 M NaCl for 30 min), prehybridized (6× SSC, 5× Denhardt’s solution, 0.5% (w/v) SDS), and hybridized with C-strand telomeric probe at 42 °C overnight. The gel was then washed and exposed to a phosphorimager screen (GE Healthcare). Average overhang sizes were calculated using the formula mean average length = ∑[Int(i)/MW(i)], where Int(i) = signal intensity and MW(i) = molecular weight of the DNA at position i. DNA in Supplementary Fig. 6a and 6i were digested with RsaI and HinfI. Generally size markers were loaded on TRF gels. However, the DNAs in Supplementary Fig. 6a and 6i were only run for a very short time so that the telomeres would remain as compact as possible to maximize the ability to detect remaining telomere resulting from Cas9-digestion. As size markers would not have been able to be resolved during this short run, they were eliminated in this in-gel hybridization. Here we focused on quantifying telomeric intensity using Alu probe (5'-CAAGAGGGATGACACGTGTTG-3') end-labeled with [γ-32P] ATP as loading controls. Gels were denatured and neutralized between C-strand telomere probe and Alu probe hybridization. Uncropped gels are shown in Supplementary Fig. 10.

Beta-galactosidase assay. Senescence-associated beta-galactosidase (β-gal) was analyzed using colorimetric β-gal staining kit (Cell Signaling) or quantified by fluorometric kit (Cell Biolabs). Total protein was measured using Precision Red protein assay reagent (Cytokeleton, Inc.).

Cell growth assays. Cells were infected with either WT or mutant hTRs at day 0, and selected with puromycin at day 2. Cells were split as needed to maintain logarithmic growth, and harvested at indicated time points and stained with trypan blue. Viable cells were scored by TC20 automatic cell counter (Bio-Rad).

Telomere dysfunction-induced foci (TIF) image analysis. Cells were washed with 1x PBS, fixed with 4% paraformaldehyde (w/v) (ThermoFisher) in 1x PBS and permeabilized with 0.5% NP-40 for 15 min. IF/FISH42 was performed with secondary antibody Alexa Fluor 488 (Molecular Probes) 1:750 for 1 h, (NB100-304; Novus Biologicals) 1:500 for 1 h. Cells were then washed and incubated with secondary antibody Alexa Fluor 488 (Molecular Probes) 1.750 for 1 h, fixed with 2% paraformaldehyde and incubated with 0.1 mg/ml RNaseA for 1 h at 37 °C. For, FISH, cells were dehydrated sequentially with ethanol (70%, 95% and 100%; 5 min each), heated in hybridization mix with 0.5 mg/ml peptide nucleic acid (PNA) telomeric probe TelC-Cy5 (PNABio) at 85 °C for 10 min to denature the DNA, followed by overnight hybridization at room temperature. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole) (Life Technologies) and mounted with Prolong Gold (Invitrogen).

Equipment and settings: Images were captured using a DeltaVision Real-time Deconvolution Microscope (Applied Precision) with a ×100 oil 1.4 NA Plan Apo objective. The Insight3 software47, which identified and fitted single molecule spots in each camera frame to determine their x, y, and z coordinates as well as photon numbers. Sample drift during data acquisition was corrected using imaging correlation analysis. The drift-corrected coordinates, photon number, and the frame of appearance of each identified molecule were saved in a molecule list for further analysis.

STORM imaging: Cells were labeled with PNA telomeric probe, TelC-Cy5 (PNABio). Individual telomeric localization signals were detected by switching the fluorophores between active and dark states stochastically. Accumulation of individual fluorophore forms a cluster of molecular positions, known as localizations, corresponding to structural characteristics of an individual telomere.

Analysis: Individual telomeres were manually selected from the STORM images. The telomeres near focal planes with good resolution were picked. These manually picked telomeres were further screened so that telomeres with more than 200 localizations were kept for the Radius of gyration (Rg) analysis.

Statistical analyses. Significance of mean was assessed by statistical analyses noted in the corresponding figure legends. These include: one-way ANOVA and Dunnett’s multiple comparison test with 95% confidence level; two-tailed unpaired t test with 95% confidence level. All graph bars were represented by means with standard error of the mean (±SEM). For STORM statistical analysis, means of Rg in the violin plots were compared using ANOVA Tukey’s multiple comparisons with 95% confidence level.

Code availability. Custom image analysis for Rg calculation were written in MATLAB 2012B. The MATLAB script is available from the authors upon request.

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**Author contributions**
T.T.C. and E.H.B. designed the experiments. T.T.C., X.S. and E.H.B. wrote the manuscript; T.T.C., J.-H.W. and G.S. performed the experiments; X.S. and J.G. performed the STORM imaging. T.T.C., X.S, J.G., G.S., B.H. and E.H.B. analyzed the data. All authors provided feedback on the manuscript.

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