Sister chromatid separation at human telomeric regions

Michal Yalon, Shoshana Gal, Yardena Segev, Sara Selig* and Karl L. Skorecki

Bruce Rappaport Faculty of Medicine and Research Institute – Technion and Rambam Medical Center, Haifa, Israel 31096

*Author for correspondence (e-mail: seligs@tx.technion.ac.il)

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Summary

Telomeres are nucleoprotein complexes located at chromosome ends, vital for preserving chromosomal integrity. Telomeric DNA shortens with progressive rounds of cell division, culminating in replicative senescence. Previously we have reported, on the basis of fluorescent in situ hybridization, that several human telomeric regions display solitary signals (singlets) in metaphase cells of pre-senescent fibroblasts, in comparison to other genomic regions that hybridize as twin signals (doublets). In the current study, we show that an additional 12 out of 12 telomeric regions examined also display metaphase singlet signals in pre-senescent cells, and that excess telomere-metaphase singlets also occur in earlier passage cells harvested from elderly individuals. In cancer cell lines expressing telomerase and in pre-senescent fibroblasts ectopically expressing hTERT, this phenomenon is abrogated. Confocal microscope image analysis showed that the telomere metaphase singlets represent regions that have replicated but not separated; this is presumably because of persistent cohesion. The introduction of mutations that interfere with the normal dissolution of cohesion at the metaphase to anaphase transition induced the *cut* (chromosomes untimely torn) phenotype in early passage fibroblasts, with predominantly telomeric rather than centromeric DNA, present on the chromatin bridges between the daughter nuclei. These results suggest that telomeric regions in animal cells may potentially be sites of persistent cohesion, and that this cohesion may be the basis for an observed excess of fluorescent in situ hybridization metaphase singlets at telomeres. Persistent cohesion at telomeres may be associated with attempted DNA repair or chromosomal abnormalities, which have been described in pre-senescent cells.

Key words: Telomeres, Sister-chromatid separation, Telomerase, Cohesin

Introduction

Telomeres are specialized nucleoprotein complexes that maintain the integrity and stability of linear eukaryotic chromosomal ends (Zakian, 1995). The concept of stable and ‘sealed’ chromosome ends was first proposed by Hermann J. Muller and Barbara McClintock in the late 1940s and early 1950s (Gall, 1995). Subsequently, the term ‘telomere capping’ emerged to describe the protective role of telomeres (McEachern et al., 2000; Blackburn, 2001; Cervantes and Lundblad, 2002; Chan and Blackburn, 2002). In multicellular organisms, in the absence of telomerase or alternative mechanisms that may be present in germ, stem and transformed cells, telomeric DNA shortens with each round of cell division. It has been proposed that the progressive shortening of telomeres subsequent to continuous rounds of cell division in normal human somatic cells forms the basis for a mitotic clock controlling the onset of replicative senescence (Harley et al., 1990; Allsopp et al., 1992). Continued cell division beyond this crucial point produces telomeres with unprotected ends, leading to the disruption of chromosomal stability as a result of end-to-end chromosomal fusions and other forms of telomeric dysfunction (Counter et al., 1992; Filatov et al., 1998). More recently, it has been shown that to maintain their integrity, telomeres bind many proteins involved in double-stranded break (DSB) repair, and that mutations in genes involved in signaling DNA damage also affect telomere stability (reviewed by Gasser, 2000; Blackburn, 2001; Chan and Blackburn, 2002).

In addition to telomere integrity, the maintenance of a stable genome relies on the faithful replication and segregation of chromosomes to daughter cells during mitosis. Concurrent with DNA replication, sister chromatids are linked by a multiprotein complex, known as cohesin, consisting of several subunits: SMC1, SMC3, Scc1 (also known as Rad21 or Mcd1) and Scc3 (SA1, SA2 in mammals) (Nasmyth, 2002). In budding yeast, cohesin abruptly dissociates from chromatin at the onset of anaphase (Michaelis et al., 1997). By contrast, in vertebrate cells, removal of the cohesin complex is achieved in a two-step process. During prophase, the bulk of cohesin dissociates from the condensing chromosome arms as a result of the action of Polo-like kinase (Losada et al., 2002; Sumara et al., 2002). However, residual cohesin remains bound to chromatin at specific sites, including centromeric regions (Warren et al., 2000; Hauf et al., 2001; Hoque and Ishikawa, 2001), and is sufficient to hold the sister chromatids together until the onset of anaphase. At this point, disruption of these complexes is mediated through the cleavage of the cohesin subunit Scc1 by the endopeptidase separase (Uhlmann et al., 1999). Securin plays a key role in the regulation of separase activity, as securin both inhibits separase and it is needed to generate its active form (Jallepalli et al., 2001) (reviewed by Uhlmann, 2003). When all of the chromosomes are aligned

properly on the metaphase spindle, the anaphase promoting complex/cyclosome (APC/C), a ubiquitin ligase, triggers the degradation of securin (Cohen-Fix et al., 1996). This action allows separase to cleave Scc1, which is followed by progression through anaphase (Ciosk et al., 1998) (reviewed by Bernard and Allshire, 2002).

Fluorescent in situ hybridization (FISH) signals from all genomic regions along chromosome arms are expected to appear at metaphase as doublet hybridization signals (Selig et al., 1992). This is consistent with the loss of cohesion along most of the length of sister chromatids during prophase. These doublet hybridization signals represent a genomic region that has replicated, and in which the replicated products have separated sufficiently to be resolved as two distinct signals (Selig et al., 1992; Boggs and Chinault, 1997). Surprisingly, Ofir et al. (Ofir et al., 2002) found a significant percentage of singlet signals in metaphase cells in five out of five different telomeric regions examined in pre-senescent human fibroblasts as opposed to early passage cells. This finding was restricted to telomeric regions and was not observed for control non-telomere regions examined. Such unexpected singlet signals in metaphase were thought to represent either incomplete replication or incomplete separation of replicated sister chromatids at telomeric regions.

In the current study we show, by examining an additional 12 telomeres in pre-senescent cells aged in vitro, that these metaphase singlet signals represent a more general phenomenon. We present data that suggests that this phenomenon is not restricted to fibroblasts aged in culture, by showing that metaphase singlets at telomeric regions also occur in earlier passage fibroblasts harvested from elderly individuals. Moreover, we find a telomerase-mediated abrogation of metaphase telomere singlets, both in cancer cell lines and shortly after ectopic expression of telomerase in primary pre-senescent fibroblasts. We also provide evidence that these telomeric metaphase singlets represent replicated but nonseparated telomeric regions, which is consistent with persistent cohesion at telomeric regions in pre-senescent cells. Finally, we show that the *cut* (chromosomes untimely torn) phenotype, in which chromatin bridges connect sister nuclei following cytokinesis (Yanagida, 1998), induced by introduction of a mutant nondegradable (ND)-SCC1 gene or a ND-securin gene, is characterized by hybridization of a telomeric probe to DNA within the bridges, and much less frequently with a centromeric probe. These findings suggest that cohesin proteins at telomeric regions persist through prophase in animal cells, and may be involved in the failure of replicated telomeric regions to separate properly in pre-senescent cells.

Materials and Methods

Cell lines and culture procedures

Human primary foreskin fibroblasts SR (Ofir et al., 2002), FSE (obtained from Shraga Blazer, Rambam Medical Center, Haifa, Israel) and BJ (Ouellette et al., 1999) (obtained from Woodring Wright, University of Texas, Southwestern Medical Center, TX, USA) were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. These cells were subcultured every 4–7 days to maintain continuous log-phase growth and propagated in culture from cell line establishment until senescence, which occurred at approximately 70 population doublings (PDs). BJ cells were obtained at PD 41 and propagated until senescence. Senescence was defined as the state at which the cells divided less than once per 2 weeks.

Primary human skin fibroblasts derived from men aged 94 and 96 years were obtained from Coriell Cell Repositories (repository numbers AG08433 and AG04059, respectively) and from one 76-year-old man during elective surgery after obtaining patient informed consent. The study was approved by the Institutional Ethics Review Board, Rambam Medical Center, Haifa, Israel. The cell culture from the skin biopsy was prepared as described previously (Blazer et al., 2002). Cells were grown in media as described above, with the exception of 20% (v/v) FCS. Beginning with the third passage, these cells were subcultured every 6–9 days to maintain continuous log-phase growth and propagated until senescence, which occurred after 15-30 PDs.

SKOV-3 and 0VCAR-3 cell lines, obtained from American Type Culture Collection, were grown in RPMI supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cell lines were grown at 37ºC in 5% CO_2.

Construction of retroviral vectors and retroviral infections

The open reading frame (ORF) of human telomerase (hTERT) cDNA was removed from GRN145 (Weinrich et al., 1997) (provided by Geron, Menlo Park, CA, USA) by digestion with *Eco*RI and cloned into the *Eco*RI site of a pBABE-eGFP (enhanced green fluorescent protein) vector [a modifed pBABE-puromycin (Morgenstern and Land, 1990) vector kindly provided by Eyal Bengal and Olga Ostrowsky, Technion Faculty of Medicine, Haifa, Israel]. This pBABE-eGFP vector was modified such that the gene for puromycin resistance was excised out with *Hin*dIII and *Cla*I enzymes and exchanged with the gene for eGFP.

The cloning of ND-SCC1 into the pBABE-eGFP vector was performed as follows: the RT-PCR product of the ORF of SCC1 was obtained from HeLa cell line RNA and subcloned into the pTZ57R vector (MBI Fermentas). The following oligonucleotides, containing *Xho*I sites at the ends (underlined), were used for this amplification: 5′-GGCCCTCGAGCCAGCCAGAACAATGTTC-3′ and 5′-CCGCTCGAGTTATATAATATGGAACCTTGG-3′. Following sequencing, one clone was found which contained only synonymous mutations (T738C, A885G, and A1170G; nucleotide positions are according to the ORF). This clone was used for site-directed mutagenesis in order to create the ND-SCC1. Amino acids 172 and 450 were mutated from Arg to Ala as described previously (Hauf et al., 2001), using the following oligonucleotides: for amino acid 172, 5′-GATGATCGTGAGATAATGGCAGAAGGCAGTGCTTTTGAG-3′, 5′-CTCAAAAGCACTGCCTTCTGCCATTATCTCACGATCATC-3′, and for amino acid 450, 5′-CCATTATTGAAGAGCCAAGCGCGCTCCAGGAGTCAGTGATG-3′, 5′-CATCACTGACTCCTGGAGCGCGCTTGGCTCTTCAATAATGG-3′. The mutations were verified by sequencing, and then the *Xho*I-*Xho*I fragment of the mutated ORF was subcloned into the *Sal*I site of the pBABE-eGFP vector.

The ND-securin gene was introduced to cells by infection with a pBABE-ND-securin-H2AGFP construct. The pBABE-H2AGFP backbone is identical to the pBABE-eGFP plasmid, with the exception of the addition of the histone H2A ORF upstream to the eGFP gene, thus targeting the GFP protein to the nucleus. The pBABE-H2AGFP plasmid containing ND-securin was constructed as follows: a PCR product was generated from the plasmid described previously (Zur and Brandeis, 2001), kindly provided by Michael Brandeis (Hebrew University, Jerusalem, Israel), using the following oligonucleotides: forward: 5′-CGGAATTCTAGAGGCTCGAGTTA-3′, and reverse: 5′-CGGGATCCATGGCTACTCTGATC-3′ (restriction sites that were added for cloning purposes are underlined). The PCR product was cloned into the *Bam*HI and *Eco*RI sites of the pBABE-eGFP vector, and its sequence was verified.

To generate retroviral particles, the viral packaging cell line GP2-

293 (stably expressing the *gag* and *pol* genes) (Burns et al., 1993), was cotransfected with the pBABE construct of interest and a plasmid containing the vesicular stomatitis virus glycoprotein gene (VSVG). Transfection was done with FuGENE 6 transfection reagent (Roche). Infection of the target cells (BJ and FSE) was conducted serially four to eight times (twice in 24 hours). 24-48 hours following the last infection, cells were split or seeded as required. The percentage of cells infected was determined either microscopically by scoring the percentage of GFP-positive cells, or by FACS analysis of whole cells.

Combined fluorescence in situ hybridization and immunofluorescence (FISH-IF)

Cells were grown for 48–72 hours on glass chamber slides (Nalge Nunc International). Pre-senescent cells were grown on chamber slides coated with fibronectin (Biological Industries, Israel). Alternatively, cells were harvested, resuspended in PBS and cytospun onto superfrost slides for 10 minutes at 400 ***g*** (1950 rpm) using a Shandon Cytospin 3 cytocentrifuge.

Combined detection of phosphorylated histone H3 (H3P) by IF and FISH was performed as previously described (Ofir et al., 2002). Combined detection of GFP by IF and FISH was performed with rabbit anti-GFP antibody (A-6455, Molecular Probes) according to the same protocol, except that the washes after the antibody detection were done with PBS only.

The following probes were used to detect telomeric regions: telomere 4q, cosmid 99561; telomere 5p, cosmid 99562; telomere 17p, cosmid 99583; telomere 2q, cosmid 99557 and telomere 15q, cosmid 99580. These cosmids were obtained from American Type Culture Collection (ATCC) and have been described previously (Ning et al., 1996). Additional probes were obtained from Vysis (TelVysion probes – #33-270000) including telomeres 5q, 10q, 10p, 16p 16q, 19q, 19p, 20q, 20p and X/Yp. These probes were already labeled with Spectrum Green or Spectrum Orange and did not require a detection step following hybridization overnight. The sizes of the probes from Vysis ranged between 75 and 191 kb. All telomeric probes were within 100-300 kb from the chromosome end, and therefore their hybridization intensity was not affected by senescence-related telomeric attrition. The cosmid probes for non-telomeric regions were cJ21 from the CFTR gene locus and HG4 from the β-globin locus (Kitsberg et al., 1993).

Scoring FISH signals in FISH-IF experiments

Percentages of singlet or doublet signals were scored per chromosome in metaphase cells. Each FISH-IF experiment was repeated at least twice, and only slides with at least 85% of nuclei displaying clear signals were scored. Scoring was performed in a blinded fashion by an individual trained for this purpose.

Telomere/centromere peptide nucleic acid (PNA)-FISH

Early passage (PD 15) FSE fibroblast cells were infected serially 4-8 times with ND-SCC1 or ND-securin as described. Forty-eight hours after the last infection, the cells were seeded on chamber slides. After an additional 48 hours, the cells were washed with PBS and fixed as follows: 1 ml of fresh PBS was added to each chamber, 1 ml of ice-cold fixative containing methanol and acetic acid (3:1) was added very slowly to the PBS and the slides were incubated in this mixture for 10 minutes. This mixture was then replaced with 1 ml of fresh fixative. After 5 minutes this procedure was repeated an additional three times. Cells were then air-dried and kept for 24-48 hours at room temperature until hybridization with the telomeric and centromeric probes was performed. Hybridization was carried out with a Cy3-labeled telomeric PNA probe and a FITC-labeled PNA probe, which detects α-satellite sequences of all centromeres, with the exception of the centromeres for the Y chromosome and chromosome 21 (gift of Peter Lansdorp, Terry Fox Laboratory, British Columbia Cancer Agency, BC, Canada). Hybridization was carried out as previously described (Lansdorp et al., 1996).

Immunofluorescence

Fibroblast cells infected with ND-SCC1 as described above, were seeded on coverslips and harvested according to the same schedule described for the PNA-FISH experiment. Cells were fixed in 3.7% formaldehyde for 10 minutes at room temperature. Following three washes with PBS, cells were incubated in ice-cold methanol for 6 minutes on ice. After three additional washes with PBS, cells were blocked for 30 minutes with 3% BSA/4xSSC and incubated for 30 minutes with CREST serum and rabbit anti-GFP antibody (described above). Following washes with PBS/0.1% triton, slides were incubated with anti-human-Cy3 and anti-rabbit-FITC antibodies (Jackson ImmunoResearch Laboratories) for 30 minutes, washed and mounted with Vectashield (Vector) anti-fade solution containing DAPI at a concentration of 200 ng/ml.

Fluorescence microscopy

Signals from FISH, FISH-IF and immunofluorescence were visualized using a Zeiss Axioscop 2 microscope using a Plan Neofluar 100/1.3 oil objective. Digital images were captured with a charged-coupled device (RTE/CCD-1300Y, Princeton Instruments, NJ), controlled by ImagePro Plus software (Media Cybernetes, MD). Analysis and pseudocolor rendering were conducted using ImagePro Plus.

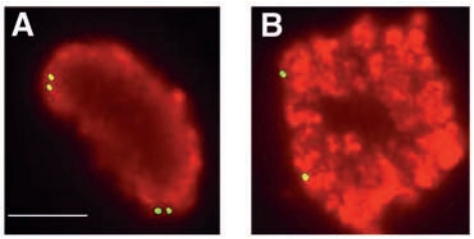
Confocal microscopy analysis

Images of FISH-labeled metaphase cells containing one singlet signal and one doublet signal (SD) were collected on a BioRad Radiance 2000 confocal system mounted on a Nikon Eclipse E600 microscope at a resolution of 512×512 pixels using a 60× objective. Eight sections were collected and maximal intensity projections of all sections were generated and used for image analysis. Quantitative analysis of FISH signals in these images was performed as follows: identically sized analysis boxes were placed and centered over the hybridization spot of the singlet and each of the two hybridization spots of the doublet signal in each cell, and the average fluorescence was determined for each spot. Analysis boxes were then placed on regions adjacent to the labeled structures, and the background fluorescence for each cell was determined. These background levels were then subtracted from the fluorescence measurements of the fluorescent spots. The fluorescence measurements of the two doublet spots were averaged, and ratios of average doublet spot fluorescence to singlet spot fluorescence were calculated for each cell. All analysis was performed with software written by Noam Ziv (Shapira et al., 2003).

Results

Senescence metaphase singlets occur at multiple telomeric regions

Most genomic regions display doublet FISH signals at metaphase (Fig. 1A), with some background levels of singlet signals attributed to technical reasons [approximately 10% of chromosomes (Lichter et al., 1990)] (Fig. 1B). By contrast, a higher than expected frequency of FISH metaphase singlets has been previously shown in five out of five different telomeric regions in pre-senescent human fibroblasts in culture (Ofir et al., 2002). To determine whether these telomere metaphase singlets represent a more general phenomenon, we examined an additional 12 telomeric regions. FISH-IF was performed on early passage and pre-senescent cells aged in vitro. FISH



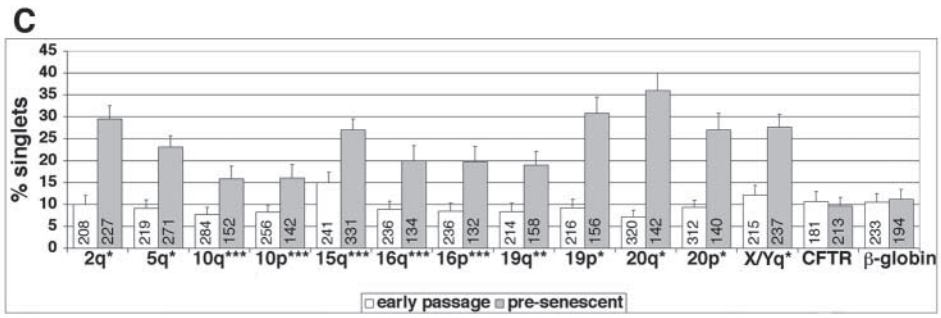


Fig. 1. (A-C) Metaphase singlets in pre-senescent fibroblast cells aged in culture. Fibroblasts from human SR foreskin fibroblast cells at PD 18 (early passage) and PD 65–67 (pre-senescent) were subjected to FISH-IF using probes for the telomeric and control regions listed and an antibody to H3P, which serves as a G2/mitosis marker. The FISH signals are detected with FITC (green) and the anti-H3P antibodies with Cy-3 (red). (A) A pre-senescent cell hybridized to a probe for β-globin, showing two doublet signals. Bar, 10 μm. (B) A pre-senescent cell hybridized to a 17p telomeric probe showing two singlet signals. (C) The percentage of chromosomes displaying metaphase singlets in pre-senescent cells aged in culture. The mean percentage (±s.d.) of chromosomes displaying a singlet signal is indicated, together with the *P* value of the binomial distribution probability analysis (**P*<0.0001, ***P*<0.001, ****P*<0.01). The number of chromosomes scored for each probe is indicated in the bar.

probes correspond to subtelomeric regions and control probes include two non-telomeric regions that are known to replicate late in S phase (CFTR and β-globin) (Ofir et al., 2002). In this assay metaphase cells are identified using both antibodies for the specific G2/mitosis marker, phosphorylated histone H3 (H3P) (Hendzel et al., 1997), and also by DAPI chromosome staining. According to the FISH replication assay, a doublet hybridization signal indicates replication and separation of the genomic region of interest, whereas a singlet indicates incomplete replication or incomplete separation of sister chromatids (Selig et al., 1992; Boggs and Chinault, 1997; Ofir et al., 2002). As shown in Fig. 1C, pre-senescent cells aged in vitro exhibit higher percentages of singlets at all tested telomeric regions compared with early passage cells, but not at the non-telomeric regions examined.

Metaphase singlets occur at telomeric regions in fibroblasts from elderly people

To determine whether or not the telomeric singlet phenomenon observed in pre-senescent cells aged in tissue culture also occurs as a manifestation of in vivo ageing, we analyzed fibroblasts derived from three elderly people. These fibroblasts were propagated in culture from cell line establishment until senescence, which occurred after only 15-30 PDs, compared with 60-80 PDs required to reach senescence in human foreskin-derived fibroblasts (Harley et al., 1990; Bodnar et al., 1998; Ofir et al., 2002). Despite fewer PDs needed to achieve senescence, FISH analysis in metaphase revealed that cells obtained from older people also had a higher percentage of singlet signals at telomeres compared with non-telomeric regions in pre-senescent cells 1-2 PDs before reaching senescence (Fig. 2), but not significantly before that point (results not shown). The singlet values at pre-senescence were similar to those seen in pre-senescent foreskin fibroblasts aged in vitro at PDs greater than 60, whereas the foreskin fibroblasts at a comparable PD in culture to that of the fibroblasts harvested from the three elderly individuals showed much lower percentages of singlets at telomeric regions (see Fig. 1C for results at PD 18). These results indicate that the telomeric singlets are not merely a manifestation of an extended period of cell growth in tissue culture but rather, that they occur also in cells that have aged primarily in vivo and been grown for comparatively much shorter periods in tissue culture.

Relation of telomeric metaphase singlets to telomerase expression

Constitutive expression of telomerase has been shown to circumvent replicative senescence in a variety of cell types (Bodnar et al., 1998). We previously reported that pre-senescent BJ fibroblasts immortalized by the introduction of

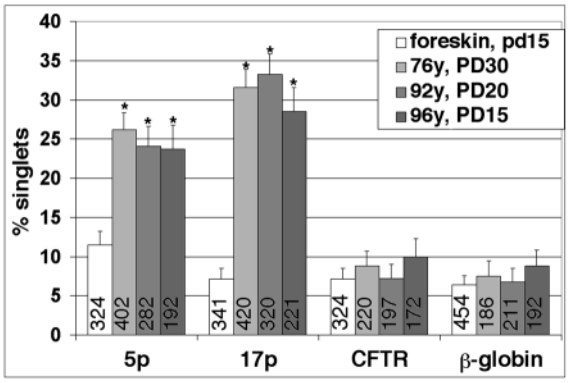


Fig. 2. Metaphase singlets in pre-senescent cells aged in vivo. Fibroblasts derived from three elderly people (y, years) were propagated in culture from cell line establishment until senescence. Pre-senescent cells (PD 15-30) were subjected to combined FISH-IF at 1-2 PDs before senescence, using probes for the telomeric and control regions indicated. The mean percentage (±s.d.) of chromosomes displaying a singlet signal is indicated, together with the *P* value of the binomial distribution probability analysis (**P*<0.0001). The number of chromosomes scored for each probe is indicated in the bar.

a viral construct expressing the hTERT catalytic component of telomerase (McChesney et al., 2000) maintain a low background level of metaphase singlets similar to that observed at non-telomeric control regions, even many PDs following infection (>150 PD) (Ofir et al., 2002). Because the telomeres in this hTERT-BJ cell line are very long (16-18 kb), in the current study we also examined the state of metaphase singlets in two telomerase-positive ovarian cancer cell lines with shorter telomeres: OVCAR-3, which has very short telomeres of ~3.5 kb, and SKOV-3, which has longer telomeres with an average length of ~12kb (Braunstein et al., 2001). In both cases, metaphase singlets at telomeres were not detected above background level and were similar in frequency to non-telomeric control regions (Fig. 3). The finding in OVCAR-3 cells in particular suggested that the effect of telomerase on the abrogation of telomeric singlets is not directly related to telomere length per se. However, transformed cell lines have accumulated numerous genetic abnormalities, and it may be difficult to draw direct inferences regarding an effect of telomerase itself to abolish excess metaphase singlets at telomeres. Accordingly, to show more directly the involvement of telomerase in the restoration of doublets, we introduced the catalytic subunit of human telomerase, hTERT, by serial retroviral infections to very late passage, pre-senescent BJ cells. Because the infection rate of pre-senescent cells is relatively low, and the mitotic index of these cells is extremely low, the mitotic-GFP-positive cells are very rare. In at attempt to collect reliable data, the experiment was repeated three times, and the telomeric region of 17p was chosen for analysis. In the presence of hTERT, the telomeric singlet phenomenon at telomere 17p is reverted in these cells as early as 4 days after infection (Table 1, Fig. 4). During this period no significant elongation of telomeres is expected. This is supported by telomere-PNA-FISH that exhibited weak signals in hTERT-infected fibroblasts similar to those in control cells (results not shown).

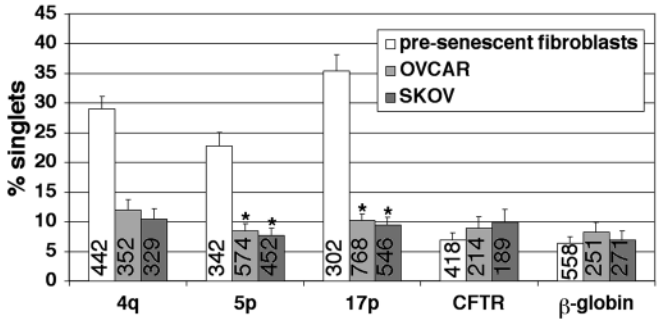


Fig. 3. Metaphase singlets at telomeres do not occur in cancer cell lines. Metaphase cells from two ovarian cancer cell lines, OVCAR-3 (telomerase positive, short telomeres) and SKOV-3 (telomerase positive, long telomeres), were subjected to combined FISH-IF using probes for the telomeric and control regions listed. The mean percentage (±s.d.) of chromosomes displaying a singlet signal in OVCAR-3 and SKOV-3 in comparison to the corresponding percentage in pre-senescent human fibroblast cells is indicated, together with the *P* value of the binomial distribution probability analysis (*P<0.0001). The number of chromosomes scored for each probe is indicated in the bar.

Table 1. Effect of telomerase on telomere-metaphase singlets

	+ hTERT		Control empty vector	
Experiment	% Singlets	No. of cells	% Singlets	No. of cells
I	11	37	50	20
II	16	66	40	30
III	12	24	31	16
Total	13.8	127	40.8	66

BJ fibroblasts at PD 67 were infected with either a pBABE-eGFP vector containing the hTERT gene, or an empty pBABE-eGFP vector. FISH with a 17p telomeric probe was conducted on these cells and GFP-positive metaphase cells were scored for the presence of singlet or doublet signals.

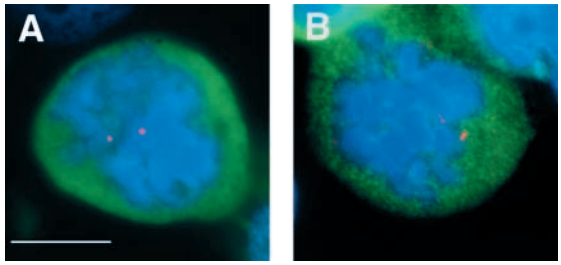


Fig. 4. (A,B) Expression of hTERT in pre-senescent cells abrogates telomere metaphase singlets. Pre-senescent BJ cells were infected serially with the pBABE-hTERT-eGFP retroviral vector and with a control empty vector. Metaphase cells at pre- and postinfection were subjected to combined FISH-IF using an antibody to GFP and probes for telomeric and control regions. Only GFP-positive cells were scored. (A) Two singlets at telomere 17p are evident in a GFP-positive pre-senescent cell after infection with a control vector. (B) A GFP-positive cell, 4 days after infection with an hTERT-containing vector, displaying two doublet FISH signals at telomere 17. The FISH signal is detected with Cy-3 (red) and the GFP with FITC (green), and the nucleus is stained with DAPI (blue). Bar, 10 μm.

Telomeric metaphase singlets in pre-senescent cells are composed of sister chromatid regions that have replicated but not segregated

The metaphase singlet signals at telomeric regions could either represent incomplete replication of DNA, or alternatively, incomplete separation of the sister chromatids at these regions, because of persisting cohesion. Both possibilities are of interest, as frustration of either process would jeopardize either chromosomal integrity or appropriate segregation of sister chromatids with cell division. Careful scrutiny of metaphase cells with one singlet signal and one doublet signal (representing each of the parental alleles) suggested that the hybridization intensity of the singlet signal was much stronger than each of the components of the doublet signal in the same cell (Fig. 5A-D). To quantitate the signal intensities systematically, confocal microscopy analysis was used to compare the intensity of the singlet signal to that of the components of the doublet signal in a large number of cells exhibiting both a singlet and a doublet signal. As shown in Table 2, the average hybridization signal intensity for singlets was twice that of the average of the two signals within a doublet. This finding strongly suggested that the singlet signals represent genomic regions in which DNA had replicated, but in which the sister chromatids at these regions had not separated.

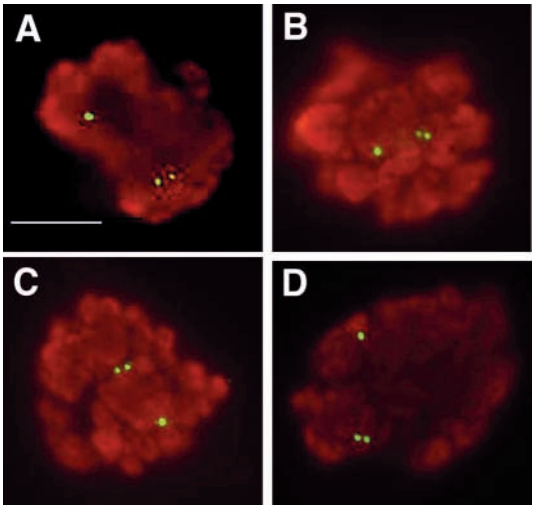


Fig. 5. (A-D) Telomeric metaphase singlets hybridize more intensely compared with the single components of a doublet signal. Combined FISH-IF utilizing a 17p telomeric probe and an antibody for H3P was performed on pre-senescent cells aged in culture (A,B) and pre-senescent fibroblasts from elderly people (C,D). The FISH signal is detected with FITC (green) and the H3P with Cy-3 (red). All four metaphase cells display one singlet signal and one doublet signal. The hybridization signal intensity of the singlet appears stronger than that of each of the two dots composing the doublet. Bar, 10 μm.

Table 2. Confocal microscope analysis of FISH signal intensity in pre-senescent cells displaying one singlet and one doublet

Cell number	Singlet intensity*	Doublet intesity‡	Doublet/singlet intensity ratio
1	737.14	335.97	0.4556
2	763.85	492.49	0.6447
3	435.79	332.14	0.7621
4	602.19	331.59	0.5506
5	428.14	132.91	0.3104
6	555.41	193.84	0.3490
7	911.86	344.53	0.3778
8	831.56	572.51	0.6884
9	775.48	362.08	0.4669
10	648.70	350.56	0.5404
11	621.91	379.60	0.6103
12	626.96	273.89	0.4368
13	628.83	436.13	0.6935
14	627.04	474.21	0.7563
15	678.06	269.12	0.3969
Average	658.19	352.10	0.53±0.038

*After reducing background (see Materials and Methods). ‡After reducing background and averaging the intensities of the twin dots comprising the doublet signal.

Chromatin bridges, induced in fibroblasts by nondegradable-Scc1 and nondegradable-securin contain telomeric DNA

Dissolution of the cohesin in prophase is mediated in animal cells by the action of polo-like kinase, and it allows sister chromatids to separate along most of their length (Uhlmann, 2003 and references therein). Persistent cohesion at centromeres enables chromosomes to maintain their appropriate alignment on the spindle until the onset of anaphase, at which stage the APC/C induces the degradation of securin, release of separase and cleavage of Scc1, enabling sister chromatids to undergo orderly segregation (reviewed by Bernard and Allshire, 2002). Certain mutations in this pathway have been shown to cause the *cut* phenotype in fission yeast (Nasmyth, 2001), and a similar phenotype has been described in cancer cell lines (Hauf et al., 2001; Zur and Brandeis, 2001; Hagting et al., 2002; Chestukhin et al., 2003). The phenotype is characterized by 'septation and/or cytokinesis occurring in the absence of normal sister-chromatid separation' (reviewed by Yanagida, 1998). In *Schizosaccharomyces pombe* telomeric DNA was shown by FISH on the chromatin bridges of *cut*1 mutants defective in the separase gene, while the centromeres had separated to the daughter nuclei (Funabiki et al., 1993). Accordingly, we were interested in assessing the possible contribution of abnormalities in telomere separation to the generation of this phenotype in human fibroblasts defective in cohesion dissolution. To this end, early passage fibroblast cells were infected with a nondegradable version of either Scc1, or a mutant version of securin previously shown to induce the *cut* phenotype in HeLa cells (Zur and Brandeis, 2001; Hagting et al., 2002). Serial infections with pBABE-ND-Scc1-eGFP induced several cellular abnormal phenotypes in early passage human fibroblasts, as shown previously in HeLa cells (Hauf et al., 2001), including abnormally shaped nuclei and micronuclei (Fig. 6A). Classical *cut* phenotype cells were also evident in the infected fibroblasts (Fig. 6B) albeit at a low frequency (0.57%, 25 *cut* configurations in a total of 4357 cells screened). By contrast, in fibroblast cells infected with the empty pBABE vector (960 cells) and control noninfected cells (1330) no *cut* configurations were detected. Two classes of *cut* configurations were observed in the ND-SCC1 infected fibroblasts; one configuration was characterized by a thin and long chromatin bridge connecting the daughter nuclei, whereas the second *cut* configuration contained a shorter and thicker chromatin bridge. Moreover, PNA-FISH using centromeric and telomeric probes revealed differences in hybridization between these classes (Table 3). The *cut* configurations with thicker chromatin bridges were rare (6.8% of total *cut* configuration analyzed by FISH), and mostly exhibited numerous telomeric FISH signals (Fig. 6C) and asymmetry, both in the size of daughter nuclei and in the corresponding distribution of FISH telomere/centromere hybridization signals between the daughter nuclei. These *cut* configurations, are consistent with failure to separate multiple chromosomes at their centromeres, as previously proposed (Hauf et al., 2001). By contrast, Fig. 6D,E shows the *cut* phenotype configuration in which nuclei are joined by a thin DNA bridge in which a few telomeric FISH signals are clearly evident in the bridge (Table 5), while no centromeric signal is detected on the bridge; this is consistent with connection of daughter nuclei by sister chromatids attached at a single or few telomeres rather than at centromere regions. In agreement with this, the size of the daughter nuclei and the distribution of telomeric and centromeric FISH signals within the daughter nuclei are symmetrical. The lack of centromeric DNA in these thin *cut* bridges was confirmed by IF using CREST antibody. Such an analysis of 36 *cut* configurations with long and thin chromatin bridges revealed that in 31 (86%) cases, no centromeric DNA was present within the bridge (Fig. 6G), and one *cut* configuration appeared to be the result of a dicentric chromosome. Two *cut* configurations with thick and

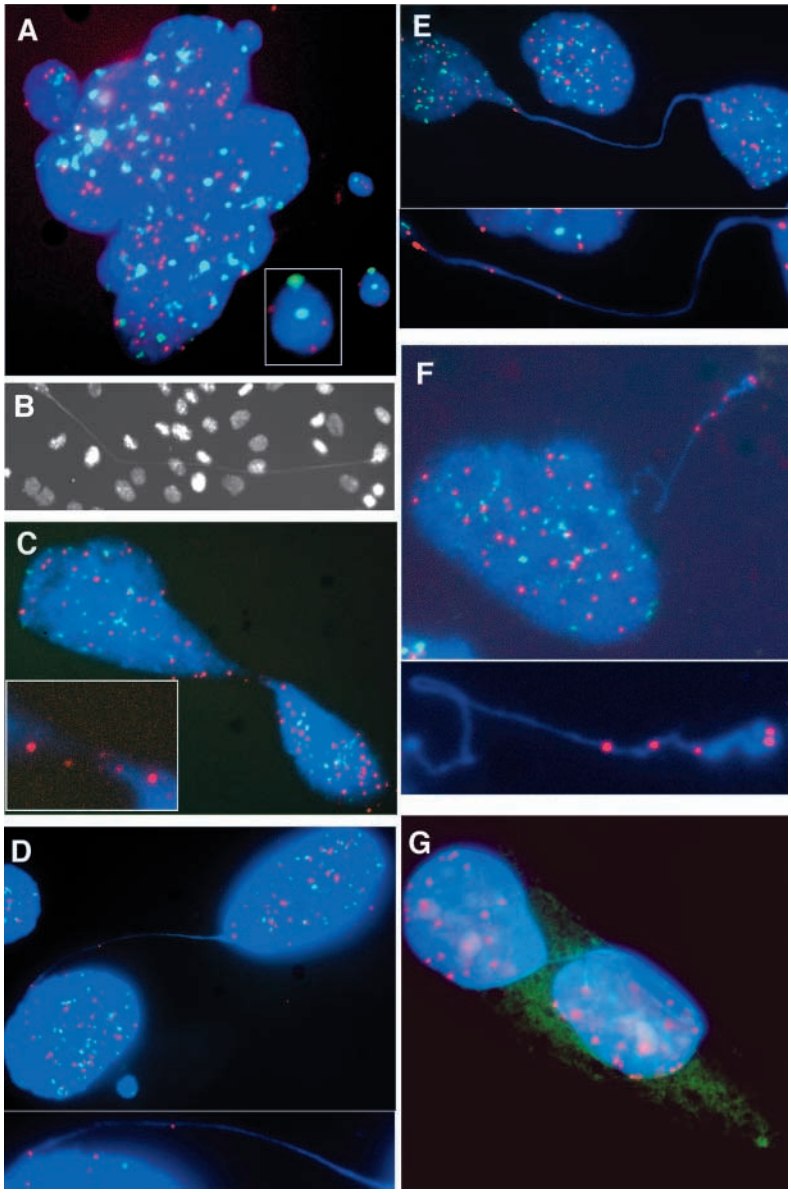


Fig. 6. (A-G) Nondegradable Scc1 induces the *cut* phenotype in early passage human fibroblasts with telomeric DNA present on the DNA bridge. Early passage fibroblasts (PD 15-18) were infected serially with ND-SCC1, and PNA-FISH with centromeric and telomeric probes was performed 2-3 days following the last infection. (A-F) Telomere signals are detected with Cy3 (red) and centromeric signals with FITC (green). Nuclei are stained with DAPI (blue). (A) An example of a nucleus with an abnormal morphology induced by the ND-Scc1mutation. In addition to the irregular nuclear shape, additional micronuclei are present in the cell, hybridizing to centromeric and telomeric probes. One of these micronuclei is shown more clearly in the inset. These nuclei are probably the result of lagging chromosomes. (B) A typical *cut* phenotype. Note that a thin strand of DNA connects the two nuclei most distantly separated in this field. (C) A *cut* phenotype with a thick DNA bridge containing multiple telomeric signals. The region of the DNA bridge, connecting the two sister nuclei, is enlarged at the bottom left. (D-E) The *cut* phenotype with a thin DNA bridge. Several telomeric signals are present on the bridge, whereas no hybridization with the centromeric probe is evident. The region of the DNA bridge is enlarged and presented in the bottom frame of each panel. (F) A nucleus containing a torn DNA bridge. An enlargement of the torn DNA strand is present at the bottom frame of the panel. (G) Staining of a GFP-positive cell with the *cut* configuration with CREST (anti-centromeric) and anti-GFP antibodies. The centromeric regions are detected with Cy3 (red) and the GFP with FITC (green). DNA is stained with DAPI (blue).

short bridges presented asymmetric daughter nuclei with no centromeric signals on the connecting bridge. Similar results were obtained following infection of early passage human fibroblasts with the securin mutant, which has been shown previously to induce the *cut* phenotype in human cancer cells (Zur and Brandeis, 2001; Hagting et al., 2002). PNA-FISH revealed that telomeric sequences were prevalent on the chromatin bridges of the *cut* configurations (Table 4), with one to two signals in most cases (Table 5). In contrast to the hybridization results after the introduction of ND-Scc1, in the case of ND-securin, 25% of the *cut* configurations displayed centromeric signals on the chromatin bridges, almost always together with telomeric signals (Table 4). Thus the *cut* configurations induced by ND-securin behave similarly to those induced by ND-Scc1, with respect to the telomeric involvement in the generation of the chromatin bridges.

Table 3. Distribution of telomere and centromere signals in *cut* bridges induced by ND-Scc1

	Thin bridges (93.2%, *n*=96)			Thick bridges (6.8%, *n*=7)	
	+ tel	– tel	Total	+ tel	– tel
+ cen	3.1%	5.2%	8.3%	*n*=1	*n*=3
– cen	52.1%	39.6%	91.7%	*n*=3	–
Total	55.2%	44.8%			

Fibroblast cells displaying the *cut* phenotype following infection with ND-Scc1 were analyzed for the presence of centromeric (cen) and telomeric (tel) signals in the chromatin bridges connecting the daughter cells. Only seven cells with *cut* phenotype had short and thick bridges.

Discussion

We have previously reported an excess of metaphase singlet FISH hybridization signals at several telomeres in pre-senescent fibroblasts (Ofir et al., 2002). In the current paper, we show that this finding extends to an additional 12 out of 12 telomeres examined and therefore appears to be a more general

Table 4. Distribution of telomere and centromere signals on *cut* bridges induced by ND-securin

	Thin bridges (95%, *n*=36)			Thick bridges (5%, *n*=2)	
	+ tel	– tel	Total	+ tel	– tel
+ cen	25%	3%	28%	*n*=1	–
– cen	55%	17%	72%	*n*=1	–
Total	80%	20%			

Fibroblast cells displaying the *cut* phenotype following infection with ND-securin were analyzed for the presence of centromeric (cen) and telomeric (tel) signals in the chromatin bridges connecting the daughter cells. Only 2/38 cells with *cut* phenotype had short and thick bridges.

Table 5. Number of telomeric signals present on *cut* bridges induced in early-passage fibroblasts by ND-Scc1 and ND-securin

	ND-Scc1		ND-securin	
No. of signals	Thin (*n*=53)	Thick (*n*=4)	Thin (*n*=29)	Thick (*n*=2)
1-2	62.3%	–	58.6%	–
3-5	32.0%	*n*=2	27.6%	*n*=2
>5	5.7%	*n*=2	13.8%	–

The percentage/number of telomeric signals present on thin and thick *cut* bridges determined by PNA-FISH with a telomeric probe.

phenomenon relevant to telomeric regions. The in vivo significance of telomere metaphase singlets in relation to cellular ageing was highlighted by the finding that excess telomere metaphase singlets were also observed at a lower number of PDs in fibroblasts harvested from elderly individuals compared with newborn foreskin fibroblasts. Hence, this phenomenon is not solely the consequence of ageing in culture, and is also evident in fibroblasts that have reached senescence in culture after significantly fewer population doublings compared with foreskin fibroblasts. It is this consistent trend under both sets of experimental conditions, in vitro and in vivo, rather than the absolute percentages of singlets and doublets, that underscores the potential physiological significance of this finding. Final validation of the in vivo significance of excess metaphase singlets would require the future development of a combined in situ FISH and IF method applicable to fresh tissues.

Confocal microscopy analysis of the singlet signal intensities suggests that the telomere metaphase singlets represent DNA that has replicated but failed to separate. To confirm this conclusion we conducted a further set of FISH hybridizations to metaphase cells with telomeric probes, but carried out cell fixation with methanol and acetic acid as opposed to formaldehyde (results not shown). Following methanol/acetic acid fixation, which disrupts nuclear protein integrity, telomere metaphase singlets were not visualized, indicating that the loss of protein-based interactions important for telomeric cohesion enables the resolution of the signals emanating from both sister chromatids. This is consistent with recent reports that indicate that under certain conditions for the nuclei preparation, replicated DNA at other genomic regions may be visualized as FISH singlets (Azuara et al., 2003; Mesner et al., 2003).

Until recently, FISH signals in vertebrate S-phase cells have been interpreted to represent principally the state of DNA replication at a genomic region that corresponds to the FISH probe, assuming that minimal separation of replicated DNA to a distance that enables visualization of two FISH signals occurs readily following replication. However, evidence stemming from the current study and others (Volpi et al., 2001; Azuara et al., 2003) suggests that not all chromosomal regions behave similarly with respect to the dynamics of sister-chromatid resolution following replication. A direct correlation between the degree of cohesion and the distance between the FISH signals of replicated chromatids in S-phase was shown in ovine and human cells either depleted of the cohesion Scc1 subunit or expressing a dominant-negative Scc1 protein (Sonoda et al., 2001; Hoque and Ishikawa, 2002) and in inactive chromatin regions in lymphocytes (Azuara et al., 2003). The current study indicates that telomeres may also be an exception to the normal dynamics of sister chromatid separation, with a delay in separation occurring as late as metaphase in pre-senescent cells. Thus, after the prophase dissolution pathway mediated by polo-like kinase has been engaged (Losada et al., 2002; Sumara et al., 2002), it appears that in addition to centromeres, regions of persistent cohesion at telomeres also remain sensitive to perturbation in the APC-initiated pathway for sister chromatid dissolution.

Perhaps the most convincing evidence for persistent cohesion at telomeric regions beyond prophase is in *S. pombe*, where telomeric DNA is shown on the chromatin bridges of *cut*1 mutants that are defective in the separase gene (Funabiki et al., 1993). On the basis of FISH analysis, the centromeres in these mutant cells have segregated to the daughter cells, whereas it is the subtelomeric regions that have failed to separate. *Cut* phenotypes in mammalian cells (Hauf et al., 2001; Zur and Brandeis, 2001; Hagting et al., 2002; Chestukhin et al., 2003) have been attributed to persistent cohesion at centromeres. However, in the current study we show that similar to fission yeast, mammalian cells with mutations in the APC/C pathway for Scc1 removal manifest a *cut* phenotype in which many of the chromatin bridges contain telomeric sequences (Tables 3 and 4). The number of telomere signals present on the chromatin bridges (Table 5) indicates that a small number of chromosomes per cell fail to separate their telomeric regions. The high percentage of anaphase bridges in HeLa cells expressing a similar Scc1 mutation (Hauf et al., 2001) also supports persistent telomere cohesion. Thus, it is likely that two distinct classes of chromatin bridges occur in *cut* phenotype cell population: those that emanate from persistent centromere cohesion, as previously considered, and those that emanate from cohesion at telomeres. The latter appear to be the more frequent basis for *cut* phenotype in primary fibroblasts, as shown in the current study.

Because persistent cohesion at centromeres is probably sufficient to ensure proper alignment of the replicated chromosomes on the spindle, it is tempting to consider the potential roles of cohesion at telomeric regions in normal mitosis. The heterochromatic nature of subtelomeric regions may be the cause of persisting cohesion (Bernard et al., 2001) (reviewed by Bernard and Allshire, 2002; Nasmyth, 2002) without implicating a specific role of telomeres per se. However, rad21 (the *S. pombe* scc1 gene) mutants were originally characterized as γ-irradiation sensitive (Birkenbihl and Subramani, 1992), and chick cells depleted of Scc1 are defective in the repair of DNA damage via homologous DNA

recombination (Sonoda et al., 2001). Thus, it is possible that rad21/Scc1 via cohesion may play a role in DNA damage repair via their binding at telomeric sites. Cellular responses to telomere uncapping are very similar to the molecular processes for double-strand break (DSB) repair (Chan and Blackburn, 2002), and DNA-damage repair proteins might be involved in the process of telomeric-DNA repair before replicative senescence (Lansdorp, 2000). Alternatively, cohesion at telomeres might facilitate the homologous recombination repair of DNA damage at other sites on the chromosome by preserving the close contact of sister chromatids along the entire length of the chromosome arms (Sonoda et al., 2001; Morrison et al., 2003). It is unclear whether senescent cells do accumulate higher levels of DSBs; however, there are indications that fibroblasts aged in culture and ageing murine kidney cells do show a significant elevation in DSBs (Singh et al., 2001; Chevanne et al., 2003). Whatever the role of cohesion at telomeres in signaling DNA damage may be, it is tempting to speculate that the presence of cohesin at telomeric regions provides a bridging mechanism between pathways for DNA damage repair and the activation of the spindle assembly checkpoint. Evidence is emerging for an ATM-independent mechanism for activation of such a checkpoint in response to DSBs in mitosis (Mikhailov et al., 2002). This could be achieved in a manner whereby so long as the need for DSB repair activity persists, the spindle assembly checkpoint prevents the initiation of the cascade of events leading to Scc1 degradation, as has been previously suggested (Uhlmann, 2003).

In addition to the DNA-damage repair proteins, telomerase plays a crucial role in capping telomeres and circumventing cellular senescence (Chan and Blackburn, 2002). Correspondingly, we found that telomerase also induces separation of the metaphase singlets at telomeres as early as 4 days after infection of pre-senescent cells with hTERT. This suggests an effect independent of substantial elongation of telomeres, as supported also by the lack of excess metaphase singlets in the telomerase-positive OVCAR cells, despite their very short average telomere length (approximately 3.5 kb). However, minimal lengthening of critically short telomeres during this short period of hTERT expression cannot be ruled out with certainty. Direct proof that this effect of telomerase is not dependent on the catalytic reverse transcription activity of hTERT, and mapping of the relevant protein domains for this effect, will require future studies with mutant versions of hTERT lacking catalytic and other functional domains.

Persistent incomplete sister chromatid separation at telomeric regions in pre-senescent cells may contribute to the functionally ‘uncapped’ state of short telomeres, and in turn it may play a role in the generation of chromosomal abnormalities with cellular ageing (Saksela and Moorhead, 1963; Thompson and Holliday, 1975; Benn, 1976; Mondello et al., 1997). Cells with unseparated telomeres may experience breakage of the chromatin bridges before cytokinesis which, in turn, may result in chromosomal abnormalities. Breakage following cytokinesis would also most probably jeopardize the subsequent survival of these cells. Conversely, telomerase-mediated abrogation of this incomplete separation may contribute to cellular immortalization. Further studies will establish whether the underlying mechanism for telomere cohesion in pre-senescent cells indeed involves the APC/C pathway, as suggested by the results obtained in the current study using early passage fibroblasts. If this pathway is involved, then this would motivate the search for mechanisms resulting in the persistent binding or recruitment of Scc1 or other members of the cohesin complex at telomeric regions, especially in senescence.

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References

Allsopp, R. C., Vaziri, H., Patterson, C., Goldstein, S., Younglai, E. V., Futcher, A. B., Greider, C. W. and Harley, C. B. (1992). Telomere length predicts replicative capacity of human fibroblasts. *Proc. Natl. Acad. Sci. USA* **89**, 10114-10118.

Azuara, V., Brown, K. E., Williams, R. R. E., Webb, N., Dillon, N., Festenstein, R., Buckle, V., Merkenschlager, M. and Fisher, A. G. (2003). Heritable gene silencing in lymphocytes delays chromatid resolution without affecting the timing of DNA replication. *Nat. Cell Biol.* **5**, 668-674.

Benn, P. A. (1976). Specific chromosome aberrations in senescent fibroblast cell lines derived from human embryos. *Am. J. Hum. Genet.* **28**, 465-473.

Bernard, P., Maure, J. F., Partridge, J. F., Genier, S., Javerzat, J. P. and Allshire, R. C. (2001). Requirement of heterochromatin for cohesion at centromeres. *Science* **294**, 2539-2542.

Bernard, P. and Allshire, R. (2002). Centromeres become unstuck without heterochromatin. *Trends Cell Biol.* **12**, 419-424.

Birkenbihl, R. P. and Subramani, S. (1992). Cloning and characterization of rad21 an essential gene of Schizosaccharomyces pombe involved in DNA double-strand-break repair. *Nucleic Acids Res.* **20**, 6605-6611.

Blackburn, E. H. (2001). Switching and signaling at the telomere. *Cell* **106**, 661-673.

Blazer, S., Khankin, E., Segev, Y., Ofir, R., Yalon-Hacohen, M., Kra-Oz, Z., Gottfried, Y., Larisch, S. and Skorecki, K. (2002). High glucose-induced replicative senescence: point of no return and effect of telomerase. *Biochem. Biophys. Res. Commun.* **296**, 93-101.

Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C.-P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S. and Wright, W. E. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349-352.

Boggs, B. A. and Chinault, A. C. (1997). Analysis of DNA replication by fluorescence in situ hybridization. *Methods* **13**, 259-270.

Braunstein, I., Cohen-Barak, O., Shachaf, C., Ravel, Y., Yalon-Hacohen, M., Mills, G. B., Tzukerman, M. and Skorecki, K. L. (2001). Human telomerase reverse transcriptase promoter regulation in normal and malignant human ovarian epithelial cells. *Cancer Res.* **61**, 5529-5536.

Burns, J. C., Friedmann, T., Driever, W., Burrascano, M. and Yee, J.-K. (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: Concentration to very high titer and efficient gene transfer into mammalian and nonmamammalian cells. *Proc. Natl. Acad. Sci. USA* **90**, 8033-8037.

Cervantes, R. B. and Lundblad, V. (2002). Mechanisms of chromosome-end protection. *Curr. Opin. Cell Biol.* **14**, 351-356.

Chan, S. W.-L. and Blackburn, E. H. (2002). New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin. *Oncogene* **21**, 553-563.

Chestukhin, A., Pfeffer, C., Milligan, S., DeCaprio, J. A. and Pellman, D. (2003). Processing, localization, and requirement of human separase for normal anaphase progression. *Proc. Natl. Acad. Sci. USA* **100**, 4574-4579.

Chevanne, M., Caldini, R., Tombaccini, D., Mocali, A., Gori, G. and Paoletti, F. (2003). Comparative levels of DNA breaks and sensitivity to oxidative stress in aged and senescent human fibroblasts: a distinctive pattern for centenarians. *Biogerontology* **4**, 97-104.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M. and Nasmyth, K. (1998). An ESP1/PDS1 complex regulates loss of sister

chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* **93**, 1067-1076.

Cohen-Fix, O., Peters, J. M., Kirschner, M. W. and Koshland, D. (1996). Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev.* **10**, 3081-3093.

Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B. and Bacchetti, S. (1992). Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* **11**, 1921-1929.

Filatov, L., Golubovskaya, V., Hurt, J. C., Byrd, L. L., Phillips, J. M. and Kaufmann, W. K. (1998). Chromosomal instability is correlated with telomere erosion and inactivation of G2 checkpoint function in human fibroblasts expressing human papillomavirus type 16 E6 oncoprotein. *Oncogene* **16**, 1825-1838.

Funabiki, H., Hagan, I., Uzawa, S. and Yanagida, M. (1993). Cell cycle-dependent specific positioning and clustering of centromeres and telomeres in fission yeast. *J. Cell Biol.* **121**, 961-976.

Gall, J. G. (1995). Beginning of the end: origins of the telomere concept. In *Telomeres* (ed. E. H. Blackburn and C. W. Greider), pp. 1-10. New York: Cold Spring Harbor Laboratory Press.

Gasser, S. (2000). A sense of the end. *Science* **288**, 1377-1379.

Hagting, A., den Elzen, N., Vodermaier, H. C., Waizenegger, I. C., Peters, J.-M. and Pines, J. (2002). Human securin proteolysis is controlled by the spindle checkpoint and reveals when the APC/C switches from activation by Cdc20 to Cdh1. *J. Cell Biol.* **157**, 1125-1137.

Harley, C. B., Futcher, A. B. and Greider, C. W. (1990). Telomeres shorten during ageing of human fibroblasts. *Nature* **345**, 458-460.

Hauf, S., Waizenegger, I. C. and Peters, J. M. (2001). Cohesin cleavage by separase required for anaphase and cytokinesis in human cells. *Science* **293**, 1320-1323.

Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett-Jones, D. P. and Allis, C. D. (1997). Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* **106**, 348-360.

Hoque, M. T. and Ishikawa, F. (2001). Human chromatid cohesin component hRad21 is phosphorylated in M phase and associated with metaphase centromeres. *J. Biol. Chem.* **276**, 5059-5067.

Hoque, M. T. and Ishikawa, F. (2002). Cohesin defects lead to premature sister chromatid separation, kinetochore dysfunction, and spindle-assembly checkpoint activation. *J. Biol. Chem.* **277**, 42306-42314.

Jallepalli, P. V., Waizenegger, I. C., Bunz, F., Langer, S., Speicher, M. R., Peters, J. M., Kinzler, K. W., Vogelstein, B. and Lengauer, C. (2001). Securin is required for chromosomal stability in human cells. *Cell* **105**, 445-457.

Kitsberg, D., Selig, S., Keshet, I. and Cedar, H. (1993). Replication structure of the human beta-globin gene domain. *Nature* **366**, 588-590.

Lansdorp, P. M., Verwoerd, N. P., van den Rijke, F. M., Dragowska, V., Little, M.-T., Dirls, R. W., Raap, A. K. and Tanke, H. J. (1996). Heterogeneity in telomere length of human chromosomes. *Hum. Mol. Genet.* **5**, 685-691.

Lansdorp, P. (2000). Repair of telomeric DNA prior to replicative senescence. *Mech. Ageing Dev.* **118**, 23-34.

Lichter, P., Tang, C.-J. C., Call, K., Hermanson, G., Evans, G. A., Housman, D. and Ward, D. C. (1990). High-resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. *Science* **247**, 64-69.

Losada, A. and Hirano, T. (2001). Shaping the metaphase chromosome: coordination of cohesion and condensation. *BioEssay* **23**, 924-935.

Losada, A., Hirano, M. and Hirano, T. (2002). Cohesion release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. *Genes Dev.* **16**, 3004-3016.

McChesney, P. A., Aisner, D. L., Frank, B. C., Wright, W. E. and Shay, J. W. (2000). Telomere dynamics in cells with introduced telomerase: a rapid assay for telomerase activity on telomeres. *Mol. Cell. Biol. Res. Commun.* **3**, 312-318.

McEachern, M. J., Krauskopf, A. and Blackburn, E. H. (2000). Telomeres and their control. *Annu. Rev. Genet.* **34**, 331-358.

Mesner, L. D., Hamlin, J. L. and Dijkwel, P. A. (2003). The matrix attachment region in the Chinese hamster dihydrofolate reductase origin of replication may be required for local chromatid separation. *Proc. Natl. Acad. Sci. USA* **100**, 3281-3286.

Michaelis, C., Ciosk, R. and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**, 35-45.

Mikhailov, A., Cole, R. W. and Rieder, C. L. (2002). DNA damage during mitosis in human cells delays the metaphase/anaphase transition via the spindle assembly checkpoint. *Curr. Biol.* **12**, 1797-1806.

Mondello, C., Riboni, R., Casati, A., Nardo, T. and Nuzzo, F. (1997). Chromosomal instability and telomere length variations during the life span of human fibroblast clones. *Exp. Cell Res.* **236**, 385-396.

Morgenstern, J. P. and Land, H. (1990). Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18**, 3587-3596.

Morrison, C., Vagnarelli, P., Sonoda, S., Takeda, S. and Earnshaw, W. C. (2003). Sister chromatid cohesion and genome stability in vertebrate cells. *Biochem. Soc. Trans.* **31**, 263-265.

Nasmyth, K. (2001). Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis. *Annu. Rev. Genet.* **35**, 673-745.

Nasmyth, K. (2002). Segregating sister genomes: the molecular biology of chromosome separation. *Science* **297**, 559-565.

Ning, Y., Roschke, A., Smith, A. C. M., Machal, M., Precht, K., Rietman, H. and Ledbetter, D. (1996). A complete set of human telomeric probes and their clinical application. National Institutes of Health and Institute of Molecular Medicine collaboration. *Nat. Genet.* **14**, 86-89.

Ofir, R., Yalon-Hacohen, M., Segev, Y., Schultz, A., Skorecki, K. L. and Selig, S. (2002). Replication and/or separation of some human telomeres is delayed beyond S-phase in pre-senescent cells. *Chromosoma* **111**, 147-155.

Ouellette, M. M., Aisner, D. L., Savre-Train, I., Wright, W. E. and Shay, J. W. (1999). Telomerase activity does not always imply telomere maintenance. *Biochem. Biophys. Res. Commun.* **254**, 795-803.

Saksela, E. and Moorhead, P. S. (1963). Aneuploidy in the degenerative phase of serial cultivation of human cell strains. *Proc. Natl. Acad. Sci. USA* **50**, 390-395.

Selig, S., Okumura, K., Ward, D. C. and Cedar, H. (1992). Delineation of DNA replication time zones by fluorescence in situ hybridization. *EMBO J.* **11**, 1217-1225.

Shapira, M., Zhai, R. G., Dresbach, T., Bresler, T., Torres, V. I., Gundelfinger, E. D., Ziv, N. E. and Garner, C. C. (2003). Unitary assembly of presynaptic active zones from Piccolo-Bassoon transport vesicles. *Neuron* **38**, 237-252.

Singh, N. P., Ogburn, C. E., Wolf, N. S., van Belle, G. and Martin, G. M. (2001). DNA double-strand breaks in mouse kidney cells with age. *Biogerontology* **2**, 261-270.

Sonoda, E., Matsusaka, T., Morrison, C., Vagnarelli, P., Hoshi, O., Ushiki, T., Nojima, K., Fukagawa, T., Waizenegger, I. C., Peters, J. M. et al. (2001). Scc1/Rad21/Mcd1 is required for sister chromatid cohesion and kinetochore function in vertebrate cells. *Dev. Cell* **1**, 759-770.

Sumara, I., Vorlaufer, E., Stukenberg, P. T., Kelm, O., Redemann, N., Nigg, E. A. and Peters, J. M. (2002). The dissociation of cohesin from chromosomes in prophase is regulated by Polo-like kinase. *Mol. Cell* **9**, 515-525.

Thompson, K. V. and Holliday, R. (1975). Chromosome changes during the in vitro ageing of MRC-5 human fibroblasts. *Exp. Cell Res.* **96**, 1-6.

Uhlmann, F., Lottspeich, F. and Nasmyth, K. (1999). Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature* **400**, 37-42.

Uhlmann, F. (2003). Chromosome cohesion and separation: from men and molecules. *Curr. Biol.* **13**, R104-R114.

Volpi, E. V., Sheer, D. and Uhlmann, F. (2001). Cohesion, but not too close. *Curr. Biol.* **11**, R378.

Warren, W. D., Steffensen, S., Lin, E., Coelho, P., Loupart, M.-L., Cobbe, N., Lee, J. Y., McKay, M. J., Orr-Weaver, T., Heck, M. M. S. et al. (2000). The Drosophila RAD21 cohesin persists at the centromere region in mitosis. *Curr. Biol.* **10**, 1463-1466.

Weinrich, S. L., Pruzan, R., Ma, L., Ouellette, M., Tesmer, V. M., Holt, S. E., Bodnar, A. G., Lichsteiner, S., Kim, N. W., Trager, J. B. et al. (1997). Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nat. Genet.* **17**, 498-502.

Yanagida, M. (1998). Fission yeast *cut* mutations revisited: control of anaphase. *Trends Cell Biol.* **8**, 144-149.

Zakian, V. A. (1995). Telomeres: beginning to understand the end. *Science* **270**, 1601-1607.

Zur, A. and Brandeis, M. (2001). Securin degradation is mediated by fzy and fzr, and is required for complete chromatid separation but not for cytokinesis. *EMBO J.* **20**, 792-801.