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Loss of hPot1 Function Leads to Telomere Instability and a *cut*-like Phenotype

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Summary

The human telomere binding protein hPot1 binds to the most distal single-stranded extension of telomeric DNA in vitro, and probably in vivo [1, 2], as well as associating with the double-stranded telomeric DNA binding proteins TRF1 and TRF2 through the bridging proteins PTOP (also known as PIP1 or TINT1) and TIN2 [2–7]. Disrupting either the DNA binding activity of hPot1 or its association with PTOP results in elongated telomeres, suggesting a role for hPot1 in telomere length regulation [2, 5, 6, 8]. However, mutations to POT1 and Cdc13p, the fission and budding yeast genes encoding the structural orthologs of this protein, leads to telomere instability and cell death [1, 9]. Thus, it is possible that the hPot1 protein may also serve to cap and protect telomeres in humans. Indeed, we now find that knocking down the expression of hPot1 in human cells causes apoptosis or senescence, as well as an increase in telomere associations and anaphase bridges, telltale signs of telomere instability [10]. In addition, knockdown cells also displayed chromatin bridges between interphase cells, reminiscent of the *cut* phenotype that was first described in fission yeast and in which cytokinesis progresses despite a failure of chromatid separation [11]. However, unlike the yeast *cut* phenotypes, we suggest that the *cut*-like phenotype observed in hPot1 knockdown cells is a consequence of the fusion of chromosome ends and that this fusion impedes proper chromosomal segregation. We conclude that hPot1 protects chromosome ends from illegitimate recombination, catastrophic chromosome instability, and abnormal chromosome segregation.

Results and Discussion

Knockdown of hPot1 Leads to a Decrease in Cell Viability in HeLa Cells

To investigate the cellular role of hPot1, we disrupted the function of this protein in human cells by using RNA interference to lower hPot1 expression [12]. Sequences encoding small hairpin RNAs (shRNA) that produce small interfering RNAs directed against hPot1 were cloned into the vector pSUPER, and the resultant constructs were transiently transfected into HeLa cells. Because of extremely low levels of endogenous hPot1 in HeLa cells (not shown), we stably expressed flag epitope-tagged hPot1 in HeLa cells to screen for shRNA constructs that could knockdown the levels of this ectopic hPot1 protein. Flag-tagged hPot1 was immunoprecipitated with an anti-Flag antibody, followed by immunoblotting with an anti-hPot1 antibody. From this analysis, we identified two constructs, shRNA-P1 and -P2, which reduced hPot1 expression by 50% or more. Neither of these shRNAs had any effect on the expression of the off-target protein YFP (Figure 1A). Lastly, these shRNAs are expected to target all known splice forms of hPot1 because the shRNAs target either exon 18 or 9, which are common to the recently described alternative splice transcripts of hPot1 [13].

In both fission and budding yeast, loss of the putative hPot1 structural orthologs, POT1 and Cdc13p, leads to a decrease in telomere stability and cell viability [1, 9, 14–16]. We therefore measured the viability upon knockdown of hPot1 in HeLa cells, a human cell line often used to characterize the cellular function of telomere binding proteins [17, 18]. HeLa cells were transiently cotransfected with a vector encoding shRNA-P1, -P2, or their corresponding scrambled controls in addition to a YFP-expressing construct, after which YFP-expressing cells, which are expected to express the shRNAs, were sorted by flow analysis cell sorting (FACS) and plated in equal numbers. Daily counting of these cells revealed that, as expected, scramble control cells proliferated in a logarithmic fashion. In contrast, the number of hPot1 knockdown cells did not increase over time (Figure 1B). This was most dramatic at day four, when few hPot1 knockdown cells were detected on plates stained with crystal violet, in contrast to the nearly confluent plate of scramble control cells (Figure 1C). Because independent targeting of either the 5′ or 3′ end of the transcript gave rise to the same phenotype, we argue that a decrease in hPot1 expression accounts for the decrease in plating efficiency.

To determine if apoptotic death accounts for the reduced number of cells observed upon treatment with hPot1 shRNAs, we performed FACS analysis on hPot1 knockdown or control HeLa cells by using Annexin V-PE and 7AAD as markers for early and late apoptosis, respectively. Specifically, HeLa cells cotransfected with shRNA-P1 or the scramble control and a YFP-expressing plasmid were sorted for YFP-positive cells and plated as described above. Three days later, these cells were double stained with Annexin V-PE and 7AAD, then subjected to a second round of FACS sorting to determine the number of cells staining positive for these markers. This analysis revealed a 3-fold increase in the number of apoptotic (Annexin V-PE- and Annexin V-PE/7AAD-positive) cells compared to scramble control cells (Figure 1D). We conclude that reduced expression of hPot1 causes apoptosis of HeLa cells.

Knockdown of hPot1 Leads to a Senescent-like Growth Arrest in HT1080 and IMR-90 Cells

Disrupting telomere stability by decreasing the amount of the telomere binding protein TRF2 on telomeres via

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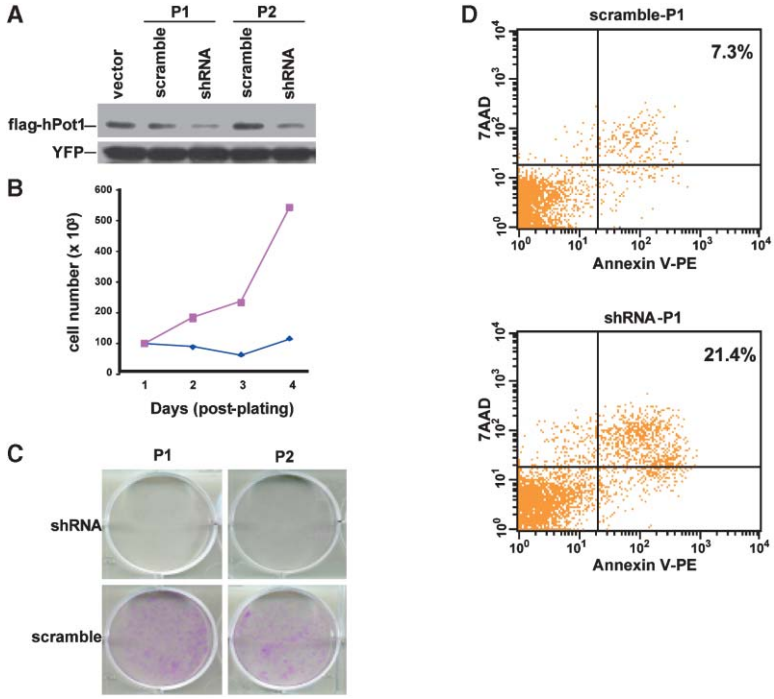

Figure 1. Knockdown of hPot1 Expression is Lethal in HeLa Cells

(A) Knockdown of hPot1 in HeLa cells. HeLa cells stably infected with a retroviral vector encoding N-terminal Flag-tagged hPot1 [8] were transiently cotransfected with 200 ng of the pEYFP-C1 (Clontech) expression plasmid and 2 μg of the pSUPER (OligoEngine) plasmid as a control (vector) or the same plasmid encoding the hPot1 shRNA-P1 target sequence (GTACTAGAAGCCTATCTCA), -P2 target sequence (TACCTCGCACTTCAAGCAA) or one of the following control scrambled sequences: scramble-P1 (ATGATCAAGCCGTATCTCA) or scramble-P2 (CATCTCACGTTCAACACGA). Cells were lysed 48 hr later, and ectopic hPot1 was detected by immunoprecipitation with the anti-Flag antibody M2 (Sigma) followed by immunoblotting with an anti-hPot1 982 rabbit polyclonal antibody generated against the peptide sequence CYGRGIRVLPESNSDVDQLKKDLES described by others to be antigenic [2]. This antibody specifically recognizes the appropriately sized band corresponding to endogenous and ectopic hPot1, of which the intensity is reduced upon introduction of the hPot1 shRNA-P1 knockdown vector. YFP proteins were coimmunoprecipitated and immunoblotted with anti-GFP antibody (Roche) as a control for gauging nonspecific knockdown as well as for transfection efficiency and gel loading.

(B) Knockdown of hPot1 inhibits cell growth. HeLa cells were transiently cotransfected as above with the pEYFP-C1 plasmid and either the shRNA-P1 or scramble-P1 control construct. Twenty-four hours later, YFP-positive cells were isolated by FACS, and 10^5 cells were plated in multiple dishes. Everyday afterward for 4 days, a plate was trypsinized and viable cells (trypan blue excluded) were counted, demonstrating a decrease in culture growth upon knockdown of hPot1. The growth curve shown is representative of three independent experiments.

(C) Knockdown of hPot1 leads to a decrease in plating efficiency. The aforementioned YFP-positive sorted HeLa cells cotransfected with the shRNA-P1, -P2, scramble-P1, or scramble-P2 at 10^5 cells/plate were plated and 4 days later were stained with crystal violet to demonstrate the decrease in cell density upon knockdown of hPot1 expression. The plates shown are representative of three independent experiments.

(D) Knockdown of hPot1 induces apoptosis in HeLa cells. The aforementioned YFP-positive sorted HeLa cells cotransfected with either the shRNA-P1 or scramble-P1 were plated, and 3 days later they were double stained with the apoptotic markers Annexin V-PE and 7AAD and subjected to a second round of FACS analysis so that apoptotic cell numbers could be determined and the increase in apoptosis upon knockdown of hPot1 expression could be demonstrated. The percent of double stained cells is shown in the upper left. Each analysis is representative of three independent experiments.

expression of a dominant-negative form of the protein, $TRF2^{\Delta B\Delta M}$, is known to cause either apoptosis or senescence, depending on whether the cell assayed has an intact p53-mediated DNA damage response or cannot execute apoptosis upon DNA damage [19]. We therefore tested the effect of knocking down hPot1 expression in HT1080 cells, which are known to enter a senescence-like state upon disruption of their telomeres [19]. Specifically, HT1080 cells were cotransfected with a YFP-encoding plasmid and one encoding hPot1 shRNA-P1 or the scramble control, after which YFP-positive cells were enriched and plated to monitor their growth. The ability of shRNA-P1 to knockdown expression of hPot1 in HT1080 cells (e.g., see Figure 4A) resulted in a decrease in culture growth and plating efficiency when these cells were compared to scramble controls (Figures 2A and 2B). This decrease was not due to an increase in apoptosis; reproducibly 5%–7% of HT1080 cells transfected with either the scramble control or shRNA-P1 vectors showed double staining with Annexin V-PE and 7AAD (Figure 2C). Instead, microscopic examination revealed a fraction of the hPot1 knockdown HT1080 cells with senescent phenotypes (not shown). Prompted by this finding, we stably infected the normal human diploid fibroblast strain IMR-90, which is more sensitive to the effects of $TRF2^{\Delta B\Delta M}$ [19], with the retroviral vector encoding shRNA-P1 or the scrambled counterpart. The Pot1 shRNA elicited a more severe senescent phenotype in these cells (Figure 2D); many cells exhibited an enlarged and flattened morphology and stained strongly positive for β-galactosidase (β-gal), a marker of senescence [20]. Thus, knockdown of hPot1 leads to either apoptosis or senescence, which based on these limited studies appears to depend upon whether the cells can mount a DNA damage response that leads to apoptosis. Future experiments are being undertaken to directly address this possibility.

Knockdown of hPot1 Induces Formation of Chromatin Bridges between Interphase Cells

Microscopic analysis of hPot1 shRNA transiently transfected cells revealed the presence of intracellular bridges between some interphase cells. To explore this phenotype further in a system in which senescent or apoptotic phenotypes did not dominate, we took advantage of the fact that cell lines could be extensively cultured upon stable knockdown of hPot1 by retroviral infection of hPot1 shRNA-expressing vectors (Figure 4B

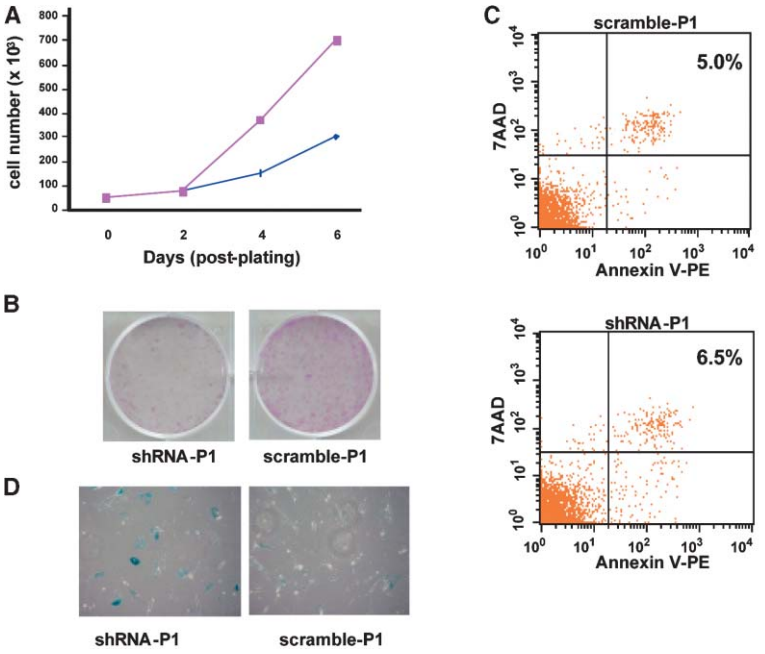

Figure 2. Knockdown of hPot1 Expression Causes a Senescence-like Phenotype in HT1080 and IMR-90 Cells

(A) Knockdown of hPot1 inhibits cell growth. HT1080 cells were transiently cotransfected at a ratio of 1 to 10 with the pEYFP-C1 plasmid and either the shRNA-P1 or scramble-P1 control construct. Twenty-four hours later, YFP-positive cells were isolated by FACS, and 5×10^4 cells were plated in multiple dishes. Everyday afterward for 6 days, a plate was trypsinized and viable cells (trypan blue excluded) were counted, demonstrating a decrease in culture growth upon knockdown of hPot1. The growth curve shown is representative of three independent experiments.

(B) Knockdown of hPot1 leads to a decrease in plating efficiency. A total of 5×10^4 of the aforementioned YFP-positive sorted HT1080 cells cotransfected with either the shRNA-P1 or scramble-P1 were plated and, 6 days later, stained with crystal violet to demonstrate the decrease in cell density upon knockdown of hPot1 expression. The plates shown are representative of three independent experiments.

(C) Knockdown of hPot1 does not induce apoptosis in HT1080 cells. The aforementioned YFP-positive sorted HT1080 cells cotransfected with either the shRNA-P1 or scramble-P1 were plated, and 5 days later they were double stained with the apoptotic markers Annexin V-PE and 7AAD and subjected to a second round of FACS analysis so that the number of apoptotic cells could be determined. The results demonstrate that knockdown of hPot1 did not lead to apoptosis in HT1080 cells. The percent of double stained cells is shown in the upper right. Each analysis is representative of three independent experiments.

(D) Knockdown of hPot1 induces senescence in normal human cells. IMR-90 normal human diploid fibroblasts were infected with retroviruses derived from plasmid pSUPER-RETRO (OligoEngine) encoding hPot1 shRNA-P1 or the scramble-P1 control sequences and selected in puromycin (0.6 μg/ml) for 7 days via methods previously described [26]. Selected cells were plated within four population doublings and 2 days later were fixed in 5% formaldehyde in PBS and incubated at 37°C for 72 hr with senescence-associated β-gal (SA-βgal) staining solution containing 1 mg/ml X-gal substrate (Invitrogen) in accordance with published protocols [20]. The plates shown are representative of three independent experiments.

and reference [6]), as opposed to the generally more robust introduction of similar vectors by the transient transfection approach. We fixed HeLa cells stably infected with a retrovirus encoding the hPot1 shRNA-P1 target sequence or, as a control, the scramble-P1 sequence and stained them with DAPI to visualize chromatin under fluorescent microscopy and thus detect the presence of chromatin bridges. We observed a 4-fold increase in the number of cells displaying interphase chromatin bridges in hPot1 knockdown cells compared to the control scramble-treated cells or cells infected with the vector alone (Figures 3A and 3B; also data not shown). These bridges were also observed at a high frequency in HT-1080 cells similarly infected with the shRNA-P1-expressing construct and in HeLa cells stably infected with retroviruses encoding other hPot1 shRNA target sequences (not shown). Because knockdown of hPot1 in two different human cancer cell lines induced chromatin bridges between interphase cells, we hypothesize that these bridges may contribute to the defects in cell growth observed upon knockdown of hPot1.

Telomere Instability Due to hPot1 Knockdown Leads to a *cut*-like Phenotype

The intracellular chromatin bridges observed in the hPot1 knockdown cells are reminiscent of those observed in the fission yeast *c*ell *u*ntimely *t*orn (*cut*) mutants, in which cytokinesis occurs despite the failure of sister chromatids to separate [11]. Typically, the two types of defects causing a *cut* phenotype are due to a failure of chromosomes to condense prior to metaphase or to resolve the protein cohesion between sister chromatids [11]. However, neither of these defects is consistent with the known function of the yeast structural orthologs of hPot1. Loss of POT1 in fission yeast has not been shown to cause a *cut* phenotype; it instead leads to catastrophic chromosome end instability [1]. We therefore hypothesized that the *cut*-like phenotype observed in hPot1 knockdown human cells could be due to a fusion of chromosome ends, physically tethering chromosomes together, even after cytokinesis.

To test this prediction, we monitored the frequency of telomere associations by conventional cytogenetic analysis. Because HT1080 cells have a near-diploid karyotype, which is ideal for cytogenetic analysis, and because these cells display chromatin bridges upon treatment with hPot1 shRNA (not shown), we chose HT1080 cells for cytogenetic analysis. To isolate enough metaphase cells needed for this analysis, we stably introduced hPot1 shRNA-P1 or the appropriate scramble control into HT1080 cells. As a result of the higher levels of endogenous hPot1 levels in HT1080 cells, it was possible to demonstrate a decrease in the level of the endogenous protein upon infection of the retroviral vector encoding shRNA-P1 but not in scramble control cells,

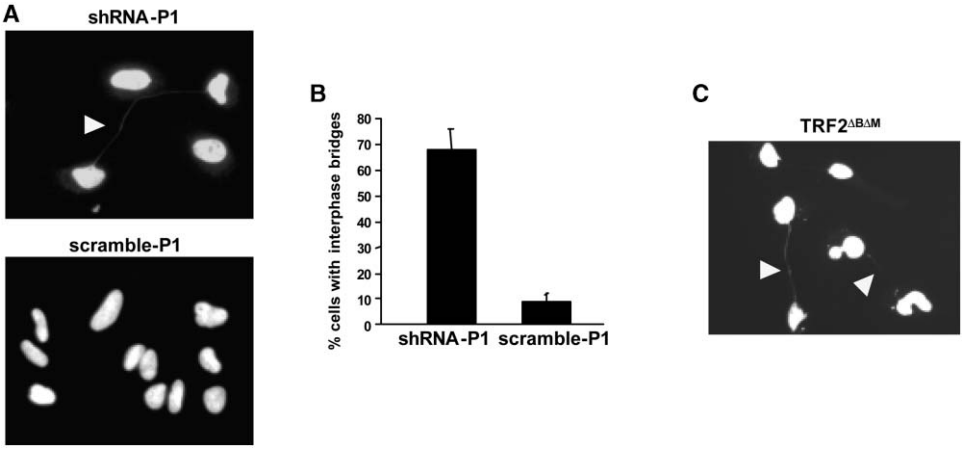

Figure 3. Knockdown of hPot1 Leads to Interphase Chromatin Bridges

(A) Visualization of intracellular chromatin bridges in hPot1 knockdown HeLa cells. HeLa cells were infected with retroviruses derived from plasmid pSUPER-RETRO (OligoEngine) encoding hPot1 shRNA-P1 target sequence or the scramble-P1 control sequence and selected in puromycin (1 μg/ml) for 5 days via methods previously described [26]. Cells were fixed in formaldehyde and stained with DAPI according to standard procedures. Microscopic visualization under 40× magnification revealed the presence of interphase bridges (arrow) in hPot1 knockdown cells.

(B) Quantitation of interphase chromatin bridges in hPot1 knockdown HeLa cells. The number of hPot1 knockdown or scramble control HeLa cells involved in an interphase bridge divided by the total number of 100 randomly chosen cells counted is shown as a percent value of the total number of cells observed. Error bars reflect SEM of three independent experiments.

(C) Visualization of intracellular chromatin bridges in HeLa cells expressing TRF2$^{\Delta B\Delta M}$. HeLa cells were transiently cotransfected at a ratio of 1 to 5 with the pEYFP-C1 plasmid and pcDNA3 encoding TRF2$^{\Delta B\Delta M}$, a dominant-negative form of the protein [10]. Twenty-four hours later, YFP-positive cells were isolated by FACS and seeded on coverslips, and one day later they were monitored for the presence of interphase bridges (arrows), as described above.

which was also confirmed at the RNA level by RT-PCR (Figure 4A). Additionally, we confirmed that as previously reported [6], knockdown of hPot1 leads to telomere elongation, as detected by Southern hybridization with a telomeric probe (Figure 4B). Cytogenetic analysis of metaphase spreads of scramble control cells revealed that the strain of HT1080 cells used for these studies had a basal level of telomere association about twice as high as that previously reported [10]. Nevertheless, the number of these associations doubled when hPot1 expression was knocked down (Figures 4C and 4D). This analysis argues against the possibility that the *cut*-like phenotype was instigated by improper chromosome condensation because knockdown of hPot1 did not cause any obvious defect in chromatin condensation upon gross inspection of metaphases (Figure 4C). Because loss of pot1 in *Aspergillus* leads to mitotic spindle and chromosome segregation defects [27] and because disrupting at least one telomere-associated protein impedes the separation of sister chromatid telomeres [28], it remains possible that other factors may yet contribute to the observed *cut*-like phenotype, although our data indicate that a major contributor to this phenotype is telomere instability. Indeed, some hPot1 knockdown cells actually displayed long strings of individual chromosomes linked at their ends, presumably due to catastrophic telomere instability (Figure 4E). This phenotype was not observed in control cells and is consistent with hPot1 performing a protective capping function that underlies the *cut*-like phenotype. In agreement with these results, we show that transient transfection of a vector encoding the TRF2$^{\Delta B\Delta M}$ dominant-negative form of the telomere binding protein TRF2, which is well documented to cause telomere instability [19], similarly caused interphase bridges (Figure 3C).

We also noted that chromosomes failed to properly resolve at anaphase when hPot1 expression was decreased, as detected by an increase in the number of anaphase bridges: chromosomes that lag behind or appear to be fused during the separation of chromosomes at anaphase. Specifically, the basal level of these events in the HT1080 cells that were stably infected with the control vector alone (not shown) or with the vector encoding the scrambled sequence (Figures 5A and 5B) was 22%, a value somewhat higher than reported for other strains of HT1080 cells [10]. However, upon knockdown of hPot1, there was a 2.5-fold increase in these aberrant segregation events (Figures 5A and 5B), as observed in the gastric cancer cell line MKN-28 when it was treated with an hPot1 antisense oligonucleotide [21]. Real-time imaging has previously shown anaphase bridges in mammalian cells to produce micronuclei when the chromosome bridges resolve and reform as small membrane bound DNA bodies [22]. We therefore assayed whether loss of hPot1 function may similarly lead to an increased number of cells displaying micronuclei. hPot1 knockdown or scramble control cells were examined microscopically for the presence of small DAPI-positive bodies distinct from the nucleus. Approximately 12% of scramble control cells had micronuclei, consistent with the number of these structures observed in other cancer cell lines [22]. Conversely, this number increased to 65% in the hPot1 knockdown cells (Figures 5C and 5D). Based on these results, we speculate that loss of hPot1 leads to fusion of chromosome ends and subsequently anaphase bridges,

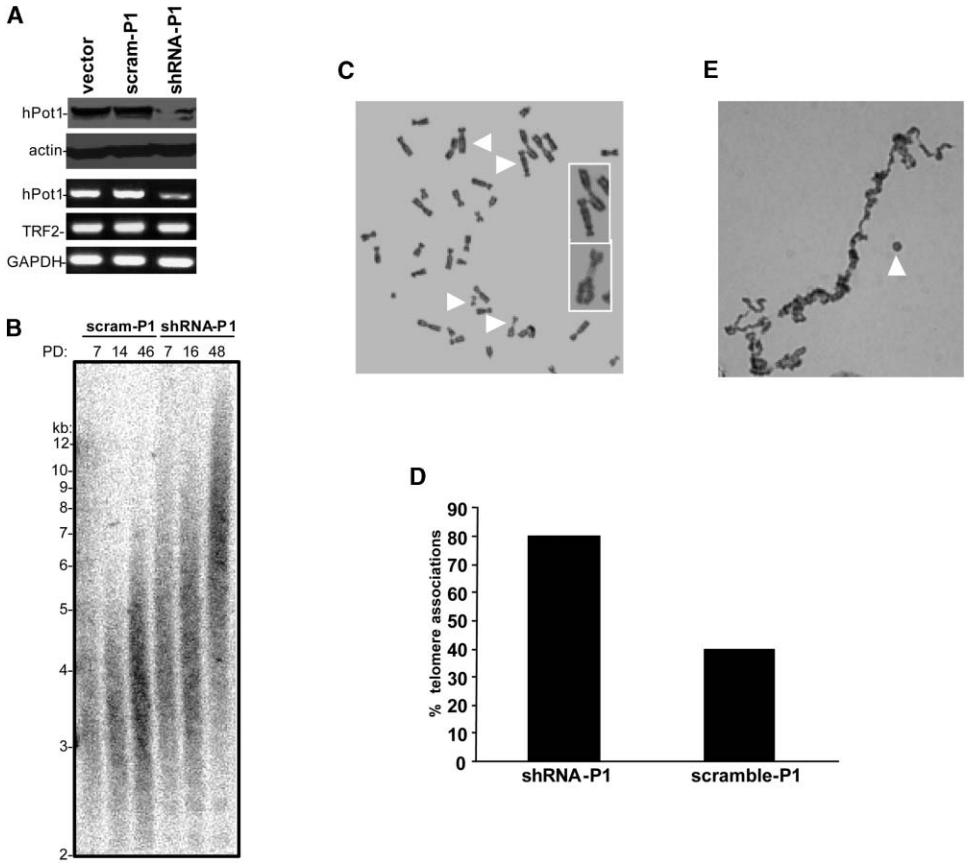

Figure 4. Knockdown of hPot1 Leads to Increased Telomere Associations

(A) RNA and protein analysis of endogenous hPot1 expression in knockdown cells. HT1080 cells were stably infected with retroviruses derived from the plasmid pSUPER-RETRO (vector) or with those encoding shRNA-P1 or scramble-P1 as before. Top panel: Immunoblotting with the rabbit polyclonal anti-hPot1 982 antibody at late passage (population doubling 40) detected endogenous hPot1 protein and confirmed stable knockdown. Actin serves as a loading control. Bottom panel: Total RNA was extracted with RNAzol B reagent (Tel-Test Inc.) from the same cells and subjected to RT-PCR amplification with primer pairs 5′-GAGACTCAGGGTATCACC and 5′-GCTGTCGTCAGGTTCTGA; 5′-GGAAGCTGCTG TCATTATTTG and 5′-AGAACCAGATCCTTCTG to amplify hPot1 and TRF2, respectively. GAPDH was amplified as previously described [26] to serve as a control for cDNA integrity and loading.

(B) Knockdown of hPot1 leads to telomere elongation. Genomic DNA isolated from HT1080 cells stably infected with the described retroviruses at regular intervals was digested with restriction enzymes to liberate telomere-containing fragments, which were detected by Southern hybridization with a telomeric probe, as previously described [26].

(C) Visualization of telomere associations. The described HT1080 cells stably expressing pSUPER-RETRO shRNA-P1 or scramble-P1 control were arrested at metaphase with 10 ng/ml colcemid (Invitrogen), incubated in hypotonic solution (0.075 M KCl), and fixed in methanol:acetic acid (3:1), after which slides were prepared and stained with Giemsa-banding solution (Invitrogen). Arrows denote telomere associations formed from at least two chromosomes viewed at 100× magnification. Insets are enlarged views of telomere associations.

(D) Quantitation of telomere associations. Telomere associations were scored as previously described [10], irrespective of ploidy, in 50 randomly chosen metaphase spreads of the described hPot1 knockdown (hPot1 shRNA-P1) versus scramble-P1 control HT1080 cells.

(E) Chromosome strings. Example of a metaphase spread derived from hPot1 knockdown HT1080 cells with multiple telomere associations, forming a string of chromosomes joined at their ends. Arrow, ring chromosome.

which can break to form micronuclei or persist during cytokinesis to form the *cut*-like chromatin bridges.

hPot1 and Chromosome Stability

We show that transient expression of hPot1 shRNA caused either senescence or apoptosis, in accord with the previously described ability of the tested cells to mount and enact upon a p53-dependant DNA damage response [19]. However, HeLa and HT1080 cells stably expressing hPot1 shRNAs could still continue to proliferate, perhaps as a result of a less efficient knockdown by stable infection methods. We suggest that the apoptosis or growth arrest was due to telomere instability because knockdown of hPot1 led to telomere associations. A failure to properly resolve such telomere associations may underlie the increase in anaphase bridges, which in turn may lead to the formation of micronuclei and *cut*-like chromatin bridges. Telomere dysfunction, rather than shortening, probably accounted for these phenotypes because they were detected in hPot1 knockdown HT1080 cells, which undergo telomere elongation (Figures 4B and 4D). With regard to the novel *cut*-like phenotype, we note that interphase chromatin bridges were induced by destabilizing telomere ends by very different means (Figures 3A–3D) and have been detected at senescence [23], a state characterized by

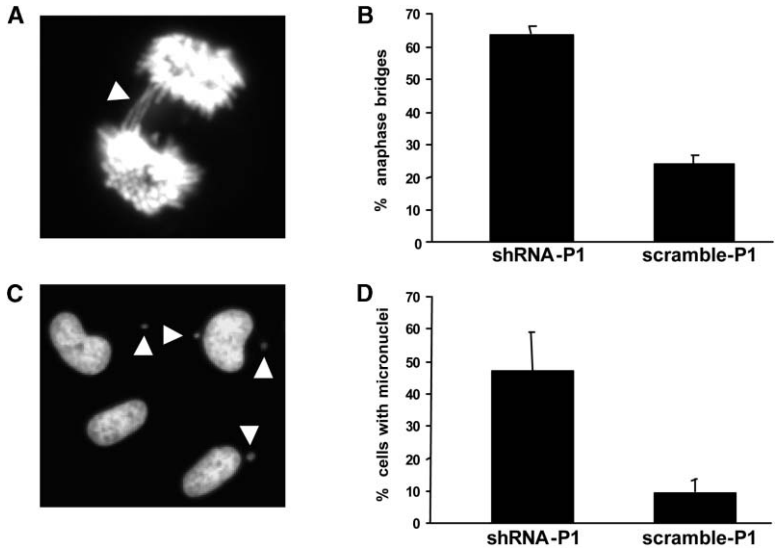

Figure 5. Knockdown of hPot1 Leads to Formation of Anaphase Bridges and Micronuclei

(A) Visualization of anaphase bridges. HT1080 cells stably infected with retroviruses derived from the plasmid pSUPER-RETRO shRNA-P1 or scramble-P1 were fixed and then stained with DAPI. Example of chromatin bridges in a single anaphase cell (arrow) observed in hPot1 knockdown cells. Magnification: 63×.

(B) Quantitation of anaphase bridges. Average and SEM percent of anaphase bridges per 50 randomly chosen anaphase cells of the described hPot1 knockdown (shRNA-P1) and scramble-P1 control HT1080 cells from three independent experiments.

(C) Visualization of micronuclei. An example of micronuclei (arrows) in the hPot1 knockdown HT1080 cells. Magnification: 63×.

(D) Quantitation of micronuclei. Average and SEM percent of micronuclei per 100 randomly chosen interphase cells of the described hPot1 knockdown (shRNA-P1) and scramble-P1 control HT1080 cells from three independent experiments.

dysfunctional telomeres. Moreover, chromatin bridges have been found at telophase of $p53^{-/-}$ mouse fibroblasts in which the *rad51* gene, which plays a role in telomere stability, is disrupted [24]. Thus, we argue that the *cut*-like phenotype can be induced by telomere instability.

Although knocking down hPot1 and disrupting the function of TRF2 both led to the same phenotypes, the effect of hPot1 shRNA was specific for hPot1 because the level of TRF2 mRNA was not altered in the knockdown cells (Figure 4A). Additionally, many of the phenotypes induced by knockdown of hPot1 could be partially rescued by expression of a shRNA-resistant hPot1 protein, although such rescues were quite variable, a topic that is currently under investigation (not shown). Because hPot1, TRF2, and other telomere binding proteins have been found to coimmunoprecipitate [2–7, 25], we argue that the similar phenotypes observed upon affecting the function of TRF2 and hPot1 may result from destabilizing a common telomere protein complex. It remains to be determined, however, if such disruptions ultimately impinge upon one critical protein or if all telomere binding proteins share an equal stake in the function of a telomere protein complex and, ultimately, the stability of chromosome ends. Whatever the case, the described results support the notion that hPot1 plays a role in chromosome end stability.

Acknowledgments

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References

1. Baumann, P., and Cech, T.R. (2001). Pot1, the putative telomere end-binding protein in fission yeast and humans. Science *292*, 1171–1175.
2. Loayza, D., and De Lange, T. (2003). POT1 as a terminal transducer of TRF1 telomere length control. Nature *423*, 1013–1018.
3. Liu, D., O'Connor, M.S., Qin, J., and Songyang, Z. (2004). Telosome—A mammalian telomere associated complex formed by multiple telomeric proteins. J. Biol. Chem. *279*, 51338–51342.
4. Ye, J.Z., Donigian, J.R., Van Overbeek, M., Loayza, D., Luo, Y., Krutchinsky, A.N., Chait, B.T., and De Lange, T. (2004). TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. J. Biol. Chem. *279*, 47264–47271.
5. Liu, D., Safari, A., O'Connor, M.S., Chan, D.W., Laegeler, A., Qin, J., and Songyang, Z. (2004). PTOP interacts with POT1 and regulates its localization to telomeres. Nat. Cell Biol. *6*, 673–680.
6. Ye, J.Z.-S., Hockemeyer, D., Krutchinsky, A.N., Loayza, D., Hooper, S.M., Chait, B.T., and de Lange, T. (2004). Pot1-interacting protein PIP1: a telomere length regulator that recruits POT1 to the TIN2/TRF1 complex. Genes Dev. *18*, 1–6.
7. Houghtaling, B.R., Cuttonaro, L., Chang, W., and Smith, S. (2004). A dynamic molecular link between the telomere length regulator TRF1 and the chromosome end protector TRF2. Curr. Biol. *14*, 1621–1631.
8. Armbruster, B.N., Linardic, C.M., Veldman, T., Bansal, N.P., Downie, D.L., and Counter, C.M. (2004). Rescue of a hTERT mutant defective in telomere elongation by fusion with hPot1. Mol. Cell. Biol. *24*, 3552–3561.
9. Garvik, B., Carson, M., and Hartwell, L. (1995). Single-stranded DNA arising at telomeres in cdc13 mutants may constitute a specific signal for the RAD9 checkpoint. Mol. Cell. Biol. *15*, 6128–6138.
10. van Steensel, B., Smogorzewska, A., and de Lange, T. (1998). TRF2 protects human telomeres from end-to-end fusions. Cell *92*, 401–413.
11. Yanagida, M. (1998). Fission yeast cut mutations revisited: Control of anaphase. Trends Cell Biol. *8*, 144–149.
12. Dykxhoorn, D.M., Novina, C.D., and Sharp, P.A. (2003). Killing the messenger: Short RNAs that silence gene expression. Nat. Rev. Mol. Cell Biol. *4*, 457–467.
13. Baumann, P., Podell, E., and Cech, T.R. (2002). Human Pot1 (protection of telomeres) protein: Cytolocalization, gene structure, and alternative splicing. Mol. Cell. Biol. *22*, 8079–8087.
14. Weinert, T.A., Kiser, G.L., and Hartwell, L.H. (1994). Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. *8*, 652–665.
15. Diede, S.J., and Gottschling, D.E. (1999). Telomerase-mediated

telomere addition in vivo requires DNA primase and DNA polymerases alpha and delta. Cell *99*, 723–733.
16. Pennock, E., Buckley, K., and Lundblad, V. (2001). Cdc13 delivers separate complexes to the telomere for end protection and replication. Cell *104*, 387–396.
17. Smogorzewska, A., van Steensel, B., Bianchi, A., Oelmann, S., Schaefer, M.R., Schnapp, G., and de Lange, T. (2000). Control of human telomere length by TRF1 and TRF2. Mol. Cell. Biol. *20*, 1659–1668.
18. van Steensel, B., and de Lange, T. (1997). Control of telomere length by the human telomeric protein TRF1. Nature *385*, 740–743.
19. Karlseder, J., Broccoli, D., Dai, Y., Hardy, S., and de Lange, T. (1999). p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science *283*, 1321–1325.
20. Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., and Pereira-Smith, O. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA *92*, 9363–9367.
21. Kondo, T., Oue, N., Yoshida, K., Mitani, Y., Naka, K., Nakayama, H., and Yasui, W. (2004). Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma. Cancer Res. *64*, 523–529.
22. Hoffelder, D.R., Luo, L., Burke, N.A., Watkins, S.C., Gollin, S.M., and Saunders, W.S. (2004). Resolution of anaphase bridges in cancer cells. Chromosoma *112*, 389–397.
23. Yalon, M., Gal, S., Segev, Y., Selig, S., and Skorecki, K.L. (2004). Sister chromatid separation at human telomeric regions. J. Cell Sci. *117*, 1961–1970.
24. Tarsounas, M., Munoz, P., Claas, A., Smiraldo, P.G., Pittman, D.L., Blasco, M.A., and West, S.C. (2004). Telomere maintenance requires the RAD51D recombination/repair protein. Cell *117*, 337–347.
25. Kim, S.H., Beausejour, C., Davalos, A.R., Kaminker, P., Heo, S.J., and Campisi, J. (2004). TIN2 mediates functions of TRF2 at human telomeres. J. Biol. Chem. *279*, 43799–43804.
26. Armbruster, B.N., Banik, S.S., Guo, C., Smith, A.C., and Counter, C.M. (2001). N-terminal domains of the human telomerase catalytic subunit required for enzyme activity in vivo. Mol. Cell. Biol. *21*, 7775–7786.
27. Pitt, C.W., Moreau, E., Lunness, P.A., and Doonan, J.H. (2004). The pot1+ homologue in Apergillus nidulans is required for ordering mitotic events. J. Cell Sci. *117*, 199–209.
28. Dynek, J.N., and Smith, S. (2004). Resolution of sister telomere association is required for progression through mitosis. Science *304,* 97–100.