Telomerase promotes formation of a telomere protective complex in cancer cells


Telomerase is a ribonucleoprotein complex that catalyzes addition of telomeric DNA repeats to maintain telomeres in replicating cells. Here, we demonstrate that the telomerase protein hTERT performs an additional role at telomeres that is independent of telomerase catalytic activity yet essential for telomere integrity and cell proliferation. Short-term depletion of endogenous hTERT reduced the levels of heat shock protein 70 (Hsp70-1) and the telomere protective protein Apollo at telomeres, and induced telomere deprotection and cell cycle arrest, in the absence of telomere shortening. Short-term expression of hTERT promoted colocalization of Hsp70-1 with telomeres and Apollo and reduced numbers of deprotected telomeres, in a manner independent of telomerase catalytic activity. These data reveal a previously unidentified noncanonical function of hTERT that promotes formation of a telomere protective complex containing Hsp70-1 and Apollo and is essential for sustained proliferation of telomerase-positive cancer cells, likely contributing to the known cancer-promoting effects of both hTERT and Hsp70-1.

INTRODUCTION

Telomerase is a ribonucleoprotein with reverse transcriptase activity that is responsible for telomere lengthening in cancer cells, germ cells, and normal tissue progenitors. In addition to this well-described role, telomerase has also been proposed to have a telomere protective function that is independent of telomere lengthening (1–3). However, a mechanism for the putative telomerase capping function has not been provided, and since it has not been determined whether catalytic activity is required, it is difficult to ascertain whether this role is truly independent of telomere lengthening.

In addition to telomere capping, a number of studies have proposed other telomere-independent functions of the catalytic component of telomerase (hTERT). There are subpopulations of hTERT that are not assembled into active telomerase, suggesting that hTERT may be sufficiently abundant for noncanonical activities (4). These activities include telomere-independent enhancement of cellular proliferation and survival, transcriptional regulation, and protection from DNA damage (5–8). Some of these noncanonical functions of hTERT, such as stimulation of cell proliferation, are thought to require catalytic activity (3, 9), while hTERT-mediated regulation of transcription driven by RNA polymerases II and III and protection against DNA damage involve a mechanism that is independent of catalytic activity (10–12). Conflicting reports on the role for telomerase catalysis in protection against apoptosis (13–17) further add to the lack of clarity.

Here, we specifically address the question of whether hTERT has a telomere protective function independent of telomerase activity. Human telomeres are protected from nucleolytic attack and DNA repair activities by the actions of a group of six proteins known as “shelterin” (18). A key member of this complex is TRF2, which binds to double-stranded telomeric DNA and helps fold telomeres into a looped-back structure known as a T-loop (19–21). Partial deple- tion of TRF2 from human telomeres results in loss of T-loops and activation of a localized ATM-dependent DNA damage response involving recruitment of DNA damage marker proteins γ-H2AX and 53BP1, representing an “intermediate state” of telomere deprotection, whereas complete loss of TRF2 results in telomeric end-to-end fusions (22–25). A basal level of spontaneous intermediate telomere deprotection is observed in many telomerase-positive immortalized cell lines, and there is an inverse correlation between the number of deprotected telomeres (“telomere dysfunction–induced foci” or TIFs) and the amount of active telomerase (26). We therefore hypothesized that high levels of telomerase (which necessitates high levels of hTERT expression) contribute to protection against intermediate-state telo- mere deprotection. Herein, we demonstrate that hTERT protects against telomere dysfunction and DNA damage signaling at telo- meres and that this protective effect does not require telomerase catalytic activity. The mechanism responsible for this telomere protective effect involves hTERT-induced telomeric localization of the chaperone protein heat shock protein 70 (Hsp70-1), which stabilizes a protective complex consisting of telomeric proteins TRF2 and Apollo.

RESULTS

Depletion of hTERT induces telomere length-independent telomere deprotection and G1 arrest

HT1080 (human fibrosarcoma) cells were used to investigate telo- mere deprotection following depletion of the endogenous pool of hTERT. HT1080 cells are well suited to these investigations due to their higher basal level of telomerase activity and fewer TIFs relative to other telomerase-positive cell lines (26). Telomere deprotection was examined using the highly quantitative metaphase-TIF (meta-TIF)
assay (26), in which colocalization of the DNA damage response marker γ-H2AX and telomeres is scored in cells arrested in metaphase. Short-term depletion of endogenous hTERT, with two separate small interfering RNAs (siRNAs) for 48 hours, significantly increased the number of meta-TIFs (Fig. 1, A and B). Telomere deprotection was independent of telomere shortening, since there was no significant difference in telomere fluorescence intensity in cells depleted of hTERT compared to the control siRNA-transfected cells (Fig. 1C). There was also no increase in the number of telomeres lacking detectable telomere signal in cells depleted of hTERT (fig. S1A), indicating that the additional meta-TIFs are not a result of very short telomeres that are not detectable by fluorescence in situ hybridization (FISH). A similar increase in telomere deprotection was observed following transient depletion of hTERT in a second cell line, transformed MRC5hTERT-NRas fibroblasts, which have stable overexpression of hTERT (fig. S1B). Cells depleted of hTERT accumulated in the G1 phase of the cell cycle (Fig. 1D), which is consistent with telomere dysfunction–induced cell cycle arrest in G1 (24).

To confirm that these results were not due to off-target effects of the siRNAs, a TERT cDNA with mutations conferring resistance to both siRNAs was overexpressed in HT1080 cells for 48 hours (Fig. 1E) with simultaneous depletion of endogenous hTERT expression (Fig. 1F). Overexpression of siRNA-resistant (sr) hTERT rescued the telomere deprotection phenotype (Fig. 1G and fig. S1C), providing further evidence that hTERT has a direct telomere protective function. There was also a partial rescue of the G1 accumulation induced by hTERT depletion upon overexpression of sr hTERT (fig. S1D).

**hTERT reduces TIFs independently of catalytic activity**

The reverse transcriptase domain of hTERT has three conserved aspartic acid residues forming the catalytic triad that is essential for the reverse transcriptase activity of telomerase; mutation of just one of these residues completely abolishes telomerase-mediated telomere lengthening (27). To specifically investigate whether hTERT can protect telomeres independently of its catalytic activity, we used a catalytically inactive hTERT, in which the essential aspartic acid residue at amino acid 712 was substituted with an alanine residue (D712A hTERT) (fig. S2A). Both wild-type (WT) and D712A hTERT were transiently overexpressed for 24 hours in two telomerase–positive cell lines that contain a large number of TIFs, GM639 and JFCF-6/T.1J/6G (Fig. 1H and fig. S2B). Overexpression of either WT or D712A hTERT resulted in a significant reduction in meta-TIFs in both cell lines (Fig. 1I and fig. S2, B and C). To ensure that this effect was not restricted to mitotic cells, the same analysis was conducted on interphase nuclei (instead of metaphase spreads) and a similar rescue of telomere deprotection was observed (fig. S2D). Overexpression of hTERT did not change hTR levels (fig. S2E), excluding the possibility that the reduction in meta-TIFs was an indirect consequence of an increase in hTR levels, leading to increased endogenous telomerase activity and consequent telomere lengthening. No change in telomere length was observed over the 24 hours of hTERT overexpression (Fig. 1J). Thus, the ability of hTERT to reduce telomeric DNA damage signaling was independent of telomerase catalytic activity and not a result of telomere lengthening. Longer-term overexpression of WT hTERT (4 days) did result in telomere lengthening (fig. S2, F and G); as expected, this further reduced the number of meta-TIFs (fig. S2H), since a portion of intermediate-state telomere deprotection is a result of telomere shortening (26). Nevertheless, overexpression of catalytically inactive hTERT for 4 days still resulted in approximately 40 to 50% reduction in telomeric DNA damage signaling compared to vector-transduced cells, representing the portion of telomere protection that cannot be attributed to telomere lengthening (fig. S2H). Moreover, no significant changes in cell cycle progression (fig. S3A) or levels of activation of the ATM-mediated DNA damage response (fig. S3B) were detected following overexpression of hTERT, demonstrating that telomere protection cannot be attributed to either of these factors.

**hTERT induces expression of heat shock protein genes through heat shock factor 1**

Several reports have shown evidence of hTERT regulating gene expression (8, 10, 28–30). To investigate potential gene expression changes involved in telomere protection by hTERT, next-generation mRNA sequencing was performed on GM639 cells transiently overexpressing WT and D712A hTERT for 24 hours (Fig. 2A). Standard differential analysis protocols detected only four genes (in addition to TERT) that were significantly up-regulated >2-fold by both WT and D712A hTERT (compared to an empty vector control), and there were no genes that were down-regulated (Fig. 2B and fig. S4A). All four of the up-regulated genes (HSPA1A, HSPA1B, HSPA6, and DNAJB1) encode heat shock proteins in the Hsp70 family (the protein Hsp70-1 encoded by the almost-identical genes HSPA1A and HSPA1B and the protein Hsp70-6 encoded by HSPA6) or the Hsp40 family (DNAJB1) (31). Up-regulation of these four genes following hTERT overexpression for 24 hours was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis (Fig. 2, C and D, and fig. S4B) and by Western blot for protein Hsp70-1 (Fig. 2E). Up-regulation of members of the Hsp70 family after expression of hTERT has also been previously reported in HeLa and U2OS cells (30).

We next investigated the mechanism behind the up-regulation of Hsp70 genes by hTERT. The increase in HSPA1A and HSPA1B mRNA and Hsp70-1 protein induced by hTERT was dependent on the presence of heat shock factor 1 (HSF1), which is a transcription factor that is transported to the nucleus to bind to heat shock elements in the promoters of these two genes and induce their expression (Fig. 2F and fig. S4, C and D) (32). In vitro studies have demonstrated a transient association between hTERT and Hsp70-1 prior to the assembly of hTERT and hTR (33). We confirmed the interaction of a small proportion of each of these proteins in human cell lysates using coimmunoprecipitation, consistent with a transient interaction in vivo (Fig. 2G), and also demonstrated colocalization between hTERT and Hsp70-1 proteins in the cytoplasm (Fig. 2H). To further confirm the interaction between hTERT and Hsp70-1, we conducted in situ proximity ligation assays (PLA) in HT1080 cells with and without hTERT overexpression. In this technique, two proteins in close proximity (within 40 nm) are detected by primary antibodies (validated in Fig. 2B and fig. S4E), leading to the ligation and amplification of oligonucleotide probes (34). A marked increase in red PLA signals was observed only in cells overexpressing hTERT (green), and PLA signals were not observed in a negative control lacking the primary antibody against Hsp70-1 (Fig. 2I). Three-dimensional (3D) reconstruction demonstrated that, while the majority of PLA signals between hTERT and Hsp70-1 resided in the cytoplasm, there were also a substantial proportion in the nucleus (Fig. 2J). The transient association between these proteins suggests that hTERT needs the chaperone activity of Hsp70-1 for proper folding. The involvement of HSF1 in the up-regulation of heat shock genes by hTERT suggests that hTERT acts like other Hsp70-1 client proteins through a well-established mechanism, in which the client protein induces dissociation of HSF1.
Fig. 1. A noncanonical function of hTERT in telomere protection. (A) Relative TERT mRNA expression measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR) after siRNA treatment of HT1080 cells for 48 hours (mean ± SE; n = 3 independent experiments). Normalized to control siRNA (siSc). *P = 0.011, **P = 0.0018. (B) Left: Representative images from the meta-TIF analysis of HT1080 cells depleted of hTERT for 48 hours. γ-H2AX immunofluorescence in red, telomere FISH in green, and chromosomes in blue. Right: Quantitation of γ-H2AX–associated telomeres from meta-TIF assays in HT1080 cells (mean ± SE; n = 3 independent experiments). Normalized to control siRNA (siSc). **P = 0.0064, ***P = 0.0002. (C) Fluorescence intensity of telomeric signals as a measure of telomere length in HT1080 cells, analyzed using the TFL-TELO program (60); 15 metaphase spreads were analyzed for each sample. One-way analysis of variance (ANOVA) was performed, P = 0.9996. (D) Left: Cell cycle profile using flow cytometry of HT1080 cells treated with control and hTERT siRNAs. Representative experiment quantifying >15,000 cells per condition. The x axis (PI-A) represents the propidium iodide intensity, while the y axis represents the cell count. Right: Quantitation of the proportion of cells in G1, S, and G2-M phases of the cell cycle profile using flow cytometry of HT1080 cells treated with control and hTERT siRNAs. Representative experiment quantifying >15,000 cells per condition. The x axis (PI-A) represents the propidium iodide intensity, while the y axis represents the cell count. (E) Western blot using whole-cell extracts from HT1080 cells showing overexpression of sR hTERT (127 kDa) for 48 hours. Actin (42 kDa) is used as a loading control. (F) Relative TERT mRNA expression after siRNA treatment of HT1080 cells for 48 hours using qRT-PCR with primers specific for endogenous hTERT (mean ± SE; n = 3 independent experiments). Normalized to control siRNA (siSc). **P = 0.0018, ***P < 0.001, ****P < 0.0001. (G) H4TERT Western blot using whole-cell extracts from HT1080 cells showing overexpression of sR hTERT (127 kDa) for 48 hours. Actin (42 kDa) is used as a loading control. (H) Relative TERT mRNA expression after siRNA treatment of HT1080 cells for 48 hours using qRT-PCR with primers specific for endogenous hTERT (mean ± SE; n = 3 independent experiments). Normalized to control siRNA (siSc). **P = 0.0018, ***P < 0.001, ****P < 0.0001, n.s., not significantly different. (I) Quantitation of the percentage of γ-H2AX–associated telomeres from meta-TIF assays in HT1080 cells expressing sR hTERT (mean ± SE; n = 3 independent experiments). **P = 0.0022, ***P = 0.0008. (J) Western blot using whole-cell extracts from GM639 cells, showing transient overexpression of WT and catalytically inactive (D712A) hTERT for 24 hours. Actin was used as a loading control. (I) Left: Representative images from meta-TIF assays using GM639 cells overexpressing either WT or D712A hTERT for 24 hours. γ-H2AX immunofluorescence in red, telomere FISH in green, and chromosomes in blue. Right: Quantitation of the percentage of γ-H2AX–associated telomeres from meta-TIF assays in GM639 cells (mean ± SE; n = 3 independent experiments). **P = 0.0021, ***P = 0.0003. (J) Fluorescence intensity of telomere signals obtained from meta-TIF assays using GM639 cells overexpressing either WT or D712A hTERT for 24 hours. γ-H2AX immunofluorescence in red, telomere FISH in green, and chromosomes in blue. Right: Quantitation of the percentage of γ-H2AX–associated telomeres from meta-TIF assays in GM639 cells (mean ± SE; n = 3 independent experiments). **P = 0.0021, ***P = 0.0003. (J) Fluorescence intensity of telomere signals obtained from meta-TIF assays using GM639 cells overexpressing either WT or D712A hTERT for 24 hours. γ-H2AX immunofluorescence in red, telomere FISH in green, and chromosomes in blue. Right: Quantitation of the percentage of γ-H2AX–associated telomeres from meta-TIF assays in GM639 cells (mean ± SE; n = 3 independent experiments). **P = 0.0021, ***P = 0.0003.
Fig. 2. hTERT induces expression of heat shock protein genes through HSF1 and interacts with Hsp70-1. (A) hTERT Western blot using whole-cell extracts from GM639 cells showing overexpression of WT or catalytically inactive (D712A) hTERT for 24 hours in triplicate samples used for RNA sequencing. Actin is used as a loading control. (B) Venn diagram showing differential gene expression resulting from transient overexpression of WT and catalytically inactive (D712A) hTERT compared to the vector control (Vec). Only five genes were up-regulated >2-fold, including TERT. For full details of gene names and fold changes, refer to fig. S4A. (C) Validation using qRT-PCR of the up-regulation of Hsp70-1 genes HSPA1A and HSPA1B upon transient hTERT overexpression for 24 hours in GM639 cells. mRNA levels are normalized to vector control (mean ± SE; n = 3 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (D) Validation using qRT-PCR of the up-regulation of Hsp70-1 genes HSPA1A and HSPA1B upon transient hTERT overexpression for 24 hours in HT1080 cells. mRNA levels are normalized to vector control (mean ± SE; n = 3 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001. (E) hTERT (127 kDa) and Hsp70-1 (70 kDa) Western blots using whole-cell extracts from GM639 cells transiently overexpressing WT and D712A hTERT for 24 hours. Actin is used as a loading control. (F) Relative mRNA expression of HSPA1A and HSPA1B using qRT-PCR, in GM639 cells overexpressing hTERT with simultaneous depletion of HSF1, 24 hours after transfection (mean ± SE; n = 3 independent experiments). Normalized to Vec + control siRNA (Vec siSc). *P < 0.05, ****P < 0.0001. (G) Immunoprecipitation using antibodies against hTERT (sheep), Hsp70-1 (mouse), and immunoglobulin G (IgG) control (mouse (top) or sheep (bottom)) from lysates of 293T cells overexpressing hTERT. Input (2 × 10^5 cell equivalents) and eluates were visualized on Western blots probed for hTERT (goat antibody) and Hsp70 (rabbit antibody). Volumes of eluate loaded correspond to the following cell equivalents: 2 × 10^5 (hTERT eluate on hTERT blot), 4 × 10^5 (Hsp70 eluate on Hsp70 blot), 1 × 10^6 (IgG control), and 4 × 10^5 (Hsp70 eluate on Hsp70 blot). (H) Representative fluorescent micrographs of GM639 cells transiently overexpressing WT hTERT for 24 hours, with immunofluorescence for Hsp70-1 (red) and GFP-tagged hTERT (green), and 4′,6-diamidino-2-phenylindole (DAPI) staining showing the nucleus. Scale bars, 10 μm. The two images shown are displaying primarily cytoplasmic localization of hTERT. (I) PLA for hTERT and Hsp70-1 (red), in HT1080 cells with and without hTERT overexpression, combined with hTERT immunofluorescence (green) and DAPI staining showing the nucleus (blue). A negative PLA control was performed by leaving out the Hsp70-1 primary antibody (−ve control). (J) 3D reconstruction of image in Fig. 2I, using the Imaris software (Bitplane), to further validate the nuclear localization of hTERT-Hsp70-1 PLA foci (red), with DAPI staining showing the nucleus (blue). See also the Supplementary Materials, fig. S4.
Fig. 3. Hsp70-1 is necessary for full telomere protection, but its overexpression is insufficient. (A) Left: Relative mRNA expression using qRT-PCR, showing knockdown of the indicated genes 48 hours after siRNA transfection of HT1080 cells (mean ± SE; n = 3 independent experiments). **P < 0.0001. Middle: Representative images from the meta-TIF analysis of HT1080 cells with depletion of HSPA1A and HSPA1B for 48 hours. γH2AX immunofluorescence in red, telomere FISH in green, and chromosomes in blue. Right: Quantitation of the percentage of γ-H2AX–associated telomeres from meta-TIF assays in HT1080 cells with depletion of the indicated Hsp70-1 mRNAs (mean ± SE; n = 3 independent experiments). *P = 0.035, **P = 0.0049. (B) Left: Representative hTERT and Hsp70-1 Western blot using whole-cell extracts from GM639 cells, with transient overexpression of hTERT and simultaneous depletion of Hsp70-1 for 24 hours. Actin is used as a loading control. Right: Quantitation of the percentage of γ-H2AX–associated telomeres from meta-TIF assays in GM639 cells overexpressing WT and R3E/R6E mutant hTERT, using qRT-PCR, following sorting for GFP fluorescence-positive cells (mean ± SE; n = 3 independent experiments). **P = 0.018. (C) Left: Representative hTERT Western blot using whole-cell extracts from GM639 cells, showing transient overexpression of D712A hTERT and Myc-tagged ZNF827 (119 kDa). Actin is used as a loading control. Right: Quantitation of Hsp70-1 protein expression following transient overexpression of D712A hTERT and Myc-tagged ZNF827 (mean ± SE; n = 3 independent experiments). *P = 0.0112. (D) hTERT and Myc Western blots using whole-cell extracts from GM639 cells, showing transient overexpression of D712A hTERT and Myc-tagged ZNF827 (119 kDa). Actin is used as a loading control. (F) Relative mRNA expression of HSPA1A (left) and HSPA1B (right) in GM639 cells overexpressing WT and R3E/R6E mutant hTERT, using qRT-PCR, following sorting for GFP fluorescence-positive cells (mean ± SE; n = 3 independent experiments). **P < 0.001. (G) Left: Hsp70-1 Western blot using whole-cell extracts from GM639 cells, showing an up-regulation of Hsp70-1 following transient overexpression of D712A hTERT and Myc-tagged ZNF827. Actin is used as a loading control. Right: Quantitation of Hsp70-1 protein expression following transient overexpression of D712A hTERT and Myc-tagged ZNF827 (mean ± SE; n = 3 independent experiments). **P = 0.004, ***P = 0.0006. (H) Left: Representative images from meta-TIF assays using GM639 cells transiently overexpressing either D712A hTERT or ZNF827. γ-H2AX immunofluorescence in red, telomere FISH in green, and chromosomes in blue. Right: Quantitation of the percentage of γ-H2AX–associated telomeres from meta-TIF assays in GM639 cells (mean ± SE; n = 3 independent experiments). *P = 0.0189. See also the Supplementary Materials, fig. S5.
from its normal sequestration by Hsp70-1, allowing HSF1 to induce expression of heat shock proteins (32, 35). This mechanism does not normally require the client protein itself to localize to the promoters of heat shock genes, so we infer that this is a different mechanism to the up-regulation of nuclear factor κB (NF-κB) pathway genes by hTERT, which involves binding of hTERT to the promoters of these genes (8).

**Hsp70-1 has a telomere protective role**

Since heat shock protein genes were the only genes up-regulated after 24 hours of hTERT overexpression, we tested whether Hsp70-1 could be involved in hTERT-mediated telomere protection. HSPA1A or HSPA1B mRNA was depleted in HT1080 cells, which have low basal levels of meta-TIFs, by siRNA treatment for 48 hours (Fig. 3A). Depletion of expression of either gene resulted in a significant increase in telomeric DNA damage signaling, demonstrating that endogenous levels of Hsp70-1 are required to protect telomeres in these cells (Fig. 3A). Depletion of HSPA1A or HSPA1B abrogated the reduction of TIFs induced by hTERT overexpression in GM639 cells (Fig. 3B and fig. S5, A and B), consistent with the interpretation that the Hsp70-1 protein is required for the hTERT-mediated telomere protective function. Western blot analyses revealed no significant changes in total exogenous hTERT protein levels following depletion of Hsp70-1 (Fig. 3B and fig. S5A), so it is unlikely that Hsp70-1 is required indirectly for hTERT-mediated telomere protection via modulation of hTERT protein levels.

**Induction of Hsp70-1 expression is insufficient to induce telomere protection**

The requirement for Hsp70-1 for full telomere protection in the presence of endogenous levels of hTERT (Fig. 3A) implies that mere induction of Hsp70-1 expression is not sufficient for telomere protection and that this is a specific function of hTERT. In support of this concept, we identified a mutated version of hTERT that retained the ability to induce Hsp70-1 expression but was unable to confer telomere protection. Expression of an hTERT variant that was reported to show altered subcellular localization [R3E/R6E (36)] resulted in up-regulation of both HSPA1A and HSPA1B expression, to a similar level as expression of WT hTERT (Fig. 3C), without resulting in a telomere protective effect (Fig. 3D). These data suggest that up-regulation of Hsp70-1 expression is insufficient for the telomere protective function of hTERT. The reason that the R3E/R6E mutation affects the ability of hTERT to protect telomeres is under further investigation.

To further confirm that induction of Hsp70-1 expression is insufficient for telomere protection, and that Hsp70-1-mediated telomere protection is a specific function of hTERT, we tested an independent Hsp70-1 client protein, also recently found to be telomere-associated, for its ability to confer telomere protection. The zinc finger protein ZNF827 has been shown to recruit the NuRD complex to telomeres in cells that have activated the alternative lengthening of telomeres (ALT) mechanism (37). Overexpression of ZNF827 in GM639 cells (Fig. 3E) resulted in a significant increase in HSPA1A and HSPA1B mRNA and Hsp70-1 protein levels compared to the empty vector control, albeit to a lesser extent than upon overexpression of D712A hTERT (Fig. 3, F and G). Similarly to hTERT, ZNF827 also induced a consistent though not statistically significant increase in expression of the heat shock genes HSPA6 and DNAJB1 (fig. S5C). However, ZNF827 overexpression did not change the level of meta-TIFs relative to the empty vector control (Fig. 3H). This provides further evidence that induction of Hsp70-1 expression alone is insufficient to induce telomere protection and that promotion of Hsp70-mediated telomere protection is a specific ability of hTERT.

**hTERT induces telomeric localization of heat shock protein Hsp70-1**

To determine whether the role of Hsp70-1 in telomere protection requires localization of the protein to telomeres, we performed immunofluorescence on HT1080 cells using a validated antibody against Hsp70-1 (fig. S6A), together with FISH with a telomere probe (Fig. 4A). A subset of telomere foci substantially overlapped with Hsp70-1 foci in three dimensions, demonstrating that Hsp70-1 physically localizes to a subset of telomeres. Furthermore, siRNA-mediated depletion of hTERT resulted in an ~2-fold reduction in Hsp70-telomere colocalizations (Fig. 4, A and B, and fig. S6B), which correlates with the ~2-fold increase in meta-TIFs in these cells upon hTERT depletion (Fig. 1B). This suggests that hTERT is needed for Hsp70-1 to localize to telomeres. As further evidence for this hypothesis, overexpression of WT hTERT resulted in an ~2-fold increase in Hsp70-telomere colocalizations (Fig. 4, A and B, and fig. S6C), demonstrating that induction of higher levels of Hsp70-1 expression is insufficient for this effect and that hTERT is directly promoting telomeric localization of Hsp70-1.

To further confirm the telomeric localization of Hsp70-1, we conducted in situ PLA between Hsp70-1 and the telomere-binding protein TRF2 in HT1080 cells with and without hTERT overexpression. PLA signals were detected between Hsp70-1 and TRF2 (antibodies validated in fig. S6, A and E), providing additional evidence for telomeric localization of Hsp70-1 (Fig. 4F). Overexpression of WT hTERT led to the appearance of nuclei with greater numbers of Hsp70-TRF2 PLA foci (Fig. 4F), consistent with the immunofluorescence results in Fig. 4C, and supportive of the hypothesis that hTERT drives the localization of Hsp70 to telomeres. Together, these data strongly suggest that Hsp70-1 mediates telomere protection by localizing to telomeres and that this localization is promoted by hTERT.

**hTERT induction of Hsp70-1 telomere localization is dependent on Apollo**

We next explored the mechanism by which Hsp70-1 reduces DNA damage signaling at telomeres. The cause of spontaneous intermediate state telomeres in telomerase-positive cells is not fully understood, but a reduction in the levels of shelterin proteins, especially TRF2, has been shown to induce TIFs (23). These observations raise the question of whether overexpression of TRF2 can suppress spontaneous TIFs in telomerase-positive cells, as it does in ALT-positive cells (26). Consistent with this notion, overexpression of TRF2 resulted in a reduction of meta-TIFs in GM639 cells (Fig. 5A and fig. S7A), demonstrating that TRF2 participates in restoration of a protective telomere structure, most likely a T-loop (25), at spontaneously arising deprotected telomeres.

TRF2 is assisted in its telomere protective function by the telomere-associated 5′ exonuclease Apollo (also known as SNM1B and encoded by DNA cross-link repair 1B gene, DCLRE1B) (38, 39). Furthermore, an interaction between Apollo and Hsp70-1 has been reported (40),...
suggesting that the telomere protective function of Hsp70-1 may involve Apollo. PLA was conducted to verify protein-protein interactions between Hsp70-1 and Apollo and determine whether these were increased upon hTERT overexpression. Reliable antibodies against Apollo are not available, so Myc-tagged Apollo was overexpressed, and PLA using anti-Myc and anti-TRF2 antibodies provided a positive control (Fig. 5B), confirming the interaction between these proteins that was previously inferred by immunofluorescence (38, 39).

Fig. 4. hTERT induces telomeric localization of heat shock protein Hsp70-1. (A) Representative fluorescent micrographs of HT1080 cells treated with two separate siRNAs targeting hTERT, with immunofluorescence for Hsp70-1 (red) and FISH for telomeres (green), and DAPI staining showing the nucleus (blue). Scale bars, 10 μm. White arrowheads indicate colocalizations between Hsp70-1 and telomeres (yellow), one of which is shown in the zoomed image on the far right. (B) Top: Relative hTERT mRNA expression measured by qRT-PCR after hTERT siRNA treatment of HT1080 cells (mean ± SE; n = 3 independent experiments). ***P < 0.001. Bottom: Quantitation of the colocalizations of Hsp70-1 and telomeres in HT1080 cells depleted of hTERT (mean ± SE; n = 3 independent experiments). ****P < 0.0001. (C) Representative fluorescent micrographs of GM639 cells sorted for cells overexpressing GFP-tagged WT hTERT or R3E/R6E hTERT, with immunofluorescence for Hsp70 (red), FISH for telomeres (green), and DAPI staining showing the nucleus (blue). Scale bars, 10 μm. White arrowheads indicate colocalizations between Hsp70 and telomeres (white), one of which is shown in the zoomed image on the far right. (D) Quantitation of colocalizations of Hsp70-1 and telomeres in GM639 cells overexpressing WT or R3E/R6E mutant hTERT (mean ± SE; n = 3 independent experiments). ****P < 0.0001. (E) Quantitation of colocalizations of Hsp70-1 and telomeres in GM639 cells overexpressing either D712A hTERT or ZNF827 (mean ± SE; n = 3 independent experiments). ****P < 0.0001. (F) PLA for detection of Hsp70-1 and TRF2 interactions (red) in HT1080 cells with and without hTERT overexpression. See also the Supplementary Materials, fig. S6.
Fig. 5. hTERT promotion of Hsp70-1 telomere localization is dependent on Apollo and assists the telomere protective function of Apollo. (A) Quantitation of the percentage of γ-H2AX–associated telomeres from meta-TIF assays in GM639 cells (mean ± SE; n = 3 independent experiments) with or without overexpression of TRF2. ***P = 0.0002. (B) Left: Representative Western blot showing transient overexpression of Myc-DDK–tagged Apollo (60 kDa) in HT1080 cells. Actin is used as a loading control. Right: PLA conducted for detection of Apollo–TRF2 interactions (red) in HT1080 cells with myc–Apollo overexpression. An anti-Myc tag antibody was used for Apollo detection. (C) PLA for detection of Apollo–Hsp70 interactions (red) in HT1080 cells overexpressing Apollo with and without hTERT overexpression. An anti-Myc tag antibody was used for Apollo detection. (D) Immunoprecipitation using antibodies against Myc tag (mouse antibody), Hsp70-1 (mouse), and IgG control (mouse) from lysates of 293T cells overexpressing Myc-DDK (FLAG)–tagged Apollo. Input (2 × 10^4 cell equivalents) and eluates were visualized on Western blots probed with antibodies against DDK (FLAG) tag (rabbit) and Hsp70 (rabbit). Volumes of eluate loaded correspond to the following cell equivalents: 5 × 10^5 (Apollo eluate on Apollo blot and Hsp70 blot), 1 × 10^6 (IgG control), 3 × 10^6 (Hsp70 eluate on Apollo blot), and 4 × 10^6 (Apollo eluate on Hsp70 blot). (E) Representative fluorescent micrographs (left) and quantitation of normalized colocalizations per cell (right) (mean ± SE; n = 3 independent experiments) of HT1080 cells overexpressing Myc-DDK–tagged Apollo and the indicated siRNAs, with immunofluorescence for Myc tag (red) and TRF2 (green), and DAPI staining showing the nucleus (blue). Scale bars, 10 µm. White arrowheads indicate colocalizations between Apollo and TRF2 (white). ***P = 0.0038, **P < 0.01. (F) Quantitation of the percentage of γ-H2AX–associated telomeres from meta-TIF assays in GM639 cells with knock-down of DCLRE1B (Apollo), with and without overexpression of D712A hTERT (mean ± SE; n = 3). ***P = 0.0056. Note that the depletion of Apollo in GM639 cells, which have a high basal level of meta-TIFs, did not further increase telomere deprotection, suggesting that this cell line is unable to tolerate higher levels of telomere dysfunction. (G) Representative fluorescent micrographs of GM639 cells overexpressing WT hTERT with immunofluorescence for Hsp70-1 (red) and TRF2 (green), and DAPI staining showing the nucleus (blue). Scale bars, 10 µm. White arrowheads indicate colocalizations between Hsp70-1 and TRF2 (yellow), one of which is shown in the zoomed image on the right. (H) Quantitation of the colocalizations of Hsp70-1 and TRF2 immunofluorescence in GM639 cells shown in (G) (mean ± SE; n = 3). ****P < 0.0001. (I) Left: Examples of normal or aberrant telomere staining on chromosomes from metaphase spreads of HT1080 cells, with FISH against telomeres (green) and centromeres (red). Right: Quantitation of numbers of “fragile telomeres” per metaphase in HT1080 cells depleted of Hsp70 (siHSPA1A + siHSPA1B), hTERT, or Apollo (mean ± SE; n = 40 metaphases counted from each of three independent experiments). *P = 0.02, ****P < 0.0001. See also the Supplementary Materials, fig. S7.
PLA foci were also observed with anti-Myc and anti–Hsp70-1 antibodies, providing in situ evidence for an interaction between Apollo and Hsp70-1, and these foci increased in number upon hTERT overexpression (Fig. 5C). We also detected interaction of a small proportion of overexpressed Apollo and Hsp70-1 in cell lysates by coimmunoprecipitation (Fig. 5D).

The interaction between Hsp70-1 and Apollo suggests that Hsp70-1 binds to and stabilizes Apollo at telomeres, allowing it to carry out its previously described telomere protective function. To provide evidence for this, we quantitated the number of Apollo foci that colocalize with telomeres (Fig. 5E). Depletion of either Hsp70-1 or hTERT significantly reduced Apollo-TRF2 colocalizations, providing evidence that both Hsp70-1 and hTERT are needed for optimal Apollo levels at telomeres.

In addition, catalytically inactive hTERT was no longer able to confer telomere protection in cells with high meta-TIF levels in the absence of Apollo (Fig. 5F and fig. S7B); this result is consistent with an involvement of Apollo in activity-independent hTERT-mediated telomere protection. To provide further evidence for this, we determined whether the absence of Apollo affected the telomeric localization of Hsp70-1 promoted by hTERT. Colocalizations between Hsp70-1 and TRF2 immunofluorescence foci increased after overexpression of catalytically inactive hTERT, and this effect was abrogated by depletion of Apollo (Fig. 5, G and H, and fig. S7, C and D). This provides evidence that Apollo bridges the interaction between Hsp70-1 and TRF2. Together, these data provide evidence for interactions between Hsp70-1, Apollo, and TRF2 at telomeres, which are increased by hTERT overexpression.

While the molecular mechanism of Apollo-mediated telomere protection has not been fully elucidated, a role for Apollo in facilitating DNA replication in human cells has been reported, both at telomeres and elsewhere in the genome (38, 41, 42). To determine whether Hsp70-1 or hTERT could facilitate this function of Apollo, we measured the numbers of aberrant telomere signals on metaphase chromosomes [known as a “fragile telomere” response and shown to be indicative of a telomere replication defect (43)]. Depletion of either Hsp70-1 or Apollo significantly increased fragile telomere numbers per cell (Fig. 5I), corroborating the role of Apollo in telomere replication, and providing evidence that Hsp70-1 facilitates this role. Depletion of hTERT induced a more subtle increase in fragile telomere numbers with one of two siRNAs; however, hTERT depletion results in loss of only one or two Hsp70-1/telomere foci per cell (Fig. 4, A and B) and there is a substantial background level of fragile telomeres in these cells (average ~4 per cell), so only a subtle effect on this phenotype would be expected. Nevertheless, together, the data in Figs. 4 and 5 support a model in which hTERT promotes Hsp70 localization to telomeres, which, in turn, stabilizes Apollo at telomeres, facilitating its telomere protective role.

**DISCUSSION**

This study demonstrates a role for the telomerase protein hTERT in telomere protection that is unequivocally independent of telomerase catalytic activity and its canonical function in telomere lengthening. Elucidating the mechanism underlying this effect revealed that hTERT enhances telomere protection by promoting localization of Hsp70-1 with the telomere-capping proteins Apollo and TRF2 (Fig. 6). Furthermore, we demonstrate that this telomere protective collaboration of Hsp70-1 and hTERT operates constitutively at endogenous telomerase levels in at least some telomerase-positive immortal cell lines, without hTERT overexpression.

We provide direct evidence that Hsp70-1, a protein implicated in the DNA damage response elsewhere in the genome (40, 44), localizes with TRF2 at telomeres (Figs. 4 and 5G). Hsp70-1 also interacts with and stabilizes the telomere protective protein Apollo (Fig. 5, B to D) (40). Several studies have reported a role for Apollo in facilitating genomic and telomeric DNA replication in human cells (38, 41, 42). Here, we demonstrate that Apollo functions as a bridge between Hsp70-1 and TRF2 and that depletion of Apollo impedes hTERT-mediated Hsp70-1 colocalization with TRF2 and hTERT-mediated telomere protection. Furthermore, we provide evidence that both hTERT and Hsp70-1 are needed to maintain optimal levels of Apollo at telomeres to assist its function in promoting telomere replication.

Our data implicate the hTERT, Hsp70, and Apollo pathway in protecting against defects in telomeric replication such as stalled replication forks, but it is possible that this pathway also protects against telomeric DNA damage responses arising from other causes. The ATM-dependent DNA damage response that occurs upon depletion of TRF2 is known to correlate with loss of T-loops from telomeres (25). The spontaneous telomeric DNA damage signals present in telomerase-positive cells are suppressed by overexpression of TRF2 (Fig. 5A), so it is likely that some of these signals are due to loss of T-loops, resulting from critical telomere shortening or other causes. Whether Hsp70 and Apollo are involved in T-loop restoration is under investigation.

Telomere protection by Hsp70-1 was not an artifact of hTERT overexpression, since depletion of endogenous hTERT in telomerase-positive cells reduced the basal number of Hsp70-1 foci localizing at telomeres (Fig. 4B), concomitant with an increase in TIFs and induction of cell cycle arrest in G1 (Fig. 1, B and D). In addition, depletion of endogenous Hsp70-1 caused telomere deprotection when hTERT was expressed at endogenous levels (Fig. 3A). These results indicate that these pathways are constitutively required in at least some telomerase-positive human cancer cell lines for evasion of telomeric DNA damage signals, providing a potential mechanism contributing to the known cancer-promoting properties of Hsp70-1 (45).

Heat shock proteins are chaperones that assist the folding of specific nascent polypeptides (46). Herein, we demonstrate that hTERT induces expression of Hsp70-1; together with multiple lines of evidence of a transient interaction between the two proteins, this suggests that hTERT is a client protein of Hsp70-1. hTERT induction of Hsp70-1 expression is mediated by transcription factor HSFI1 but does not appear to be due to a generalized proteotoxic stress response, since only a specific subset of four heat shock response genes are induced. All experiments in this study were carried out at 37°C, i.e., not under heat shock conditions; HSFI1 has been shown to protect telomere integrity during heat shock (47), but whether this effect involves Hsp70-1 remains to be investigated. Nevertheless, proteins of the Hsp70 family are well known to function in nonstressed cells, in which they bind newly synthesized polypeptides and assist their folding [reviewed in (32)]. HSFI1 is also known to modulate levels of Hsp70-1 and cochaperones, including HSPA6 and DNAJB1, under nonstressed conditions (48, 49). Thus, it is likely that induction of these four chaperones by hTERT plays a specific physiological role in folding of the hTERT protein in nonstressed cells. In further support of a physiological role for interactions between hTERT and Hsp70-1, cells from Hsp70-1 knockout mice have reduced telomerase activity.
and show telomere abnormalities, again suggesting the involvement of this chaperone in telomerase stabilization in vivo (50). These data support a model whereby Hsp70-1 stabilizes hTERT that is not associated with hTR, and induction of Hsp70-1 expression by hTERT allows a positive feedback loop to exert functions independent of telomere lengthening (Fig. 6).

However, our data also demonstrate that, while Hsp70-1 is necessary for full telomere protection, up-regulation of its expression is not sufficient for this effect, since a mutant version of hTERT and the unrelated protein ZNF827 are able to up-regulate Hsp70-1 expression, yet cannot reduce TIFs or induce an increase in Hsp70-1 localization to telomeres. The additional property of hTERT that results in increased Hsp70-1 localization is under investigation.

Our data are consistent with the possibility that cancer cells use additional molecules of hTERT, not assembled into active telomerase complexes (4), to promote telomere stability. While the number of hTERT molecules in immortal human cells is reported to be <1000 per cell (4, 51), the technical limitations in quantitating these low levels of protein necessitate the use of indirect calculations based on amounts of assembled, active telomerase. Cancer cells also express catalytically inactive isoforms of hTERT arising from alternative splicing of its mRNA, levels of which can far exceed those of the active isoform (52); it is therefore likely that the total amount of hTERT protein molecules greatly exceeds 1000 per cell, providing sufficient hTERT for its noncanonical functions in addition to telomere maintenance.

The DNA-protective effects of hTERT may not be restricted to telomeres, but since telomeric DNA damage is not as efficiently repaired as damage at other sites in the genome (53, 54), and normal somatic cells only need to acquire five TIFs to initiate p53-dependent senescence (55), cancer cells must overcome the telomeric DNA damage response to avoid growth arrest or apoptosis. While hTERT is not transcribed in most somatic cells, up-regulation of oncogenic transcription factors such as Myc, AP1, SP1, and NF-κB, or mutation of the hTERT promoter, results in transcriptional reactivation of hTERT in cancer cells, which not only endows cells with replicative immortality but also provides the other cancer-promoting properties of telomerase that collectively contribute to the hallmarks of cancer (5, 56). This has implications for telomerase-based cancer therapeutics. One of the disadvantages of inhibitors of telomerase catalytic activity is the lag time taken for cancer cell growth arrest due to the time taken for telomeres to shorten to a critical length. However, targeting hTERT expression would remain specific to cancer cells, while simultaneously suppressing both its canonical telomere lengthening effects and its telomere-independent effects.

**Materials and Methods**

**Plasmid construction**

hTERT mutations were introduced into the plasmid pApex-3P-hTERT (57) using several cloning methods. Single (D712A) or multiple amino acid (R3E/R6E) point mutations were introduced using site-directed mutagenesis (QuickChange II XL Site-Directed Mutagenesis kit) following instructions from the manufacturer (Agilent Technologies). pApex-3P-hTERT constructs with a green fluorescent protein (GFP) tag (immediately N terminal of the hTERT open reading frame) were designed to overcome the low transfection efficiency of some constructs to allow for fluorescence-activated cell sorting (FACS) to select GFP-positive (i.e., hTERT overexpressing) cells. To make the GFP-tagged constructs, the In-Fusion HD cloning kit (Clontech Laboratories Inc.) was used, following the manufacturer’s instructions. For the sR hTERT, a synthetic double-stranded DNA fragment (Integrated DNA Technologies) containing silent mutations in the hTERT sequence targeted by the two siRNAs used in the study was ligated into restriction-digested pApex-3P-hTERT plasmid. For retroviral transductions, pMIG retroviral vectors that encode either WT or catalytically inactive (D868A/D869A) hTERT and a GFP downstream of an internal ribosomal entry site were used. The complete hTERT gene was sequenced in all constructs to confirm the absence of unwanted changes. Other constructs used were myc-DDK-tagged ZNF827 (37), myc-DDK-tagged DCLRE1B (catalog no. RC205744; OriGene), and pLXSN or pLXSN-TRF2 (26).

**Cell culture**

Immortalized cell lines HT1080 (fibrosarcoma; American Type Culture Collection), human embryonic kidney (HEK) 293T [adenovirus-immortalized human embryonic kidney, T. Adams; Commonwealth Scientific and Industrial Research Organisation (CSIRO)], JFCF6/T.1/J6G [SV40-immortalized jejunal fibroblasts, P. Bonnefin and R. Reddel; Children’s Medical Research Institute (CMRI)], GM639...
Immunoprecipitation

Events were gated. Figures were generated using FlowJo 7.2.2 software. As well as propidium iodide fluorescence. A total of 20,000 counting events were excluded from the analysis based on forward and side scatter signals, flow cytometry (BD Biosciences, USA). Cell debris and dead cells were analyzed the following day by flow cytometry using a FACSCanto II (Merck Millipore), with rotation for 1 hour at 4°C. Lysates were heated at 65°C for 10 min and loaded onto Nupage 12% bis-tris gels for lower–molecular weight proteins; Life Technologies, which were electrophoresed at 150 V for 70 to 100 min. Protein was transferred onto a nitrocellulose membrane using degassed transfer buffer (Towbin Buffer: 25 mM tris-base, 192 mM glycine, 20 mM NaCl, and 5 mM MgCl2, 300 mM sucrose, and 0.5% Triton X-100, 100 mM NaCl, 2 mM MgCl2, 0.1% (v/v) Triton X-100, 100% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), Complete Protease Inhibitor Cocktail (Roche), and Phosphatase Inhibitor Cocktail Set II (Merck Millipore)], with rotation for 1 hour at 4°C. Lysates were clarified with centrifugation at 16,000 g for 1 hour at 4°C, and supernatants were precleared with protein G agarose beads (25 µl per ml of lysate) with rotation overnight at 4°C. Beads were washed three times in lysis buffer and lysed in 4 × LDS buffer (see below) in preparation for Western blot analysis.

Western blots

Cells were lysed with 4 × LDS buffer (2% lithium dodecyl sulfate, 106 mM tris-HCl, 141 mM tris-base, 40% glycerol, 0.075% SERVA Blue G50, and 0.025% phenol red). Per 100 µl of 4 × LDS buffer, 2 µl of β-mercaptoethanol (Sigma-Aldrich) and 2 µl of Benzonase nuclelease (Novagen) were added to make a final concentration of 1 × 10^4 cells/ml. Lysates were heated at 65°C for 10 min and loaded onto Nupage 4-16% Bis-Tris gels (3 to 8% tris acetate for high–molecular weight proteins and 4 to 12% bis-tris gels for lower–molecular weight proteins; Life Technologies), which were electrophoresed at 150 V for 70 to 100 min. Protein was transferred onto a nitrocellulose membrane using degassed transfer buffer (Towbin Buffer: 25 mM tris-base, 192 mM glycine, and 10% methanol) at 100 V for 60 min. Membranes were blocked with 5% milk/TBST (15.4 mM tris-HCl, 4.6 mM tris-base, 137 mM NaCl, and 1% Tween 20), and primary antibody incubations were conducted at 4°C overnight. Membranes were washed with TBST and incubated with a secondary antibody conjugated to horseradish peroxidase (Dako) for 1 hour at room temperature. Blots were again washed with TBST and then exposed to appropriate ratios of ECL plus detection kit (GE Healthcare) and visualized using the Fujifilm LAS4000 Multigauge imager.

Antibodies used for Western blotting include anti-hTERT (goat polyclonal, catalog no. SC7215, 1:1000; Santa Cruz Biotechnology), anti-pS1981 ATM (rabbit polyclonal, catalog no. AB81929, 1:1000; Abcam), anti-ATM (rabbit polyclonal, catalog no. AB32420, 1:2000; Abcam), anti-pT68 Chk2 (rabbit polyclonal, catalog no. 2661, 1:1000; Cell Signaling), anti-Chk2 (mouse monoclonal, catalog no. 05-649, 1:1000; Merck Millipore), anti-Hsp70/Hsp72 2H9 (mouse monoclonal, catalog no. MABE1130, 1:2000; specific for Hsp70-1; Merck Millipore), anti-Hsp70 (rabbit polyclonal, catalog no. ab79852, 1:10,000; Abcam), anti-TRF2 (rabbit polyclonal, catalog no. NB11-57130, 1:400; Novus), anti-Myc tag (7D10) (rabbit polyclonal, catalog no. 2278, 1:1000; Cell Signaling), anti-DYKDDDDK Tag (rabbit polyclonal, catalog no. 23685, 1:1000; Cell Signaling), anti-vinculin (mouse monoclonal, catalog no. V9131, 1:10000; Sigma-Aldrich), and anti-actin (rabbit polyclonal, catalog no. A2103, 1:10000; Sigma-Aldrich).

Immunofluorescence and FISH

Cell cycle analysis

Cells were harvested and fixed with ice-cold 100% ethanol and incubated at 4°C overnight. The following day, cells were collected by centrifugation at 800 g for 5 min, and the cell pellet was washed in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). A total of 1 × 10^6 cells were resuspended in 250 µl of propidium iodide staining solution [1% BSA, 3.8 mM Tri-sodium citrate, propidium iodide (40 µg/ml), and ribonuclease A (RNase A) (50 µg/ml) in PBS] and then incubated at room temperature for 30 min to promote RNase digestion. Cells were incubated overnight at 4°C and were analyzed the following day by flow cytometry using a FACS Canto flow cytometer (BD Biosciences, USA). Cell debris and dead cells were excluded from the analysis based on forward and side scatter signals, as well as propidium iodide fluorescence. A total of 20,000 counting events were gated. Figures were generated using FlowJo 7.2.2 software.

Immunoprecipitation

Cells were lysed at a concentration of 2 × 10^6 cells/ml in lysis buffer [20 mM Hepes–KOH (pH 7.9), 100 mM NaCl, 2 mM MgCl2, 0.1% (v/v) Triton X-100, 100% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM diithiothreitol (DTT), Complete Protease Inhibitor Cocktail (Roche), and Phosphatase Inhibitor Cocktail Set II (Merck Millipore)], with rotation for 1 hour at 4°C. Lysates were clarified with centrifugation at 16,000 g for 1 hour at 4°C, and supernatants were precleared with protein G agarose beads (25 µl per ml of lysate; Roche) for 15 min at 4°C prior to incubation with antibody for 1 hour at 4°C. Antibodies used for immunoprecipitation include polyclonal sheep anti-hTERT (catalog no. abx120550, 20 µg/ml; Abbexa), anti-Hsp70/Hsp72 2H9 (mouse monoclonal IgG1, catalog no. MABE1130, specific for Hsp70-1; 20 µg/ml; Merck Millipore), and anti-Myc tag [mouse monoclonal IgG2ax, catalog no. 2276S (9B11), 1:1000; Cell Signaling]. Immunoglobulin G (IgG) isotype-specific negative controls used with each antibody were sheep polyclonal IgG (catalog no. ab37385; Abcam), mouse IgG1 (catalog no. ab81032; Abcam), and mouse IgG2ax (catalog no. ab18415; Abcam). Immunoprecipitates were recovered with protein G agarose beads (25 µl per ml of lysate) with rotation overnight at 4°C. Beads were washed three times in lysis buffer and lysed in 4 × LDS buffer (see below) in preparation for Western blot analysis.

Meta-TIF assay

Cells were arrested in metaphase using colcemid (20 ng/ml) (Karyomax) added 3 hours prior to harvesting. Following cytocentrifugation, cells were permeabilized using preextraction buffer [20 mM Hepes–KOH (pH 7.9), 20 mM NaCl, 5 mM MgCl2, 300 mM sucrose, and 0.5% (v/v) NP-40] for 10 min at room temperature and then fixed in 4% formaldehyde in PBS for 10 min. Immunofluorescence for γ-H2AX and telomere FISH were conducted as previously described (26) using a mouse monoclonal antibody against γ-H2AX (catalog no. 05-636, diluted 1:1000; Merck Millipore). Imaging of slides for the meta-TIF assay was conducted as previously described (26). Approximately 100 to 150 metaphase spreads were counted for each treatment, with three biological replicates performed for each experiment.

Telomere FISH on metaphase spreads

Cell cultures were treated with colcemid (20 ng/ml) for the last 4 hours of culture to accumulate mitotic cells. Cells were harvested by trypsinization and incubated in hypotonic buffer (0.2% KCl and 0.2% per ml of lysis buffer) with rotation overnight at 4°C. Beads were washed three times in lysis buffer and lysed in 4 × LDS buffer (see below) in preparation for Western blot analysis.
Tri-sodium citrate for 10 min at 37°C. Ice-cold fixative (3:1 methanol/acetic acid) was then added, and the cells were incubated for 5 min on ice. The cells were then centrifuged at 277g for 8 min and washed three times in ice-cold fixative. Concentrated cells in fixative were then dropped onto clean, dry microscope slides held over a water bath at 75°C for 3 s and left to dry for 48 hrs. Slides were then treated with deoxyribonuclease (DNase)–free RNase A (100 μg/mL) (Sigma-Aldrich) in 2 × SSC (0.3 M NaCl and 30 mM sodium citrate) at 30 min at 37°C, rinsed in PBS, and postfixed in 4% (vol/vol) formaldehyde in PBS at room temperature for 10 min. Slides were then dehydrated in a graded ethanol series [75% (v/v) for 3 min, 85% (v/v) for 2 min, and 100% for 2 min] and allowed to air dry. Slides were rinsed in water, denatured in 70% formamide/30% 2× SSC for 2 min at 80°C, and immediately dehydrated with another ethanol series, and then telomere FISH was carried out as previously described (26).

**Interphase immunofluorescence and FISH**

Cells were cultured on sterile glass coverslips in a 12-well plate. Following transfection and necessary treatments, cells were washed twice with PBS. Cells were preextracted as in meta-TIF assay and then fixed in 2% paraformaldehyde in PBS for 10 min. Coverslips were blocked with ABDIL (antibody dilution buffer) (20 mM tris-Cl (pH 7.5), 2% BSA, 0.2% Fish Gelatin (Sigma-Aldrich, USA), 150 mM NaCl, 0.1% Triton X-100, and DNase-free RNase A (100 μg/mL) (Sigma-Aldrich) at room temperature for 1 hour. Primary antibody incubation was conducted at 4°C overnight or at room temperature for 1 hour. Secondary antibodies (conjugated to a fluorophore) were diluted 1:1000 in ABDIL, added to the coverslips, and incubated at 37°C for 30 min or at room temperature for 1 hour. For telomere FISH experiments, coverslips were fixed with 2% paraformaldehyde in PBS for 10 min, slides were ethanol-dehydrated, and then telomere FISH was carried out as previously described (26). Staining was visualized on a Zeiss Axio Imager M1 microscope, with a Plan Apochromat 63× oil objective (numerical aperture, 1.4), and an AxioCam MR digital camera (Carl Zeiss). Exposure times between treatments were consistent. Image brightness and contrast were adjusted in Zen (Carl Zeiss) and Adobe Photoshop for presentation, equally across all figure panels. Antibodies used include anti-colin (rabbit polyclonal, catalog no. Sc32860, 1:500; Santa Cruz), anti-TRF2 (rabbit polyclonal, catalog no. NB110-57130, 1:500; Novus), anti-γ-H2AX (catalog no. 05-636, 1:1000; Merck Millipore), anti-Hsp70 S5 (mouse monoclonal, catalog no. ab2787, 1:200; Abcam), and anti-Myc [mouse monoclonal, catalog no. 2276S (9B11); Cell Signaling]. For most quantitative analyses, colocalizing foci in a minimum of 100 cells were manually counted for each treatment, with three biological replicates performed for each experiment. For the experiment in Fig. 5E, colocalizing foci were quantitated using automated image analysis. ZEN microscopy images (.czi) were processed into extended projections of z-stacks using ZEN desk 2011 software (Carl Zeiss) and imported into CellProfiler v2.1.1 (59) for analysis. The 4′,6-diamidino-2-phenylindole (DAPI) channel was used to mask individual nuclei as primary objects. Foci within each segmented nucleus were identified using an intensity threshold-based mask. Any given object was considered to be overlapping another object when at least 80% of the first object’s area was enclosed within the area of a second object. **Proximity ligation assay**

Cells were cultured on sterile glass coverslips in a 12-well plate, and following transfection and necessary treatments, cells were washed twice with PBS and fixed with 2% paraformaldehyde for 10 min at room temperature, followed by PBS washes, and then permeabilized using KCM buffer [120 mM KCl, 20 mM NaCl, 10 mM tris (pH 7.5), and 0.1% Triton X-100]. PLA was performed using DUOLINK In Situ Red Starter (Mouse/Rabbit) PLA Kit (DUO92101; Sigma-Aldrich), according to the manufacturer’s instructions. The following were the antibodies used: anti-Hsp70/Hsp72 2H9 (mouse monoclonal, catalog no. MABE1130, 1:200; Merck Millipore), anti-TRF2 (rabbit polyclonal, catalog no. NB110-57130, 1:400; Novus), anti-Myc (9B11) (mouse monoclonal, catalog no. 2276, 1:1000; Cell Signaling), and anti-hTERT (rabbit polyclonal, catalog no. 600–401–252S, 1:500; Rockland).

**Telomere length measurement**

Telomere fluorescence intensity (proportional to telomere length) was quantified using the TFL-TELO Software (from P. Lansdorp, Vancouver, Canada) (60), according to the instruction manual provided. The frequency of chromosome ends with particular fluorescence intensity was determined from 15 metaphase spreads used in the meta-TIF assay. These were binned into categories and plotted using GraphPad Prism.

**Telomerase activity assay**

To verify the absence of telomerase catalytic activity of hTERT mutant (D712A), a direct telomerase activity assay was performed, as previously described (61). HEK293T cells were transfected with telomerase overexpression constructs and harvested and lysed using ice-cold hTERT lysis buffer [20 mM Hepes-KOH (pH 7.9), 300 mM KCl, 2 mM MgCl2, 0.1% (v/v) Triton X-100, 10% (v/v/v) glycerol, 1 mM PMSF, and 1 mM DTT] to make lysates equivalent to 2 × 105 cells/mL. Immunopurification of telomerase was performed as previously described (61) using a polyclonal anti-hTERT antibody and elution with a competing peptide (both available from Abbelexa Ltd., Cambridge, UK). Activity of the eluted telomerase was measured in a 20 μl extension reaction containing 20 mM Hepes-KOH (pH 7.9), 2 mM MgCl2, 5 mM DTT, 1 mM spermidine, 0.1% Triton X-100, 0.5 mM dTTP, 0.5 mM dATP, 5 μM α-32P-dGTP (198 Ci/mmol), and 1 μM DNA primer Bio-L-18GGG [5′-Biotin-CTAGACCTGTCATCA(TTAGGG)3] for 60 min at 37°C. The reaction was terminated with EDTA, and the products were isolated on Dynabead M280 streptavidin beads (Life Technologies), together with a 12-nt biotinylated control DNA, as previously described (61). Products were electrophoresed and imaged with phosphorimaging as previously described (61).

**RNA-sequencing and data analysis**

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) as per the manufacturer’s instructions, with in-column DNase digestion. RNA was confirmed to be of sufficient quality [with RNA integrity number (RIN) scores of 10] and quantity by the Australian Genome Research Facility before sequencing. For sequencing, cDNA libraries were generated from the total RNA of each sample, and sequencing was performed on the Illumina HiSeq platform generating 50- and 100-base pair reads, averaging approximately 23 million reads per biological replicate. Trimmomatic software was used to first trim universal and index adaptors from the raw reads (62); each read was then scanned with a 4-base wide sliding window, cutting when the average quality per base dropped below a Phred score of 20, and reads were then dropped if the length was less than 36 bases. The quality of raw and trimmed reads was assessed using the FastQC software (www.bioinformatics.babraham.ac.uk/projects/fastqc/; Babraham Institute).
The trimmed and filtered sequences (36 to 50 bases) were mapped to an unmasked human reference (hg19) using the Bioconductor Rsubread package (63) in the R statistical language (version 3.0.3). Voom-limma (64) was used to perform differential expression analysis on normalized counts and was confined to genes that had greater than 1 read per million mapped reads in at least one-third of the sample libraries. Control group (empty vector) and hTERT overexpression groups (WT and D712A) each contained three biological replicates. Genes that exhibited a greater than twofold change in expression, with P value ≤0.05, in all three biological replicates were considered to be differentially expressed.

**Gene expression analysis by qRT-PCR**

RNA extraction was conducted using RNase Mini Kit (Qiagen) as per the manufacturer’s instructions and quantitated on a Nanodrop. Gene expression analysis by qRT-PCR with SYBR Green PCR Master Mix (Thermo Fisher Scientific), as per the manufacturer’s instructions. cDNA synthesis was conducted using 5 µM Oligo(dT)20 as a primer, as per the manufacturer’s instructions. Gene expression analysis was conducted using SYBR Green PCR Master Mix (Thermo Fisher Scientific), 2 µl of cDNA synthesis reaction, 400 nM forward and reverse primers (listed in table S2), and cycling conditions listed in tables S3 and S4 using a RotorGene 6000 qRT-PCR machine. The average C(T) value of three technical PCR replicates was expressed relative to the C(T) of reference gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) which was verified to not vary across conditions by the ∆∆C(T) method using Rotor Gene 6000 software. Three biological experiments were conducted, and the average relative expression compared to the control (vector or control siRNA) was shown.

**Statistical analysis**

Statistical analyses were performed with GraphPad Prism. Two-tailed unpaired Student’s t tests were conducted on all experiments, unless otherwise specified.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/10/eaav4409/DC1

**REFERENCES AND NOTES**


