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Phosphorylation of H2AX at Short Telomeres in T Cells and Fibroblasts*

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Ling-Yang Hao‡, Margaret A. Strong§, and Carol W. Greider§¶

From the ‡Graduate Program in Human Genetics and §Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Eukaryotic cells undergo arrest and enter apoptosis in response to short telomeres. T cells from late generation mTR$^{-/-}$ mice that lack telomerase show increased apoptosis when stimulated to enter the cell cycle. The increased apoptosis was not inhibited by colcemid, indicating that the response did not result from breakage of dicentric chromosomes at mitosis. The damage response protein γ-H2AX localized to telomeres in metaphases from T cells and fibroblasts from mTR$^{-/-}$ cells with short telomeres. These data suggest that the major mechanism for induction of apoptosis in late generation mTR$^{-/-}$ cells is independent of chromosome segregation and that loss of telomere function through progressive telomere shortening in the absence of telomerase leads to recognition of telomeres as DNA breaks.

The telomeres consist of tandem sequence repeats bound by specific telomere proteins and are required for chromosome stability. Telomere sequence loss during replication (1, 2) is balanced by sequence addition by telomerase. Telomerase contains an essential RNA and a catalytic protein component (3). Telomere-binding proteins bind to the repeat sequence to help carry out telomere function. Loss of either telomere-binding proteins or telomere sequence causes telomere dysfunction, although the mechanisms by which this occurs are not yet clear. When a dominant negative version of the telomere-binding protein TRF2, which removes endogenous TRF2 from telomeres, is expressed, cells undergo chromosome fusions, cellular senescence, and apoptosis (4). In contrast to this rapid effect caused by the removal of telomere-binding proteins, telomere sequence loss that occurs in the absence of telomerase generates a more gradual response. Yeast grown in the absence of functional telomerase show progressive decline in growth potential (5). Once sufficient telomeres are critically short, cells arrest at G_2/M and show increased genomic instability (6–8). Mice that lack the RNA component of telomerase (mTR$^{-/-}$) have decreased fertility, increased T cell apoptosis, and an increased number of chromosome fusions after successive interbreeding for four to six generations (9, 10).

The mTR$^{-/-}$ mice revealed that telomeres play two different roles in tumorigenesis. Short telomeres trigger cell death that may contribute to reduction in tumor progression (11); on the other hand, short telomeres also lead to an increase in tumor formation in some mouse models (12–14). This increase is likely to be the result of genomic instability initiated by short dysfunctional telomeres (6, 15, 16).

Cells employ multiple pathways to sense and repair DNA double-stranded breaks. Broken ends are recognized and repaired by specific proteins including DNA-PKcs, Ku, the MRX complex (MRE11, RAD50, and NBS1/Xrs2), RPA, ATM, ATR, ATRIP, and others (reviewed in Ref. 17). Unrepaired DNA breaks can lead to chromosome instability (reviewed in Ref. 18). Thus, when cells encounter irreparable damage, they may undergo programmed cell death to protect the organism. Failure to eliminate damaged cells may result in chromosome rearrangements, often leading to further instability (reviewed in Refs. 17 and 19). One of the earliest events at the site of DNA damage is the phosphorylation of histone variant H2AX, γ-H2AX (20). The presence of γ-H2AX provides the platform for other damage proteins such as p53BP1, Mre11, and Brca1 to localize to the break site (21).

The loss of telomere function has been proposed to mimic a DNA double-stranded break. However, the relationship between telomere function and the DNA damage response pathway is complex. A major role of the telomere is to distinguish natural chromosome ends from DNA breaks. At the same time, many proteins involved in DNA damage response such as Ku, MRX complex, and DNA-PKcs normally localize to telomeres (22–24). In yeast, when an endogenous DNA break is created, some of the telomere-bound DNA repair proteins relocalize from the telomere to the DNA break site. In addition, the cells that are deleted for these damage recognition/repair genes often have altered telomere length (25–29).

When dysfunctional telomeres are generated either through removal of telomere-binding proteins or through telomere shortening in the absence of telomerase, DNA damage pathways are activated. Yeast with critically short telomeres activate DNA damage checkpoints and cells arrest at the G_2/M phase of the cell cycle (7, 8, 30). Overexpression of dominant negative TRF2 in human cells triggers p53- and ATM-dependent apoptosis (4). Short telomeres trigger a p53 response in late generation mTR$^{-/-}$ mice (15), and cells with short telomeres have an enhanced sensitivity to ionizing radiation (31, 32). Thus, although it is clear that dysfunctional telomeres send a signal similar to DNA damage, the mechanism that recognizes telomere dysfunction is not clear.

The increased apoptosis in late generation mTR$^{-/-}$ cells may be due to recognition of critically short telomeres as double-stranded DNA breaks. Alternatively, it may be due to chromosome fusions and subsequent breakage of dicentric chromosomes. To distinguish between these mechanisms, we studied apoptosis in late generation mTR$^{-/-}$ T lymphocytes. We found that apoptosis was independent of M phase progression, suggesting that the breakage of dicentric chromosomes is not a major factor in triggering apoptosis. In addition, we found that

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¶ To whom correspondence should be addressed: Dept. of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, 617 Hunterian Bldg., 725 N. Wolfe St., Baltimore, MD 21205. Tel.: 410-614-6506; Fax: 410-614-2987; E-mail: cgreider@jhmi.edu.

the damage marker γ-H2AX localized to the telomere in late generation mTR$^{-/-}$ cells. Our results suggest that critically short telomeres are recognized directly as DNA breaks.

MATERIALS AND METHODS

Mice—Two independent lines of mTR$^{-/-}$ mice were used. Line 1 used for spectral karyotyping analysis was 60% C57BL/6, 37.5% 129/Sv, and 2.5% SJL as described (9). Line 2 used for T cell and MEF[1] studies was derived from line 1 by backcrossing to C57BL/6J as described (33).

Cell Culture and T Lymphocyte Purification—To purify T cells, splenocytes were isolated through negative selection with T cell enrichment gravity flow through kit (13031; StemCell Technologies). The purified T cells were stimulated with 4 μg of ConA (Sigma) and 20 units of IL-2 (Roche Applied Science) at 2 million cells/ml of medium.

TUNEL Analysis—Isolated T cells were stimulated with ConA and IL-2 in culture for 48 h prior to TUNEL analysis. The cells were analyzed by an *in situ* cell death detection kit (1 684 795; Roche Applied Science).

Spectral Karyotyping—Spectral karyotyping was performed on late generation mTR$^{-/-}$ splenocyte metaphases as described (34). The splenocytes were cultured and stimulated with 1 mg of lipopolysaccharide (Sigma), 400 μg of ConA, and 200 units of IL-2 for 48 h. The cells were arrested in colcemid for 1 h and then swelled in 4% KCl for 4 min at 37 °C. The cells were then washed six times in 3:1 methanol:aceitic acid fixative. The metaphase spreads were hybridized and analyzed according to the manufacturer's protocol (Applied Spectral Imaging).

Cell Staining and FACS Analysis—Unfixed cells were washed with PBS and stained prior to FACS analysis. For cell apoptosis analysis, Annexin V-Alexa 568 was used as suggested by the vendor (1985485; Roche Applied Science), and fluorescein isothiocyanate-coupled anti-BrdU (556028 PharMingen) was used to detect S phase progression of the cells. Cell cycle analysis was carried out by washing cells with PBS once and then incubating in staining solution (in 10 ml of PBS with 0.025% Nonidet P-40, 1 mg of RNase A, and 50 mg of propidium iodide) for 30 min in the dark. For CFSE (C1157 Molecular Probe) analysis, T cells were washed once with PBS and then resuspended at 4 million cells/ml in PBS and stained with 2 μM CFSE for 8 min in the dark at room temperature. 5 ml of culture medium was then added to the cells and incubated for 5 min in dark. Stained cells were washed three times with PBS and then cultured at 2–4 million cells/ml of culture medium with ConA and IL-2 in the dark.

Cytospin Preparation and Immunostaining—The cells were washed once with PBS and then treated with 0.075 M KCl for 2 min, and the metaphases were prepared by spinning cells on to slides in a cytospin centrifuge (Thermo Shandon Cytospin 3) at 1000 rpm for 5 min. The cells attached to the slides were fixed with methanol or ethanol for 1 h at −20 °C. The cells were stained with 1:4000 γ-H2AX antibody (16193; Upstate Biotechnology, Inc.) or 1:400 p53BP1 (a gift from Dr. Junjie Chen). A secondary antibody was used at 1:10,000 Cy3 or 1:1000 fluorescein isothiocyanate (Jackson Immunoresearch). Both primary and secondary antibodies were diluted in KCM (120 mM KCl, 20 mM NaCl, 10 mM Tris-HCl, pH 7.7, 0.1% Triton X-100). For staining with both γ-H2AX and telomere FISH, the cells were treated with RSB buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$) for 10 min at 37 °C, then cytospun at 2000 rpm for 5 s, and then fixed in 2% formaldehyde for 10 min. The slides were washed with PBS and treated with 0.5% Nonidet P-40 in PBS. Because cytospun metaphases often contain partial metaphase chromosome sets, we quantitated the average number of chromosomes/spread and found no significant difference between wild type and late generation mTR$^{-/-}$ ($p > 0.05$). γ-H2AX signals were scored first before telomere FISH.

Immunoblot Analysis—The cells were lysed in radioimmune precipitation assay buffer (150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with 1× protease inhibitor mixture (1836153; Roche Applied Science) and 1× phosphatase inhibitor mixture 1(P2850; Sigma) after 48 h of stimulation. The antibodies used were: anti-Actin (A2066, 1:2000; Sigma), anti-Ser^{15} phospho-p53 (9284, 1:1000; Cell Signaling), anti-Ser^{317} phospho-Chk1 (A300–163A, 1:500; Bethyl), anti-Bax (2772, 1:2000; Cell Signaling), and anti-cleaved Caspase 3 (9661, 1:1000; Cell Signaling). The band intensity was normalized to the actin level with the ImageGauge program.

Mouse Embryonic Fibroblasts—MEFs were generated as described (9). The metaphases were generated from passage 3 MEFs from wild type and mTR$^{-/-}$ as described above.

RESULTS

Late Generation mTR$^{-/-}$ Mice T cells Undergo Apoptosis in Response to Mitogen—To examine the role of short telomeres in apoptosis of lymphocytes, we stimulated late generation mTR$^{-/-}$ mouse T cells *in vitro* for 48 h and stained with annexin V to identify apoptotic cells and with propidium iodide to determine DNA content. Late generation mTR$^{-/-}$ T cells showed an increase in apoptotic cells compared with age-matched wild type cells (Fig. 1*A*) in agreement with a previous report (35). Three independent experiments with mTR$^{-/-}$ mice showed a consistent 2-fold increase in the total number of annexin V-positive mTR$^{-/-}$ T cells compared with wild type.

After 48 h in culture without stimulation, both wild type and mTR$^{-/-}$ T cell cultures showed a high level of apoptosis (36) (Fig. 1*A*). At 48 h post stimulation, the cells had identifiable G_1, S, and G_2 peaks typical of proliferating cells (Fig. 1*B*); thus, cells were not entering apoptosis because of a lack of stimulation. The unstimulated cultures exhibit a predominant G_1/G_0 peak and substantial sub-G_1/G_0 peak that represents the apoptotic cells (Fig. 1*B*). There was an increase in the sub-G_1/G_0 peak in stimulated mTR$^{-/-}$ culture compared with wild type T cells (Fig. 1*B*), consistent with the increase apoptosis observed by annexin V staining (Fig. 1*A*). To further examine the apoptosis in T cells, we quantitated the number of TUNEL-positive cells in wild type and mTR$^{-/-}$ mice. 26% of the mTR$^{-/-}$ T cells were TUNEL-positive cells, whereas only 14% of T cells from wild type cultures were positive. To test whether this increase in apoptosis results from increased DNA damage response in mTR$^{-/-}$ cells, we used Western analysis to examine the level of several markers of DNA damage response. We found an increase in phosphorylated form of Chk1 in mTR$^{-/-}$ T cells (Fig. 3*D*). We further found a 2-fold increase in p53 phosphorylated at serine 15, cleaved caspase 3, and BAX in mTR$^{-/-}$ protein extract (data not shown).

T Cell Apoptosis Is Independent of Chromosome Segregation—To determine whether the apoptosis and induction of a DNA damage response in late generation mTR$^{-/-}$ T cells was due to short telomeres or to chromosome breakage at metaphase, we examined the cell cycle dependence of apoptosis. When mTR$^{-/-}$ T cells were grown in the presence of BrdU for 48 h, 89% the cells were both BrdU- and annexin V-positive. This result indicates that annexin V-positive cells in the late generation mTR$^{-/-}$ culture have replicated at least once (Fig. 2*A*). Telomere dysfunction causes end-to-end fusion in mTR$^{-/-}$ cells (9); therefore, the apoptosis seen may be the consequence of the breakage of dicentric chromosomes during segregation. To examine this possibility, we determined whether apoptosis in these cells requires the completion of M phase. T lymphocytes from wild type and late generation mTR$^{-/-}$ mice were first stimulated to cycle and then arrested with colcemid, a drug that inhibits spindle formation. The initial stimulation was required to distinguish those cells in the population that were capable of dividing initially from those that were not. If the increased level of apoptosis in mTR$^{-/-}$ cells requires chromosome segregation and breakage of dicentrics, then colcemid arrest should reduce the overall number of annexin V-positive cells. The percentage of annexin V-positive cells in the mTR$^{-/-}$ cultures did not decrease in the presence of colcemid in three independent experiments (Fig. 2*C*). To test the completeness of the colcemid block, we used both FACS analysis and CFSE staining. Colcemid arrest increased the number of G_2/M cells by 2.5-fold for both wild type and mTR$^{-/-}$ T cells (Fig. 2*B*).

[1] The abbreviations used are: MEF, mouse embryonic fibroblast; ConA, concanavalin A; IL, interleukin; TUNEL, TdT-mediated dUTP nick end labeling; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; BrdU, bromodeoxyuridine; CFSE, carboxy-fluorescein diacetate, succinimidyl ester; FISH, fluorescent *in situ* hybridization.

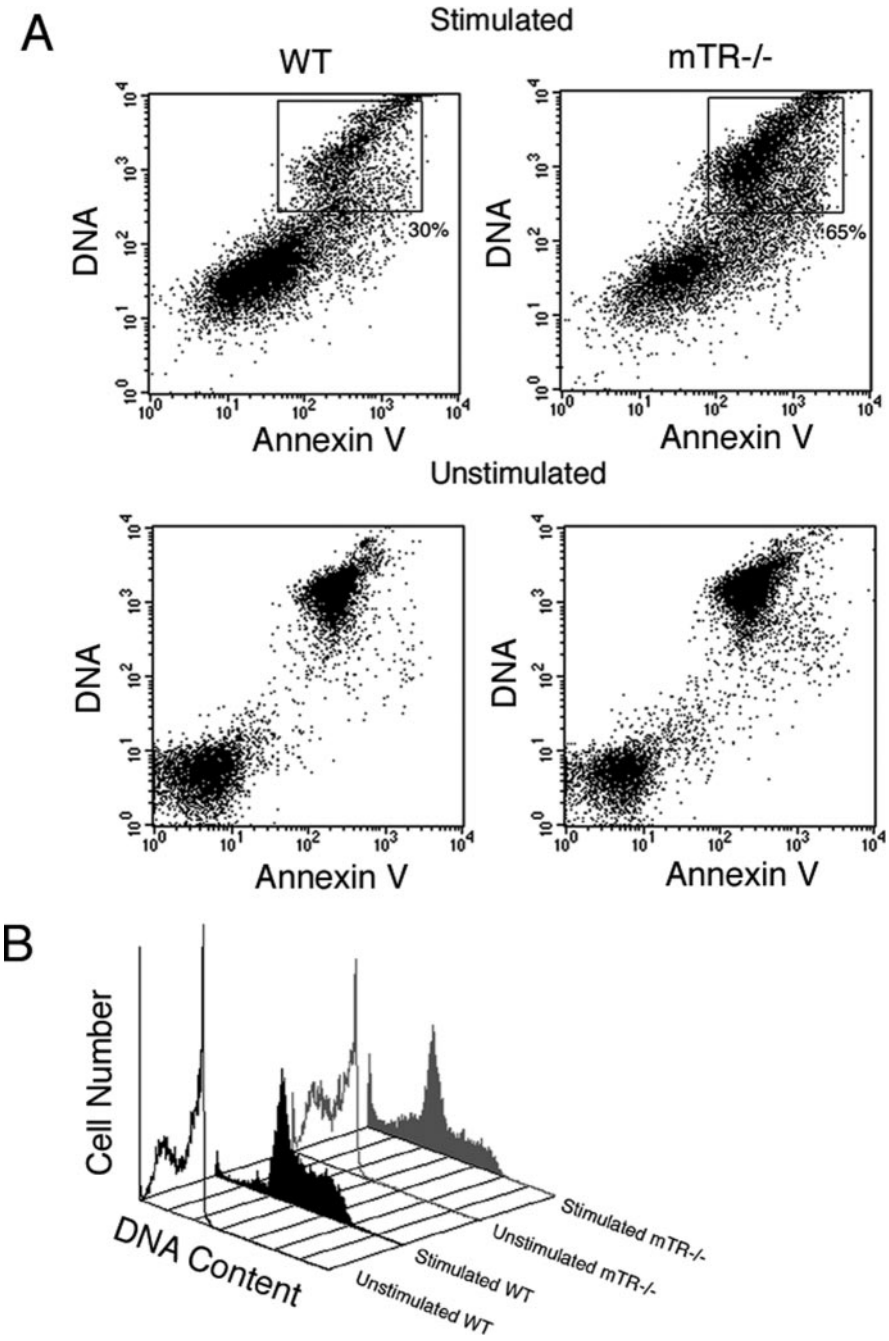

FIG. 1. **Increased annexin V-positive cells in mTR$^{-/-}$ cultures.** T cells from age-matched wild type (*WT*) and late generation mTR$^{-/-}$ mice were stimulated (*top panels*) *in vitro* for 48 h and assayed for apoptosis via annexin V staining. The *bottom panels* show the unstimulated cells after 48 h. There was a significant increase of apoptotic T cells from mTR$^{-/-}$ animals compared with wild type ($p < 0.05$). mTR$^{-/-}$ T lymphocytes had increased annexin V-positive cells (65%) compared with wild type sample (30%). *B*, cell cycle profile of stimulated and unstimulated mTR$^{-/-}$ (*gray*) and wild type (*black*) T cells after staining with propidium iodide.

CFSE is a cytoplasmic fluorescence dye that reduces its intensity by half every time the cell divides. We used this dye to determine the number of cell cycles that cells completed after stimulation (Fig. 2*D*, *black bars*). When cells were arrested with colcemid from 36 to 42 h after stimulation, the percentage of cells that had undergone four divisions decreased 2–4-fold in wild type and mTR$^{-/-}$ cells (Fig. 2*D*). Although FACS analysis and CFSE staining showed an efficient colcemid arrest, there was no comparable decrease in the level of apoptosis in the arrested cell population. This indicates that breakage of dicentric chromosomes at mitosis is not the predominant mechanism that leads to apoptosis in mTR$^{-/-}$ cells.

If apoptosis in mTR$^{-/-}$ cells was due to the accumulation of chromosome breaks in the cell divisions prior to arrest, we would expect to see translocations indicative of breakage, fusion, and bridge cycles. To address this possibility, we examined chromosome structure using spectral karyotyping. In agreement with previous reports (9), chromosome fusions were seen in the metaphase spreads from mTR$^{-/-}$ T cells (Table I). However, although fusions were seen, only 3 of 1100 late generation mTR$^{-/-}$ metaphases showed translocations. Because cycles of breakage, fusion, and bridge formation would lead to chromosome translocations, the low level of such rearrangements suggests that the accumulation of broken chromosomes through breakage, fusion, and bridge in the first few cell cycles is not the major mechanism that triggers apoptosis in mTR$^{-/-}$ cells.

Short Telomeres Are Recognized as DNA Breaks—To further understand the role of short telomeres in the induction of apoptosis, we tested whether short telomeres are recognized as DNA breaks. We examined whether phosphorylated H2AX, called γ-H2AX, one of the earliest modifications that occur at sites of double-stranded DNA breaks, is localized to telomeres. As a positive control we irradiated wild type cells with γ radiation, and these irradiated cells showed dosage-dependent γ-H2AX staining of metaphase chromosomes (Fig. 3*A*). We then

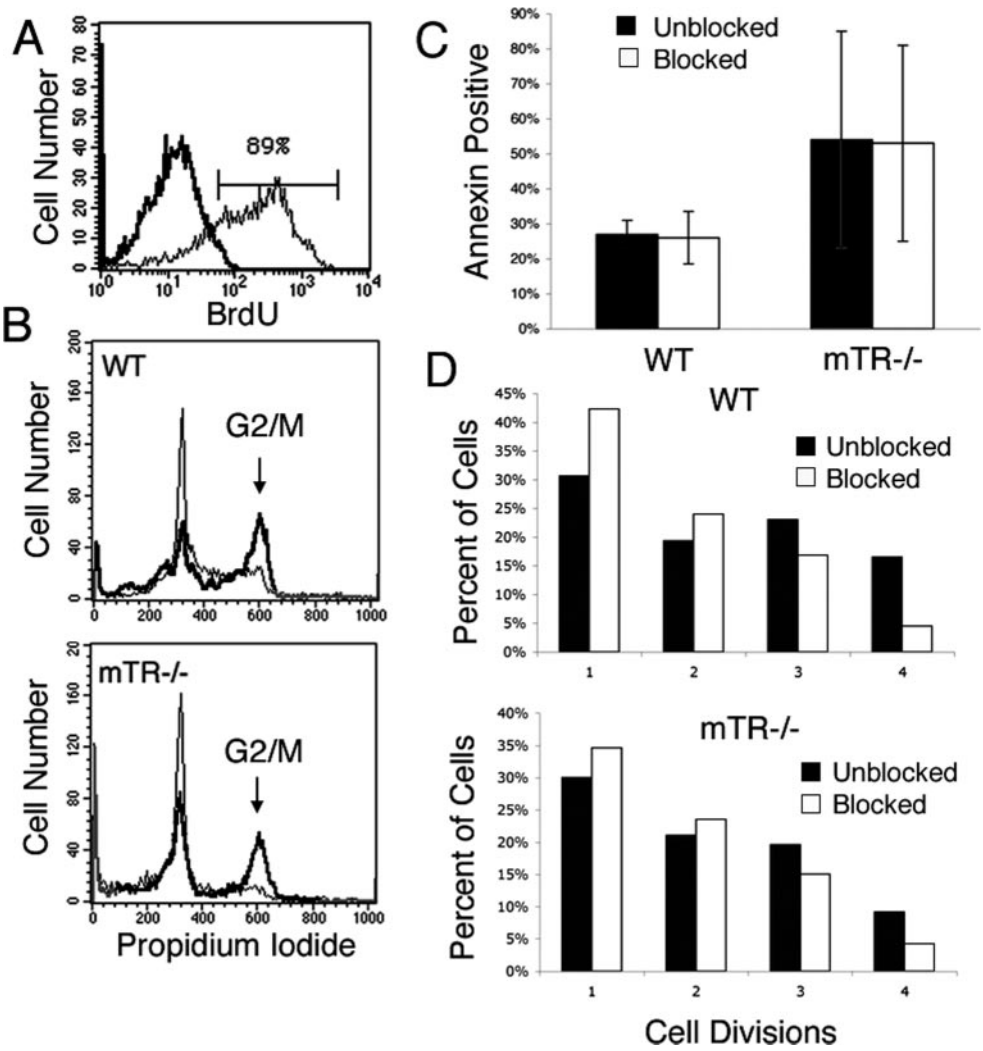

FIG. 2. **Late generation mTR$^{-/-}$ annexin V-positive cells are M phase independent.** *A*, analysis of cell cycling by BrdU incorporation. Late generation mTR$^{-/-}$ cells were grown in the presence (*thin black lines*) or absence of BrdU (*bold black lines*). The annexin V-positive cells were gated and tested for BrdU incorporation. 89% of the annexin-positive cells were BrdU-positive. *B*, the DNA content of T lymphocytes stimulated in culture for 44 h (*thin black line*) compared with cultures treated with colcemid for 6 h (*bold black line*) showed enrichment of G_2/M cells (indicated by *arrow*). *C*, cultures grown in the presence (*white bars*) or absence (*gray bars*) of colcemid for 6 h showed similar levels of annexin V-positive cells. The average was taken from three independent cell cultures of each genotype. *D*, the number of cell cycles completed and the effectiveness of colcemid arrest assayed by CFSE staining. The *top panel* shows number of divisions WT cells underwent as assayed by CFSE staining. *Black bars* represent cultures without arrest at 42 h post stimulation; *white bars* represent cells blocked with colcemide from 36 to 42 h post stimulation between the third and fourth divisions. The *bottom panel* shows the number of divisions mTR$^{-/-}$ cells underwent as assayed by CFSE staining. Two independent cultures of cells were assayed with similar results. *WT*, wild type.

TABLE I
Karyotype analysis of splenocytes from late generation mTR$^{-/-}$ mice

Metaphases examined	Translocations[a]	Fusions
968[b]	3	193[c]
162[d]	0	100[e]

[a] Translocation scored through spectral karyotype analysis.
[b] Total number of metaphases from BL/6 and BL/6 129 mix background strains.
[c] Ref. 33.
[d] Total number of metaphases from BL/6 and 129S6/SvEvTac mix background.
[e] Ref. 48.

generated metaphases from mature T cells from wild type and late generation mTR$^{-/-}$ mice. γ-H2AX staining was observed near the termini of chromosomes in the mTR$^{-/-}$ cells (Fig. 3*B*). We quantitated the γ-H2AX staining in wild type and late generation mTR$^{-/-}$ T cells from three independent mice. The chromosome termini from late generation mTR$^{-/-}$ T cells showed more γ-H2AX staining than wild type T cells ($p < 0.05$) (Fig. 3*C*). The low level of γ-H2AX staining observed on wild type chromosome ends may represent a low level of dysfunctional telomeres in wild type cells or may be due to telomere proximal signal that cannot be distinguished from telomeres in this assay.

γ-H2AX Staining of Short Telomeres in MEFs—To test whether short telomeres from other cell types are also bound by γ-H2AX, we generated independent MEF lines from late generation mTR$^{-/-}$ and wild type animals. Interphase MEFs showed an increase of γ-H2AX and p53BP1 foci staining in late generation mTR$^{-/-}$ compared with wild type cells (data not shown). MEF metaphases were stained for γ-H2AX. Late generation mTR$^{-/-}$ MEFs showed a higher frequency of γ-H2AX staining at telomeres than wild type MEFs (Fig. 4). To determine whether the increase in γ-H2AX foci in late generation mTR$^{-/-}$ MEFs was specific to telomeres, we quantitated the γ-H2AX signal that was not telomeric. The ratio of telomeric signal to nontelomeric signal was 2.7-fold higher in late generation mTR$^{-/-}$ MEFs compared with wild type MEFs (Table II). This result further suggests that γ-H2AX is localized to short telomeres. The chromosome preparations from MEF cultures

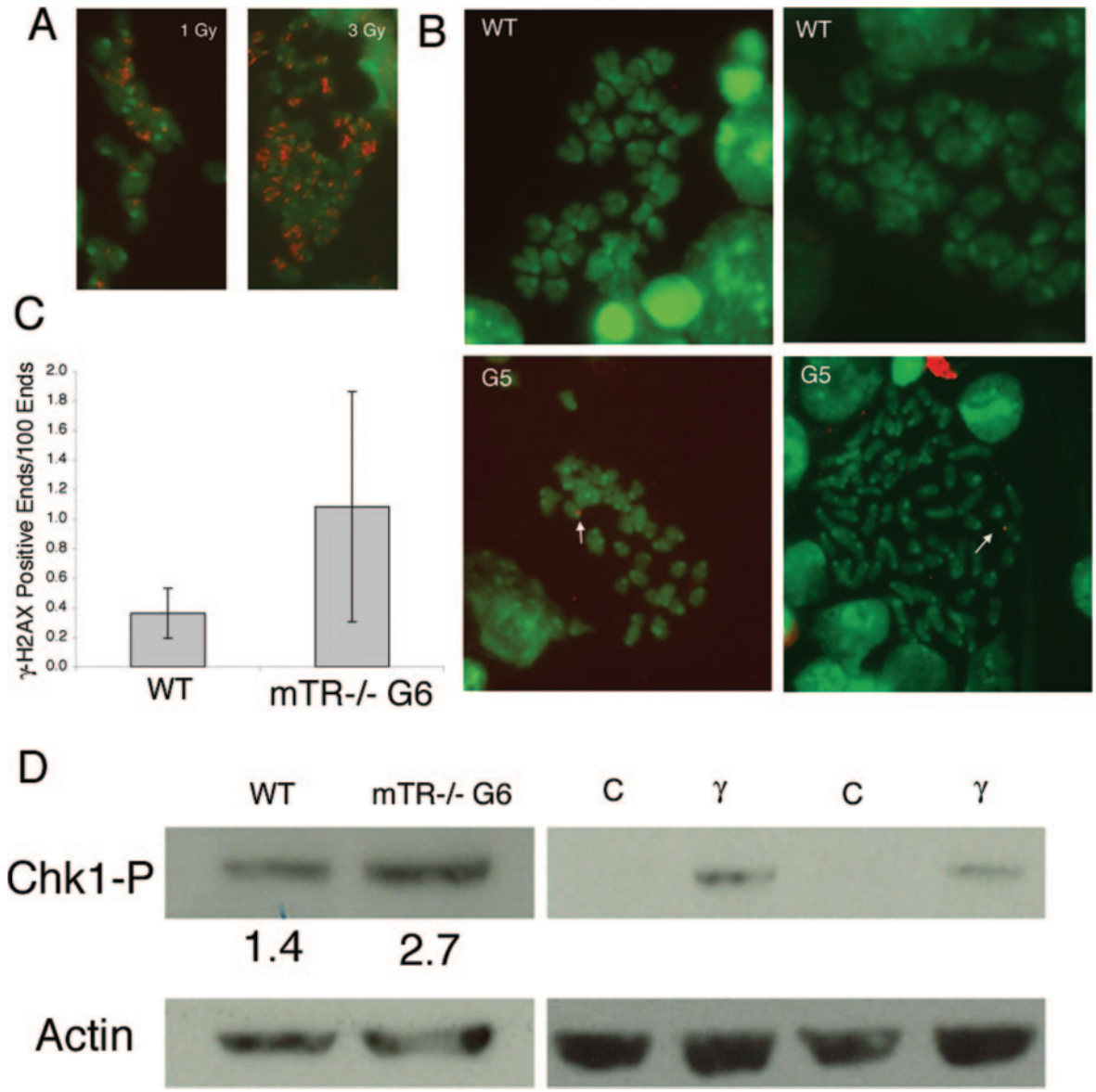

FIG. 3. **Late generation mTR$^{-/-}$ T cells show γ-H2AX at telomeres.** *A*, γ irradiation dosage dependent staining of γ-H2AX on T cells. DNA breaks were generated by γ irradiation with a Cs^{137} source, and cells were stained 20 min post-irradiation. γ-H2AX signal is visualized using a Cy3 filter (*red*), and DNA is visualized by 4′,6-diamidino-2′-phenylindole-dihydrochrolide stain (*green*). *B*, metaphase spreads from aged matched T cells from wild type or mTR$^{-/-}$ G5 animals. *White arrows* depict examples of γ-H2AX-positive ends. *C*, quantitation of γ-H2AX at the telomeres from mTR$^{-/-}$ shows increased γ-H2AX signals in late generation mTR$^{-/-}$ T cells. The values of WT (17 positive ends of 4724 ends) and mTR$^{-/-}$ (95 positive ends of 8232 ends) were significantly different ($p < 0.05$, Fisher's exact test). Analysis of the metaphase spreads was scored blinded. *D*, Western analysis showed an increase of serine 317 phosphorylated Chk1 in mTR$^{-/-}$ T cells than wild type (*WT*). The ratio of Chk1 phosphoprotein to actin is given below the figure. γ irradiated MEFs (*lanes* γ) and control MEFs (*lanes C*) are shown as a positive control for Chk1 phosphorylation.

were more easily resolved and had a better morphology than those isolated from T cells. Thus, for technical reasons, we cannot compare the absolute number of γ-H2AX-positive telomeres in mTR$^{-/-}$ T cells *versus* MEFs.

H2AX Signals Localize to Short Telomeres—The shortest telomeres in the population show no telomere signal in FISH and are referred to as signal free ends (33). Although telomere dysfunction may occur on telomeres that contain some telomere signal by FISH, telomeres that lack signals are more likely to be recognized as dysfunctional. To determine whether the γ-H2AX-positive chromosome ends correlated with short telomeres, we combined telomere FISH analysis with γ-H2AX staining. γ-H2AX-positive spreads were probed with telomere FISH to identify signal free ends. 67% of signal free ends from late generation mTR$^{-/-}$ cells showed co-localization with γ-H2AX, whereas wild type cells did not have signal free ends (Table III). This suggests that the shortest telomeres in the population are more likely to be recognized as DNA damage.

DISCUSSION

Loss of telomere function has been suggested to mimic a double-stranded DNA break. Dysfunctional telomeres may cause rearrangements through homologous recombination and end-to-end chromosome fusions (33, 37). Loss of telomere function can also cause cell cycle arrest (7, 8) and apoptosis (4, 10, 35). Because chromosome breaks also cause arrest and apoptosis (reviewed in Ref. 38), damage induction caused by telomere dysfunction may be a secondary effect of breakage of dicentric chromosomes. In human cells that have lost telomere function through overexpression of dominant negative TRF2, apoptosis is not dependent on the S phase, suggesting that breakage of dicentrics is not responsible for apoptosis in these cells (4). In

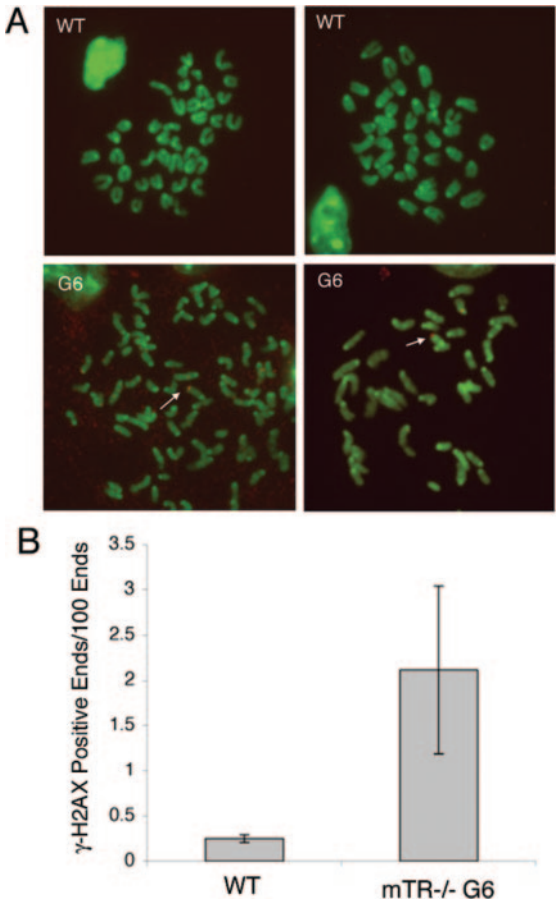

FIG. 4. **Late generation mTR$^{-/-}$ MEFs showed increased γ-H2AX at telomeres.** *A*, wild type (*WT*) and late generation mTR$^{-/-}$ MEFs from passage 3 were probed with γ-H2AX antibody (*red*) and 4′,6-diamidino-2′-phenylindole-dihydrochrolide (*green*), the *white arrows* indicate telomeric γ-H2AX staining. *B*, quantitation of γ-H2AX localized to chromosome ends in MEFs for three wild type MEF lines (12 positive ends of 4752 ends) and three late generation mTR$^{-/-}$ MEF lines (110 positive ends of 4876 ends examined). All of the studies were performed on passage 3 cells, and more than 20 metaphase spreads were scored for each independent line. The difference was significant, $p < 0.05$ as determined by Fisher's exact test.

TABLE II
Telomeric and nontelomeric γ-H2AX staining in MEFs

	Number of chromosomes[a]	Nontelomeric γ-H2AX signal[b]	Telomeric γ-H2AX signal[c]	Ratio[d]
Wild type	2376	9 (43%)	12 (57%)	1.3
mTR$^{-/-}$	2468	31 (22%)	110 (78%)	3.5

[a] Total number of chromosomes analyzed for each genotype.
[b] Percent represents nontelomeric γ-H2AX signal divided by total γ-H2AX signal.
[c] Percent represents telomeric γ-H2AX signal divided by total γ-H2AX signal.
[d] Ratio represents telomeric γ-H2AX signal divided by nontelomeric γ-H2AX signal.

this study we tested whether a different form of telomere dysfunction, generated by telomere shortening in the absence of telomerase, is due to recognition of telomere damage directly or due to breakage of dicentric chromosomes in cycling mitotic cells.

Mitosis Is Not Required for Apoptosis in Late Generation mTR$^{-/-}$ T Cells—We had previously reported that meiotic spermatogonia cells apoptosis is not due to chromosome breakage in telomerase knockout mouse (10). Here we found that in mitotic cells, the disruption of the spindle did not reduce the apoptosis in late generation mTR$^{-/-}$ T cells. Because colcemid

TABLE III
Co-staining of γ-H2AX on SFE in mTR$^{-/-}$ cells
SFE, signal free ends.

	SFE	γ-H2AX on SFE	γ-H2AX with signal
	%	%	%
mTR$^{-/-a}$	1.9	1.2	7.7
Wild type[b]	0	0	5.7

[a] 312 total chromosomes scored. 95% confidence interval does not overlap with wild type by exact binomial confidence interval test.
[b] 194 total chromosomes scored.

blocks microtubule polymerization, it is unlikely that the integrity of the chromosomes was compromised. Furthermore, the low fraction of metaphases that had translocations cannot explain the number of apoptotic cells observed. This suggests that the repeated breakage of fused chromosomes is unlikely to be responsible for the apoptosis in the late generation mTR$^{-/-}$ cells. We cannot, however, exclude the possibility that some cells undergo apoptosis in response to chromosome breaks, although this does not seem to be the predominant pathway.

Short Telomeres and DNA Breaks Illicit Similar Response—Later generation mTR$^{-/-}$ mice and cells show an increased sensitivity to ionizing radiation (31, 32), suggesting that there is an additive effect of short telomeres and double-stranded DNA breaks. Cells with only a few short telomeres may not show a cellular response or may adapt to this low level of damage (39, 40). However, when the overall level of damage increases above a threshold through induction of double-stranded breaks, a cellular response is triggered. This suggests that the pathways responding to telomere dysfunction and DNA breaks overlap.

Although the checkpoint pathways that are activated by DNA breaks and telomere dysfunction are similar, it is not clear whether there might be proteins that specifically recognize telomere damage. Many different proteins that respond to DNA double-stranded breaks including Ku, DNA-PKcs, Nbs1, and Mre11 also associate with telomeres (22–24, 41), We chose to examine γ-H2AX, which is thought to be a very early event in damage signaling. The localization of γ-H2AX to dysfunctional telomeres suggests that pathways for signaling damage are conserved. In addition to these conserved proteins, there may be specific proteins not yet discovered that distinguish the signal sent from a dysfunctional telomere and the signal sent from a DNA break. It would be interesting to determine whether the response to telomere dysfunction may be different in subtle ways from the response to double-stranded breaks.

Different Types of Telomere Dysfunction Signal DNA Damage—Telomere dysfunction is a term often used to describe many different physical changes to telomeres. Loss of telomere function can be brought about in several ways including alteration of telomere-binding proteins (4, 42) and progressive telomere shortening (5, 9, 43). Both mechanisms for creating dysfunction result in chromosome fusions, cell death, or cell cycle arrest. A recent study showed that telomere shortening in primary human cells leads to binding of γ-H2AX, p53BP1, and other damage recognition proteins on telomeres (44). In another study interphase human cells overexpressing dominant negative TRF2 also showed localization of γ-H2AX as well as other DNA damage response proteins to interphase telomere foci (44, 45). Together with these two reports, our study further supports the idea that when telomeres become dysfunctional, either through removal of binding proteins or the shortening in the absence of telomerase, they are recognized as DNA breaks directly.

Despite the similarity in the downstream response, the fundamental molecular components of telomere function that are disrupted by dominant negative TRF2 and loss of telomerase

may be different. Telomere shortening may lead to a loss of binding sites for essential proteins or may physically disrupt the ability of telomeres to form T loops (46). In contrast, overexpression of dominant negative TRF2 removes endogenous TRF2 and its binding partners from the telomere (4) and also TRF2 interacts with damage response pathways (23, 47). Although chromosome fusion and apoptosis occur in response to both methods of telomere dysfunction, the pathways that respond may differ in subtle ways that could have profound cellular consequences. It will be interesting to decipher the similarities and differences in how cells respond to different kinds of telomere dysfunction.

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REFERENCES

1. Olovnikov, A. M. (1973) *J. Theor. Biol.* **41,** 181–190
2. Watson, J. D. (1972) *Nat. New Biol.* **239,** 197–201
3. Greider, C. W., and Blackburn, E. H. (1985) *Cell* **43,** 405–413
4. Karlseder, J., Broccoli, D., Dai, Y., Hardy, S., and de Lange, T. (1999) *Science* **283,** 1321–1325
5. Lundblad, V., and Szostak, J. W. (1989) *Cell* **57,** 633–643
6. Hackett, J. A., Feldser, D. M., and Greider, C. W. (2001) *Cell* **106,** 275–286
7. Enomoto, S., Glowczewski, L., and Berman, J. (2002) *Mol. Biol. Cell* **13,** 2626–2638
8. IJpma, A., and Greider, C. W. (2003) *Mol. Biol. Cell* **14,** 987–1001
9. Blasco, M. A., Lee, H.-W., Hande, P. M., Samper, E., Lansdorp, P. M., DePinho, R. A., and Greider, C. W. (1997) *Cell* **91,** 25–34
10. Hemann, M. T., Rudolph, K. L., Strong, M. A., DePinho, R. A., Chin, L., and Greider, C. W. (2001) *Mol. Biol. Cell* **12,** 2023–2030
11. Greenberg, R., Chin, L., Femino, A., Lee, K.-H., Gottlieb, G., Singer, R., Greider, C. W., and DePinho, R. A. (1999) *Cell* **97,** 515–525
12. Artandi, S. E., Chang, S., Lee, S. L., Alson, S., Gottlieb, G. J., Chin, L., and DePinho, R. A. (2000) *Nature* **406,** 641–645
13. Rudolph, K. L., Millard, M., Bosenberg, M. W., and DePinho, R. A. (2001) *Nat. Genet.* **28,** 155–159
14. Meeker, A. K., Hicks, J. L., Platz, E. A., March, G. E., Bennett, C. J., Delannoy, M. J., and De Marzo, A. M. (2002) *Cancer Res.* **62,** 6405–6409
15. Chin, L., Artandi, S., Shen, Q., Tam, S., Lee, S.-L., Gottlieb, G., Greider, C. W., and DePinho, R. A. (1999) *Cell* **97,** 527–538
16. O'Hagan, R. C., Chang, S., Maser, R. S., Mohan, R., Artandi, S. E., Chin, L., and DePinho, R. A. (2002) *Cancer Cell* **2,** 149–155
17. Khanna, K. K., and Jackson, S. P. (2001) *Nat. Genet.* **27,** 247–254
18. Melo, J., and Toczyski, D. (2002) *Curr. Opin. Cell Biol.* **14,** 237–245
19. Rich, T., Allen, R. L., and Wyllie, A. H. (2000) *Nature* **407,** 777–783
20. Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., and Bonner, W. M. (1998) *J. Biol. Chem.* **273,** 5858–5868
21. Celeste, A., Fernandez-Capetillo, O., Kruhlak, M. J., Pilch, D. R., Staudt, D. W., Lee, A., Bonner, R. F., Bonner, W. M., and Nussenzweig, A. (2003) *Nat. Cell Biol.*
22. Hsu, H. L., Gilley, D., Blackburn, E. H., and Chen, D. J. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 12454–12458
23. Zhu, X. D., Kuster, B., Mann, M., Petrini, J. H., and de Lange, T. (2000) *Nat. Genet.* **25,** 347–352
24. d'Adda di Fagagna, F., Hande, M. P., Tong, W. M., Roth, D., Lansdorp, P. M., Wang, Z. Q., and Jackson, S. P. (2001) *Curr. Biol.* **11,** 1192–1196
25. Boulton, S. J., and Jackson, S. P. (1996) *Nucleic Acids Res.* **24,** 4639–4648
26. Porter, S. E., Greenwell, P. W., Ritchie, K. B., and Petes, T. D. (1996) *Nucleic Acids Res.* **24,** 582–585
27. Ritchie, K. B., Mallory, J. C., and Petes, T. D. (1999) *Mol. Cell. Biol.* **19,** 6065–6075
28. Tsukamoto, Y., Taggart, A. K., and Zakian, V. A. (2001) *Curr. Biol.* **11,** 1328–1335
29. Riha, K., Watson, J. M., Parkey, J., and Shippen, D. E. (2002) *EMBO J.* **21,** 2819–2826
30. Johnson, F. B., Marciniak, R. A., McVey, M., Stewart, S. A., Hahn, W. C., and Guarente, L. (2001) *EMBO J.* **20,** 905–913
31. Wong, K. K., Chang, S., Weiler, S. R., Ganesan, S., Chaudhuri, J., Zhu, C., Artandi, S. E., Rudolph, K. L., Gottlieb, G. J., Chin, L., Alt, F. W., and DePinho, R. A. (2000) *Nat. Genet.* **26,** 85–88
32. Goytisolo, F. A., Samper, E., Martin-Caballero, J., Finnon, P., Herrera, E., Flores, J. M., Bouffler, S. D., and Blasco, M. A. (2000) *J. Exp. Med.* **192,** 1625–1636
33. Hemann, M. T., Strong, M. A., Hao, L. Y., and Greider, C. W. (2001) *Cell* **107,** 67–77
34. Liyanage, M., Coleman, A., du Manoir, S., Veldman, T., McCormack, S., Dickson, R. B., Barlow, C., Wynshaw-Boris, A., Janz, S., Wienberg, J., Ferguson-Smith, M. A., Schrock, E., and Ried, T. (1996) *Nat. Genet.* **14,** 312–315
35. Lee, H.-W., Blasco, M. A., Gottlieb, G. J., Horner, J. W., Greider, C. W., and DePinho, R. A. (1998) *Nature* **392,** 569–574
36. Lenardo, M., Chan, K. M., Hornung, F., McFarland, H., Siegel, R., Wang, J., and Zheng, L. (1999) *Annu. Rev. Immunol.* **17,** 221–253
37. Smogorzewska, A., Karlseder, J., Holtgreve-Grez, H., Jauch, A., and de Lange, T. (2002) *Curr. Biol.* **12,** 1635–1644
38. Friedberg, E. C. (1985) *DNA Repair*, 1st Ed., W. H. Freeman and Company, New York
39. Sandell, L. L., and Zakian, V. A. (1993) *Cell* **75,** 729–739
40. Lee, S. E., Moore, J. K., Holmes, A., Umezu, K., Kolodner, R. D., and Haber, J. E. (1998) *Cell* **94,** 399–409
41. Gravel, S., Larrivee, M., Labrecque, P., and Wellinger, R. J. (1998) *Science* **280,** 741–744
42. van Steensel, B., Smogorzewska, A., and de Lange, T. (1998) *Cell* **92,** 401–413
43. Riha, K., McKnight, T. D., Griffing, L. R., and Shippen, D. E. (2001) *Science* **291,** 1797–1800
44. d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003) *Nature* **426,** 194–198
45. Takai, H., Smogorzewska, A., and de Lange, T. (2003) *Curr. Biol.* **13,** 1549–1556
46. Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H., and de Lange, T. (1999) *Cell* **97,** 503–514
47. Zhu, X. D., Niedernhofer, L., Kuster, B., Mann, M., Hoeijmakers, J. H., and de Lange, T. (2003) *Mol. Cell* **12,** 1489–1498
48. Qi, L., Strong, M. A., Karim, B. O., Armanios, M., Huso, D. L., and Greider, C. W. (2003) *Cancer Res.* **63,** 8188–8196