

# DNA Ligase IV-Dependent NHEJ of Deprotected Mammalian Telomeres in G1 and G2

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## Summary

**Background:** Telomeres are required to prevent end-to-end chromosome fusions. End-to-end fusions of metaphase chromosomes are observed in mammalian cells with dysfunctional telomeres due to diminished function of telomere-associated proteins and in cells experiencing extensive attrition of telomeric DNA. However, the molecular nature of these fusions and the mechanism by which they occur have not been elucidated.

**Results:** We document that telomere fusions resulting from inhibition of the telomere-protective factor TRF2 are generated by DNA ligase IV-dependent nonhomologous end joining (NHEJ). NHEJ gives rise to covalent ligation of the C strand of one telomere to the G strand of another. Breakage of the resulting dicentric chromosomes results in nonreciprocal translocations, a hallmark of human cancer. Telomere NHEJ took place before and after DNA replication, and both sister telomeres participated in the reaction. Telomere fusions were accompanied by active degradation of the 3' telomeric overhangs.

**Conclusions:** The main threat to dysfunctional mammalian telomeres is degradation of the 3' overhang and subsequent telomere end-joining by DNA ligase IV. The involvement of NHEJ in telomere fusions is paradoxical since the NHEJ factors Ku70/80 and DNA-PKcs are present at telomeres and protect chromosome ends from fusion.

## Introduction

The consequences of telomere loss in human cells are of interest since replicative telomere shortening can impair telomere function during tumorigenesis and cellular aging. Human tumors are thought to experience a transient phase of diminished chromosome end protection prior to the activation of telomerase (reviewed in [1]), and diminished telomere function due to telomere attrition causes replicative senescence in human cells [2, 3]. In dyskeratosis congenita, reduced telomerase activity is

correlated with a premature depletion of self-renewing tissues [4, 5]. In most settings, deprotection of chromosome ends is inferred from the occurrence of telomere associations, which are cytogenetically defined as end-to-end associations of two or more intact chromosomes forming dicentric or multicentric chromosomes. However, the exact nature of these end-to-end associations and the mechanism by which they are generated are not known.

An impediment in the study of telomere dysfunction in cells undergoing telomere attrition is the low frequency of the informative events and their stochastic occurrence. Furthermore, it is difficult to distinguish the immediate consequences of telomere dysfunction from subsequent damage associated with the rupture of dicentric chromosomes. One solution to this problem is to investigate the consequences of inhibition of TRF2, a telomeric DNA binding protein that protects mammalian telomeres (reviewed in [6]). Expression of a dominant-negative allele of TRF2, TRF2<sup>ΔBΔM</sup>, results in immediate deprotection of chromosome ends in every cell and allows detailed dissection of how chromosomes lacking telomere function behave and how cells respond to this insult.

A prominent phenotype of TRF2 inhibition is the occurrence of chromosome end fusions in metaphase and chromatin bridges in anaphase [7]. These fusions occur even though there is no overt loss of the telomeric DNA; telomeric sequences are retained at the sites of fusion, as demonstrated by fluorescence in situ hybridization (FISH). When telomere fusions are frequent, they can be detected in genomic blots as new (TTAGGG)<sub>n</sub>-containing restriction fragments. These telomere fusion fragments have a molecular weight that is approximately twice that of the original telomeres [7], which is consistent with the preservation of telomeric DNA at the fusion sites.

A mechanism that could be responsible for the formation of end-to-end telomere fusions is nonhomologous end joining (NHEJ) (reviewed in [8]). The ligase responsible for NHEJ is DNA ligase IV/XRCC4, and its action is enhanced by the Ku70/80 heterodimer and DNA-PKcs. Both chromosome-internal DNA breaks and chromosome ends lacking telomeric DNA can be processed by NHEJ [9]. However, a paradoxical result emerged from the analysis of mouse cells lacking Ku70/80 or DNA-PKcs function. Such cells were found to harbor telomere fusions with preservation of the telomeric DNA, suggesting that Ku70/80 and DNA-PKcs protect chromosome ends from fusions and arguing against a role for NHEJ in the processing of deprotected telomeres [10–16]. A protective role for Ku70/80 and DNA-PKcs at telomeres was further suggested by the association of these proteins with telomeric DNA, as monitored by chromatin immunoprecipitation [12, 17].

A recent report employing chromosome orientation fluorescence in situ hybridization (CO-FISH) established that most chromosome end fusions after TRF2 inhibition are postreplicative events and involve telomeres repli-

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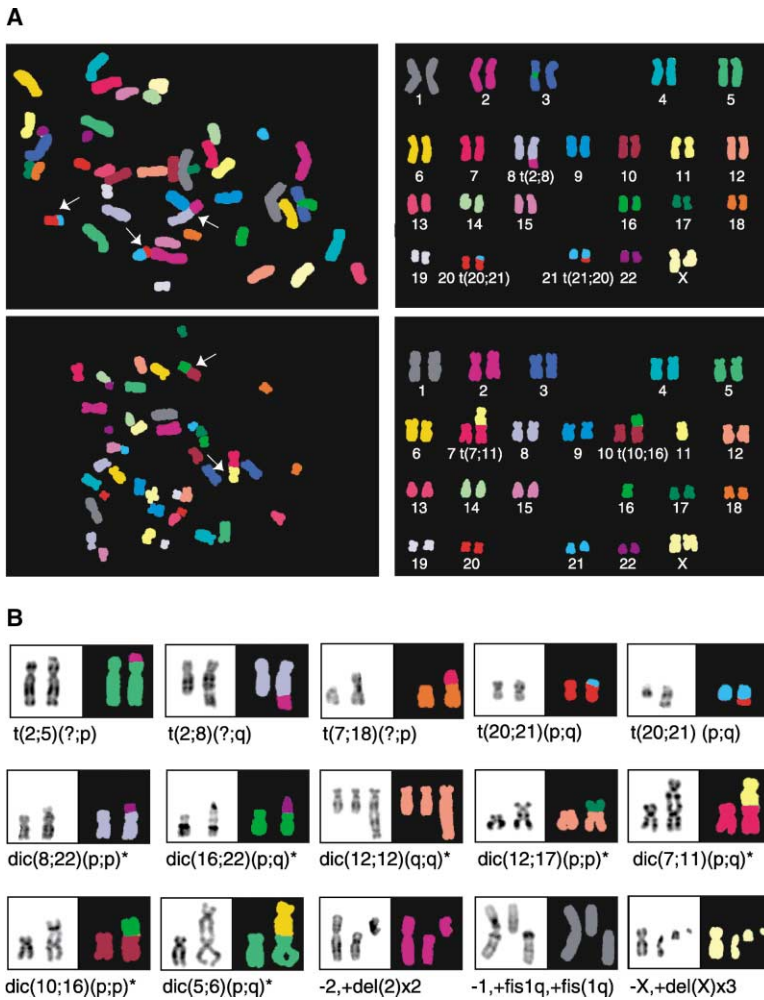


Figure 1. Nonreciprocal Translocations in IMR90 Fibroblasts Expressing TRF2<sup>ΔBΔM</sup>

Multiplex fluorescence in situ hybridization (M-FISH) on metaphase spreads of IMR90 fibroblasts infected with a retrovirus expressing TRF2<sup>ΔBΔM</sup>. Spreads were prepared 6 days after infection (including 3 days of puromycin selection). Cells were treated with colcemid for 12 hr before harvest.

(A) Examples of full metaphase spreads of cells expressing TRF2<sup>ΔBΔM</sup>.

(B) Examples of translocation (top row), dicentric chromosomes formed by telomere fusion (indicated by an asterisk), and other chromosomal abnormalities (three right panels in the bottom row). Abbreviations: del, deletion; dic, dicentric; fis, centric fission; t, translocation.

cated by leading-strand synthesis [10]. These observations suggested that TRF2 protects telomere ends in G2 and that TRF2 is primarily required for protection of leading-strand ends. Here, we examine these issues further and demonstrate that TRF2 protects all chromosome ends both in G1 and G2 and that the main threat to dysfunctional mammalian telomeres is inappropriate repair by NHEJ.

## Results

### Nonreciprocal Translocations Resulting from TRF2<sup>ΔBΔM</sup>-Dependent Telomere Uncapping

Expression of the TRF2<sup>ΔBΔM</sup> dominant-negative allele was previously shown to cause chromosome end fusions in a variety of telomerase-positive tumor cell lines [7, 10, 18] and telomerase-negative primary human fibroblasts [19]. Chromosome painting with multiplex fluorescence in situ hybridization (M-FISH) [20, 21] showed that the telomere fusions in primary human IMR90 fibroblasts primarily involved different chromosomes; although, dicentrics formed between homologs were also noted (Figures 1A and 1B). As expected, these chromosome end fusions were only observed in fibroblasts infected with a TRF2<sup>ΔBΔM</sup> retrovirus and did not occur in metaphases from control cultures infected with the retroviral

vector or with a retrovirus expressing full-length TRF2 (Table 1). We and others have speculated that chromosome end fusions could give rise to nonreciprocal translocations and deletions (see for review [1]). In agreement with this prediction, cells lacking normal TRF2 function showed several nonreciprocal translocations that were not observed in control cells (Figures 1A and 1B). The most likely source of these translocations is the rupture of dicentric chromosomes formed through telomere fusions. However, we cannot exclude the possibility that they are created by break-induced replication initiated by a deprotected chromosome end. We note that the M-FISH data did not show evidence for repeated breakage-fusion-bridge (BFB) cycles, which should yield multicolored chromosomes. It is possible that the M-FISH analysis was carried out too soon after the insult (within 2–4 cell divisions) to allow multiple BFB cycles, or that the checkpoint status of the primary cells used here prevented continued cell divisions with an unprotected end.

### Inhibition of TRF2 Results in Covalent Telomere Fusions

The molecular nature of telomere fusions has not been established. Inspection of end-to-end fused metaphase chromosomes does not provide information on whether

Table 1. Chromosomal Aberrations in IMR90 Metaphase Spreads Analyzed by M-FISH

Retrovirus	n	Translocations	Telomere Fusions	Deletions and Fragments	Other
TRF2 <sup>ΔBΔM</sup>	44	t(2;5);t(7;18), t(2;8), t(20;21), t(20;21)	dic(8;22), dic(16;22), dic(12;12), dic(12;17), dic(7;11), dic(10;16), dic(5;6)	del(2)x2, fis(1p), fis(1q), del(X)x3,del(5p), del(5q), del(11p), del(11q), fra(1p)	chrg(12)
TRF2	10	t(2;7)	--	--	--
Vector	35	--	--	del(2p), del(10p)	--

"n" indicates the number of metaphases analyzed. All chromosomal abnormalities were clonal events. Abbreviations: chrg, chromosome gap; del, deletion; dic, dicentric; fis, centric fission; fra, fragile site; t, translocation.

the associations are covalent or mediated by protein or nucleic acid interactions. As telomere fusions were detected in genomic blots of protein-free DNA soon after inhibition of TRF2 [7], a protein-mediated association is unlikely. However, the possibility that the new fragments were composed of two telomeres held together by non-covalent nucleic acid interactions, such as G-G base pairing of the single-stranded telomeric overhangs or the invasion of the overhang of one telomere into the duplex array of another, was not excluded. To determine whether the telomere fusion fragments represented covalently joined telomeric ends, we examined them by fractionation under alkaline conditions. Like all base pairing, Hoogsteen pairing of G residues is disrupted in NaOH, whereas ligated DNA ends are stable under these conditions. DNA was isolated from the HTC75 cell lines T4, T19, and T22 [7], which are three previously characterized Tet-inducible telomerase-positive tumor cell lines that accumulate chromosome end fusions after induction of TRF2<sup>ΔBΔM</sup>. As reported previously, TRF2<sup>ΔBΔM</sup> induced a class of larger (TTAGGG)<sub>n</sub>-containing fragments (Figure 2A, left panel). These TTAGGG repeat fragments were also detectable when the DNA from these cells was denatured in NaOH and fractionated on an alkaline gel (Figure 2A, right panel). To verify that the NaOH treatment had fully denatured the DNA, a telomeric DNA ladder of self-ligated TTAGGG repeat fragments was denatured and fractionated in parallel with the genomic DNAs and then hybridized to a TTAGGG repeat probe without additional denaturation. As expected, this control DNA hybridized to the probe, verifying that the alkali treatment had denatured the telomeric DNA (data not shown). These data demonstrate that the telomeric fusions induced by the removal of TRF2 involve covalent ligation of telomeres.

#### DNA Ligase IV Is Required for Telomere-Telomere Ligation

As the telomeric DNA repeat tracts always have the same cen-5'-TTAGGG-3'-tel orientation at chromosome ends, homologous recombination (HR) between telomeres does not give rise to covalent end-to-end fusions. Instead, telomere fusions must be generated by some form of nonhomologous end-joining. To test the involvement of NHEJ in TRF2<sup>ΔBΔM</sup>-induced telomere fusions, we used mouse embryonic fibroblasts (MEFs) lacking DNA ligase IV (Lig4<sup>-/-</sup>) [22], which is essential for all NHEJ but has no known role in telomere protection. Because Lig4<sup>-/-</sup> MEFs grow poorly, MEFs from Lig4/p53 double KO embryos were used in our studies [23]. Lack of p53 rescues the lethality of DNA ligase IV defi-

ciency and suppresses the slow-growth phenotype but has no effect on NHEJ [23, 24]. In addition, absence of p53 allows for continual proliferation of mouse cells in spite of deprotected telomeres [19].

MEFs obtained from Lig4<sup>+/+</sup>p53<sup>-/-</sup> and Lig4<sup>-/-</sup>p53<sup>-/-</sup> embryos were infected with a retrovirus expressing TRF2<sup>ΔBΔM</sup> or the pLPC retroviral vector, and the resulting chromosomal abnormalities were assessed in metaphase cells. Peptide nucleic acid FISH (PNA FISH) with a telomeric probe served to distinguish telomere fusions from fusions of randomly broken chromosomes, which occur at low frequency in DNA ligase IV null cells [24]. Lig4<sup>+/+</sup>p53<sup>-/-</sup> mouse cells contained frequent telomere fusions when forced to express TRF2<sup>ΔBΔM</sup>. In 30 metaphases examined, 14 had telomere fusions with a total of 51 fusions (median of 1.5 fusion per metaphase) (Figures 2B–2D). These telomere fusions either involved single chromatids (chromatid-type fusions) or both chromatids (chromosome-type fusions). In contrast to cells containing an intact NHEJ pathway, cells lacking DNA ligase IV did not accumulate telomere fusions in response to TRF2<sup>ΔBΔM</sup>; the frequency of telomere fusions was ~25-fold lower, and the occurrence of dicentrics was independent of TRF2 inhibition. As reported previously [24], Lig4<sup>-/-</sup>p53<sup>-/-</sup> MEFs had extensive chromosomal damage, including breaks, fragments, and a few chromosome fusions without a telomeric signal (Figures 2B–2D), but these aberrations were observed regardless of TRF2 status. Taken together, the results indicate that DNA ligase IV-dependent NHEJ is the main mechanism by which telomeres are fused after inhibition of TRF2. The long-term consequences of telomere uncapping in Lig4<sup>-/-</sup>p53<sup>-/-</sup> MEFs is currently under investigation.

#### NHEJ of Sister Telomeres

Chromatid-type fusions are the most frequent chromosome aberration in cells lacking normal TRF2 function (see Figures 2 and 3). Bailey and coworkers used CO-FISH on HTC75 cells induced to express TRF2<sup>ΔBΔM</sup> and found that chromatid-type fusion always involved the joining of telomeres generated by leading-strand DNA synthesis [10]. Based on this result, fusions of sister telomeres should not occur. However, sister fusions made up a substantial fraction of the fusions events when TRF2<sup>ΔBΔM</sup> was expressed in primary human IMR90 fibroblasts and hTERT-immortalized human BJ fibroblasts (Figures 3B–3D). The fusion of sister telomeres is obvious from both the single telomeric signal and the contiguous chromatin connection between the sisters (Figure 3C). Analysis of telomere fusions in 20 metaphases of TRF2<sup>ΔBΔM</sup>-expressing IMR90 cells revealed 9

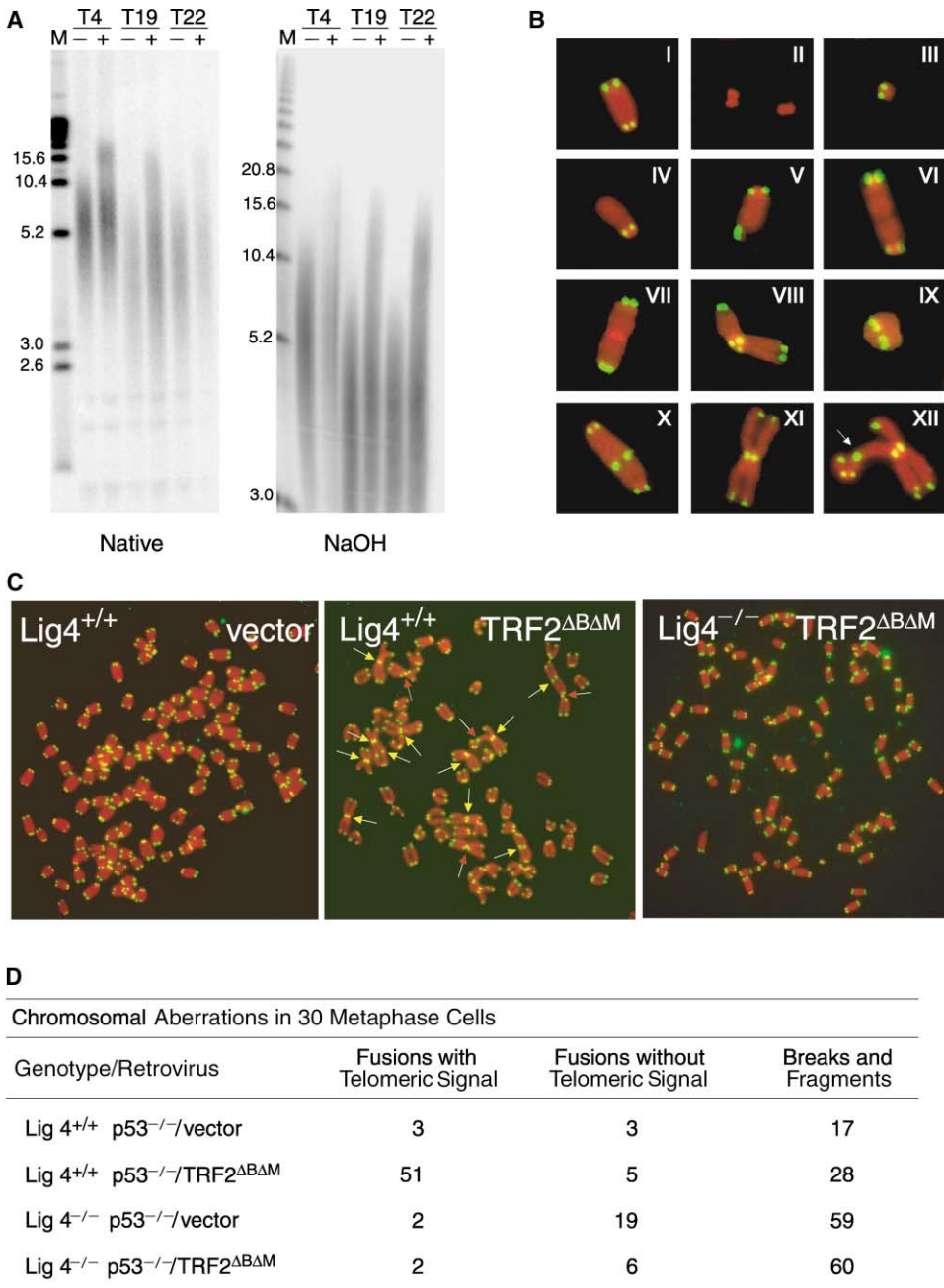


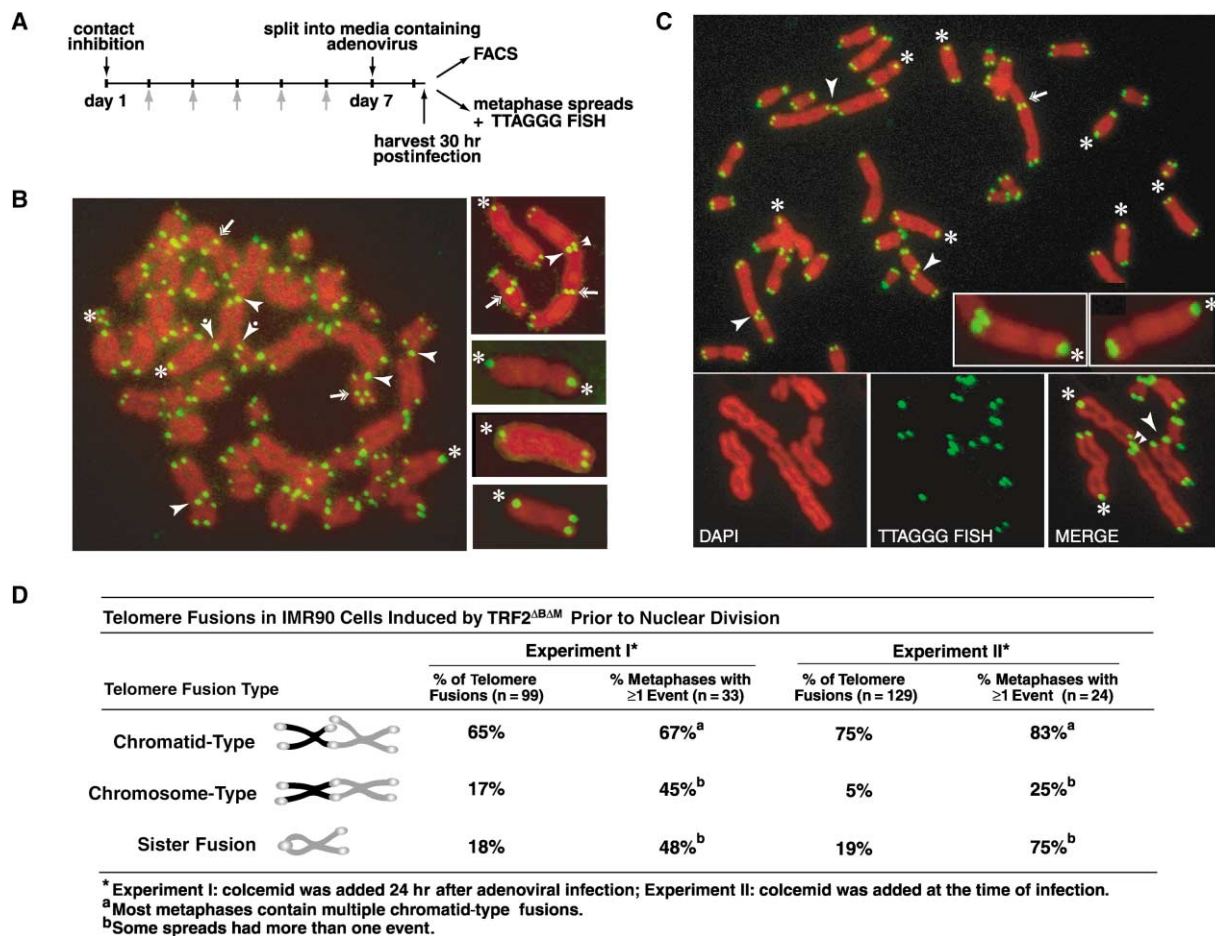
Figure 2. Covalent Fusion of Dysfunctional Telomeres by DNA Ligase IV

(A) Inhibition of TRF2 results in covalent joining of telomeric DNA fragments. A Southern blot of telomeric restriction fragments resolved under native (left) and alkaline (right) conditions. TRF2<sup>ΔBΔM</sup>-inducible cell lines, T4, T19, and T22, were grown under repression (-) or induction (+) conditions. HinfI/RsaI-digested genomic DNA was fractionated under native conditions (left) or under alkaline denaturing conditions (right). After blotting, telomeric DNA was detected with a TTAGGG repeat probe. "M" indicates a telomeric DNA MW marker; fragment sizes are given in kilobases.

(B) Examples of chromosomal abnormalities observed after TRF2 inhibition in MEFs. Metaphase spreads were prepared after 3 days of puromycin selection of retrovirally infected MEFs and were processed for telomeric PNA FISH. DNA was stained with DAPI and false colored in red. (I) Normal chromosome. (II–XII) Aberrant chromosomes from Lig4<sup>+/+</sup>p53<sup>-/-</sup> MEFs expressing TRF2<sup>ΔBΔM</sup>; (II) fragment without a telomeric signal; (III) fragment with a telomeric signal; (IV) telomeric signal missing from the long arms; (V) chromatid break; (VI) long-arm chromosome fusion without a telomeric signal; (VII) short-arm chromosome fusion without a telomeric signal; (VIII) short-arm chromatid fusion with a telomeric signal; (IX) two-chromosome ring with a telomeric signal at the fusion sites; (X) long-arm chromosome fusion with a telomeric signal; (XI) short-arm chromosome fusion with a telomeric signal; (XII) long-arm chromatid fusion with a telomeric signal (arrowhead) and short-arm chromosome fusion with a telomeric signal (as in [XI]). Lig4<sup>-/-</sup>p53<sup>-/-</sup> MEFs expressing TRF2<sup>ΔBΔM</sup> only showed a single short-arm chromatid fusion (as in [VIII]), but its appearance was independent of TRF2<sup>ΔBΔM</sup>.

(C) Metaphase spreads from Lig4<sup>+/+</sup>p53<sup>-/-</sup> and Lig4<sup>-/-</sup>p53<sup>-/-</sup> mouse cells infected with control (vector) or TRF2<sup>ΔBΔM</sup>-carrying retroviruses. The yellow arrows indicate chromosome-type fusions; and the red arrows indicate chromatid-type fusions.

(D) A summary of chromosome aberrations in Lig4<sup>+/+</sup>p53<sup>-/-</sup> and Lig4<sup>-/-</sup>p53<sup>-/-</sup> cells infected with a control or a TRF2<sup>ΔBΔM</sup>-expressing retrovirus. Note that chromosome end fusions lacking telomeric signals are not dependent on the expression of TRF2<sup>ΔBΔM</sup>.



**Figure 3. Induction of Sister Telomere Fusions by TRF2<sup>ΔBΔM</sup> and Generation of Chromosome-Type Fusions in One Cell Cycle**  
 (A) Experimental strategy to examine chromosomal abnormalities generated in one cell cycle. Gray arrows indicate times of feeding.  
 (B and C) Metaphase spreads of (B) IMR90 and (C) hTERT-BJ1 cells expressing TRF2<sup>ΔBΔM</sup>. Asterisks indicate sister chromatid fusions, arrowheads indicate single chromatid fusions, double-headed arrows indicate chromosome-type fusions, an arrowhead with a dot indicates fusions formed independently by two sister chromatid arms of the same chromosome, and two arrowheads side by side indicate an example of two closely juxtaposed telomeres that have not fused. The inserts in (C) show magnified sister fusions from two additional metaphase spreads. The lower panels in (C) show the DAPI and FISH images separately to allow viewing of the continuous DAPI signal through the sister fusion site.  
 (D) Summary of fusion events in IMR90 cells expressing TRF2<sup>ΔBΔM</sup>.

sister fusions and 57 non-sister chromatid fusions (also see Figure 3D). These data show that NHEJ threatens telomeres after DNA replication and that TRF2 is required to prevent these events from happening. The fact that sister telomeres can fuse demonstrates that TRF2 protects telomeres regardless of whether they are replicated by leading- or lagging-strand synthesis. Expression of telomerase did not appear to alleviate the need for TRF2 protection since TRF2<sup>ΔBΔM</sup>-induced sister fusions are also frequent in hTERT-BJ1 cells that express this enzyme (Figure 3C).

#### Loss of Telomeric Overhangs and Telomere NHEJ in G1

In addition to numerous chromatid-type fusions, human and mouse fibroblasts expressing TRF2<sup>ΔBΔM</sup> showed frequent chromosome-type fusions (Figures 2 and 3). The simplest explanation for chromosome-type fusions is the NHEJ of two telomeres in G1 and subsequent repli-

cation of the dicentric in S phase. However, chromosome-type fusions can also arise from duplication of a chromatid-type dicentric created in a preceding cell division, although their high frequency (for instance, see Figure 2C) would argue against this scenario. We addressed this question directly by asking whether chromosome-type fusions can be generated in a single cell division (see Figure 3A for an experimental schematic). Primary human IMR90 fibroblasts were arrested by contact inhibition for 6 days, resulting in a homogeneous population of G0 cells with a 2n DNA content, as deduced from FACS analysis (data not shown). These cells were then infected with either a TRF2<sup>ΔBΔM</sup> adenovirus [18] or the vector control and were prompted to enter G1 by plating at reduced density. The cells were prevented from progressing beyond metaphase by the addition of colcemid at the time of their entry into G1. After 24 hr, only 1 telomere fusion was present in 15 metaphases from the control cell population (data not shown), while

numerous fusions, including many chromosome-type fusions, were found in the TRF2<sup>ΔBΔM</sup>-expressing cells (Figures 3B–3D). Since the cells did not progress beyond metaphase, the occurrence of chromosome-type fusions is most easily explained by NHEJ in G1. Alternatively, they could have resulted from the simultaneous fusion of four telomeres (two pairs of sisters), but such events should be rare.

Further evidence for prereplicative NHEJ of telomeres was obtained by molecular analysis of the telomeric DNA. Contact-inhibited IMR90 cells were infected with the TRF2<sup>ΔBΔM</sup> adenovirus or the vector control, and the cells were split into media containing aphidicolin to prevent their progression through S phase (see Figure 4A for an experimental schematic). Within 20 hr, the cells were processed, and the status of the telomeres was examined by genomic blotting (Figure 4B). The results showed that such cell populations contain a minor class of telomeric restriction fragments that are larger than the original telomeres, and this is consistent with telomere-telomere fusion events. FACS analysis as well as examination of the mitotic index (<1/600) confirmed that the cells had not escaped the aphidicolin block. Thus, the data suggest that uncapped telomeres can fuse before DNA replication.

NHEJ of telomeres is likely to require degradation of the G-strand 3' telomeric overhang, which is about 150 nt in human cells [25–27]. It is predicted that telomere overhangs can be lost passively during DNA replication since leading-strand DNA synthesis is expected to generate blunt ends. However, NHEJ of telomeres before DNA replication would require an active degradation of G-strand overhangs. Therefore, we asked whether G-strand overhang signals were affected in TRF2<sup>ΔBΔM</sup>-expressing cells that were not going through S phase. IMR90 cells were contact inhibited for 7 days and were then infected with adenoviruses expressing TRF2<sup>ΔBΔM</sup>, TRF2, or the vector control, and the cells were maintained in G0 for 30 hr (see the schematic in Figure 4C). Changes in the abundance of the G-strand overhang DNA were assayed by a quantitative in-gel hybridization technique. The data showed that inhibition of TRF2 resulted in a modest but significant reduction of the overhang signal compared to cells infected with the adenoviral vector (Figures 4D and 4E). The reduced overhang signal could signify complete loss of the G strand from a subset of telomeres or a shortening of the overhang at many telomeres (or both). Interestingly, the larger DNA fragments representing fused telomeres were not observed in these experiments, suggesting that progression into G1 may be required for NHEJ of uncapped telomeres. These data suggest that, in the absence of normal TRF2 function, the G-strand overhang is vulnerable to nucleolytic attack resulting in a partial loss of overhang DNA even before DNA replication. This active removal of the 3' overhangs is likely to be a prerequisite for the NHEJ of deprotected telomeres in G1.

## Discussion

The genetic and physical evidence presented here demonstrate that dysfunctional telomeres are repaired by DNA ligase IV-dependent nonhomologous end joining

(Figure 5). This finding is paradoxical since two factors that facilitate NHEJ at random DNA breaks, the Ku70/80 heterodimer and DNA-PKcs, are known to reside at natural chromosome ends [12, 17]. Moreover, cells lacking normal Ku70/80 or DNA-PKcs function display a mild telomere fusion phenotype [10–16], indicating that these proteins contribute to the protection of telomeres. Thus, Ku70/80 and DNA-PKcs may play opposing roles at chromosome ends depending on the status of the telomeres. On the one hand, these factors mediate a telomere-capping function, since cells lacking their function have end-to-end chromosome fusions. On the other hand, as facilitators of NHEJ, the DNA-PKcs complex may contribute substantially to the joining of uncapped telomeres by DNA ligase IV. In this regard, it is likely that the magnitude of the telomere uncapping phenotype of Ku70/80 and DNA-PKcs deficiency has been underestimated in studies monitoring end-to-end fusions, since impaired DNA-PK function would lead to loss of telomere protection but would also diminish the efficiency of telomere-telomere ligation by DNA ligase IV.

NHEJ of telomeres can take place in G1 and G2 (Figure 5B). This process is presumably dependent on the removal of the single-stranded 3' overhang of TTAGGG repeats (Figure 5A). Passive loss of the 3' telomeric overhang is expected to occur during DNA replication, since leading-strand DNA synthesis would generate blunt-ended telomeres [28]. Regeneration of the 3' overhang has been proposed to require processing of the C-rich telomeric DNA strand by a nuclease [26]. If TRF2 recruits this putative nuclease, inhibition of TRF2 will result in the persistence of blunt-ended telomeres, and preferential fusion of leading ends may be anticipated. Indeed, chromatid-type telomere fusions with preferential involvement of leading ends has been noted [10]. However, the occurrence of sister telomere fusions argues that the telomere generated by lagging-strand DNA synthesis can also partake in the NHEJ reaction (Figure 5B). The lagging-strand end is predicted to carry a short overhang representing the site of the last RNA primer, and active removal of this overhang may be required for NHEJ of lagging-end telomeres in G2. Similarly, active degradation of the long 3' overhangs present at telomeres in G1 may be required for NHEJ of telomeres before DNA replication. Physical evidence suggests that the 3' overhang can be degraded in G0, indicating the presence of cellular nucleases that threaten the telomere terminus after TRF2 is inhibited.

Interestingly, the relative frequency of chromosome-type fusions and chromatid-type fusions varies substantially in different settings ([7, 10], this study). One possibility is that the processing of uncapped telomeres in G1 requires a cell cycle arrest and that the checkpoint status of the cells determines the efficacy and duration of this arrest. For instance, chromosome-type fusions are frequent in checkpoint-proficient primary human fibroblasts (this study) but are rare in HT1080 cells [10], which lack a functional p53 pathway and therefore may have a diminished ability to arrest before S phase when telomeres are uncapped. However, chromosome-type fusions are also quite frequent in p53-deficient mouse cells, indicating that p53 status is not the only parameter influencing the frequency of G1 NHEJ of uncapped telo-

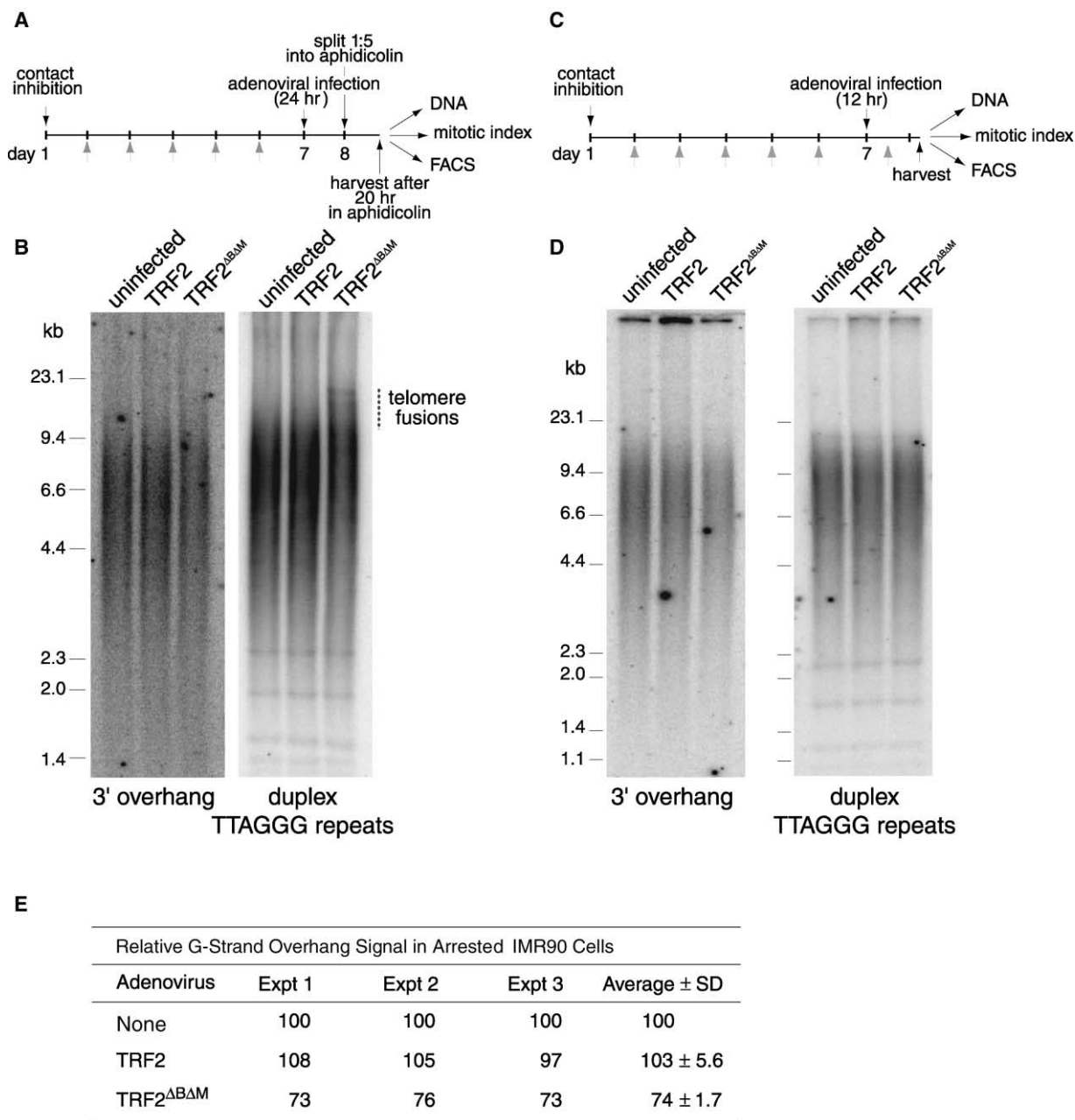


Figure 4. Physical Detection of TRF2<sup>ΔBΔM</sup>-Induced Telomere Fusions and Loss of the Telomeric Overhang Prior to S Phase

(A) Strategy and timeline of the experiment depicted in (B). Gray arrows indicate times of feeding.

(B) Detection of telomeric fusions arising before S phase. DNA from aphidicolin-blocked cells was isolated, digested, and fractionated under native conditions. Telomeric DNA containing 3' (TTAGGG)<sub>n</sub> overhangs is visualized by in-gel hybridization under native conditions (left panel). Total telomeric DNA was visualized after denaturation of the same gel, followed by rehybridization with the same probe. Telomere fusions can be seen in TRF2<sup>ΔBΔM</sup>-expressing cells under denaturing conditions (right panel), but not under native conditions.

(C) Strategy and timeline of the experiment depicted in (D). Gray arrows indicate times of feeding.

(D) An in-gel G-overhang assay on G0 cells. DNA from G0 cells was isolated, digested with MboI and AluI, and fractionated under native conditions. Telomeric 3' (TTAGGG)<sub>n</sub> overhangs were detected by in-gel hybridization under native conditions (left panel). Total telomeric DNA was visualized after denaturation of the DNA in the gel, followed by in-gel hybridization with the same probe (right panel).

(E) Quantification of G-overhang signals in arrested IMR90 cells. Data derived from three parallel experiments (one of them shown in [D]) using the method shown in (D). The level of the G-overhang signal was normalized to the signal of duplex telomeric DNA obtained in the same lane. For each experiment, the normalized values of infected cells are presented relative to the normalized values of noninfected cells.

meres. Other variables that might affect the occurrence of G1 telomere fusions in different cell lines are the stability of the 3' overhang prior to S phase, the activity of the NHEJ pathway in G1, and the efficacy of TRF2 inhibition in G1.

Whereas the single-stranded 3' overhang is degraded, the duplex part of the telomere is stable. This result was unexpected based on studies in budding yeast, where the processing of DNA ends has been documented in detail. In that organism, exposed DNA

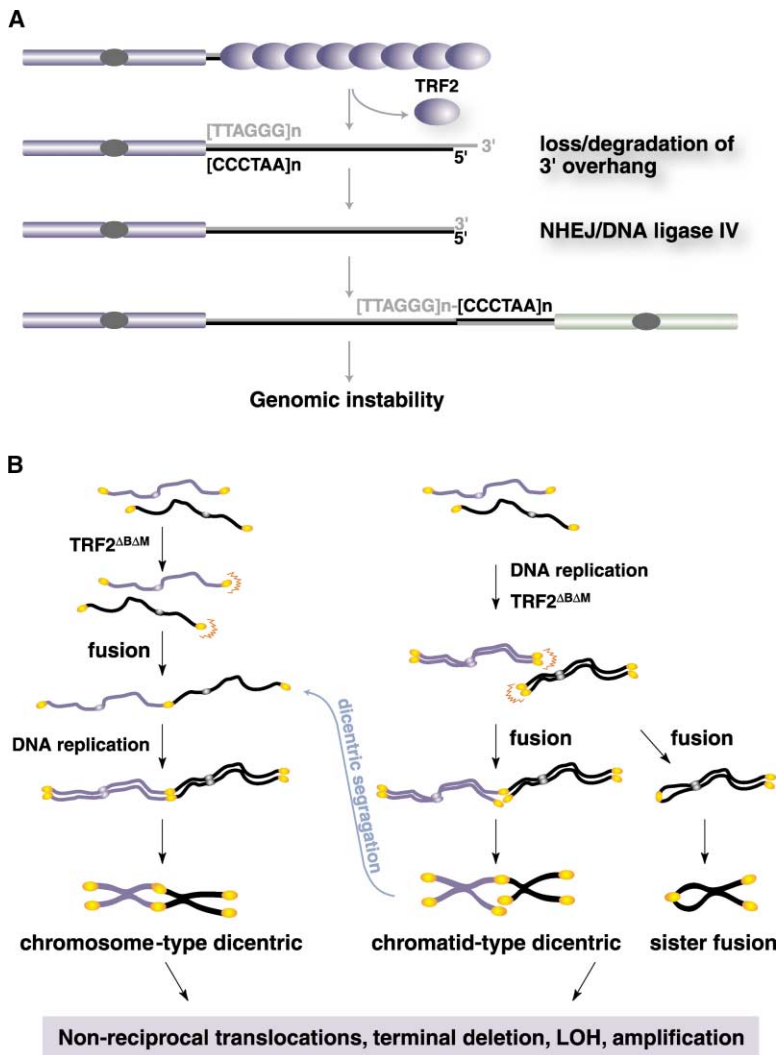


Figure 5. Molecular and Chromosomal Consequences of Telomere Uncapping

(A) Molecular events at chromosome ends after inhibition of TRF2. Expression of TRF2<sup>ΔBΔM</sup> removes TRF2 from telomeres, resulting in nucleolytic attack on the exposed 3' overhang. Overhang loss could also occur during DNA replication if the 3' overhang is not regenerated at the leading-strand end. DNA ligase IV ligates the G strand of one telomere to the C strand of another, resulting in dicentric chromosomes.

(B) A schematic showing the consequences of telomere NHEJ before and after DNA replication. Chromosome-type dicentrics can arise when a telomere fusion occurs in G1 or may result from segregation of a dicentric generated by a chromatid-type fusion in a previous round of cell division. Telomere NHEJ after DNA replication results primarily in chromatid-type fusions involving telomeres replicated by leading-strand DNA synthesis, although sister fusions involving lagging ends also occur.

ends, created either by HO endonuclease or through loss of telomere protection, undergo extensive degradation from their 5' ends, while the other strand is resistant to degradation, resulting in long single-stranded 3' tails [29–32]. The situation at the exposed mammalian telomeres studied here appears to be different, with the 3' single-stranded overhang being processed by nucleolytic attack and the 5'-ended strand of duplex DNA being stable. Whether the stability of the 5' telomeric end is due to additional telomere protection factors (for instance, single-stranded telomeric DNA binding factors such as Pot1 [33]) remains to be determined.

Important insights into the first events after telomere deprotection have emerged from studies in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The former protects its chromosome ends by using a sequence-specific telomeric DNA binding complex composed of Cdc13p, Stn1p, and Ten1p [31, 34–39]. Loss of either of these factors exposes telomeres to degradation of the 5' strand, while the 3' strand persists. There is no evidence for NHEJ of budding yeast telomeres in these and other settings. Loss of the protective factor Pot1 from the telomeres of *S. pombe* also leads

to extensive degradation of the telomeric DNA, but, in this case, both strands are affected and chromosome end fusions are observed [33]. Similar end-to-end fusions arising in telomerase-deficient fission yeast are not dependent on Ku70/80 or DNA ligase IV [40]. In contrast, when the *S. pombe* telomere-protective factor Taz1 is lost, the telomeric DNA is not attacked by nucleases but instead undergoes nonhomologous end joining in a Ku70/80/lig4-dependent reaction, and this event more closely mimics the uncapping of human telomeres reported here [41]. Interestingly, NHEJ of Taz1-deficient fission yeast telomeres only occurs before DNA replication. In the G2 phase of the cell cycle, fission yeast processes uncapped telomeres preferentially by homologous recombination. These findings suggest that, in yeast, uncapped telomeres are processed by the DNA repair pathway that prevails when the telomere becomes defective [41]. Similarly, the prevalence of NHEJ at defective mammalian telomeres may reflect that this pathway is active in mammalian cells. It is also possible that the presence of Ku70/80 at mammalian telomeres represses the HR pathway as it does at two-ended breaks [42].



Soon after the first demonstration of telomere attrition in human cells, it was speculated that loss of telomere protection could be an important contributor to genome instability in human cancer [1, 43–45]. Dicentric chromosomes generated by end-to-end fusion generate anaphase bridges that can be resolved by chromosome breakage. It was proposed that the resulting terminal deletions and nonreciprocal translocations are potential sources of oncogenic changes such as LOH and gene rearrangements. Additional BFB cycles could result in more complex rearrangements, including gene amplification. The data presented here demonstrate the occurrence of nonreciprocal translocations and associated terminal deletions soon after loss of telomere function. Moreover, we document that cells with telomere dysfunction contain fusions between sister chromatids, and this is a prerequisite for gene amplification. Further dissection of the consequences of telomere dysfunction in human cells should illuminate the full spectrum of the chromosomal abnormalities that can result from telomere uncapping and the significance of these events to human cancer.

### Conclusions

In this study, the inhibition of the telomere-protective protein TRF2 is used to study the fate of dysfunctional telomeres in mammalian cells. A main pathway by which these uncapped telomeres are processed is through DNA ligase IV-mediated NHEJ. Several lines of evidence indicate that telomeres lacking TRF2 resemble the critically shortened telomeres of senescence cells [19, 46]. Therefore, the conclusion that dysfunctional telomeres are treated like DNA breaks and repaired by NHEJ is likely to also pertain to the shortened telomeres of primary human cells nearing replicative senescence or those of transformed cells experiencing telomere crisis. Although NHEJ may threaten the integrity of shortening telomeres, some factors that stimulate NHEJ, such as Ku70/80 and DNA-PKcs, also protect chromosome ends from fusion. These findings underscore the emerging view that DNA repair complexes have a dual role at chromosome ends.

### Experimental Procedures

#### Cell Culture, Retroviral Infection, and Adenoviral Infection

The T4, T19, and T22 cell lines are described in [7]. Primary Lig4<sup>+/+</sup>p53<sup>-/-</sup> and Lig4<sup>-/-</sup>p53<sup>-/-</sup> MEFs (obtained from the Alt laboratory [23]) were used at early passage (p3–p5) and were grown in DMEM/10% fetal calf serum (FCS), with nonessential amino acids, glutamine, and antibiotics [7]. IMR90 fibroblasts (ATCC) were grown in the same media, but with 15% FCS. hTERT-BJ1 cells were purchased from Clontech. Retroviral vectors and infection protocols are described in [46]. Adenoviral infections with Ad-TRF2 and Ad-TRF2<sup>ΔBΔM</sup> were performed as described in [18].

#### Multiplex Fluorescence In Situ Hybridization (M-FISH)

M-FISH was performed as described previously [20, 21], with minor modifications. Five pools of flow-sorted whole-chromosome painting probes (kindly provided by Ferguson-Smith) were amplified and labeled with five different fluorochromes: FITC, Cy3, Cy3.5, Cy5, and Cy5.5. About 100 ng of each probe was precipitated in the presence of 30 μg Cot-1 DNA, dissolved in 10 μl hybridization mixture (15% dextrane sulfate, 2× SSC), and hybridized for 48 hr. Microscopic evaluation was performed with a Leica DMRXA microscope (Leica) equipped with a Sensys CCD camera (Photometrics) with a

Kodak KAF 1400 chip. Images for each fluorochrome were acquired separately with highly specific filter sets (Chroma Technology) and were processed with the Leica MCK software (Leica Microsystems Imaging Solutions).

#### Metaphase Chromosome Analysis

Metaphase spreads were prepared as described previously [7]. For fluorescence in-situ hybridization (FISH), slides were aged overnight in a chemical hood. PNA FISH was performed as described by [47], using a FITC-conjugated PNA probe (FITC-5'-CCCTAACCTAACCTAA-3', PerSeptive Biosystems); DNA was counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Images were captured with an Axioplan2 Zeiss microscope with a Hamamatsu digital camera supported by OpenLab software.

#### Alkaline Gel Electrophoresis and In-Gel Detection of Telomeric DNA

Genomic DNA [46] was digested with HinfI/RsaI and was fractionated on a native 0.7% agarose gel or adjusted to 10 mM EDTA, mixed with 0.2 volumes of 6× alkaline loading buffer (0.3 M NaOH, 6 mM EDTA, 18% Ficoll, 0.15% bromocresol green, 0.25% xylene cyanol FF), and run in duplicate on a 0.5% alkaline agarose gel in 50 mM NaOH/1 mM EDTA (pH 8.0) for 27 hr at 50V at 4°C with two changes of running buffer. Half of the alkaline gel, as well as the native gel, was subsequently treated with 0.25 N HCl, denatured, neutralized, blotted in 20× SSC onto a nylon membrane, and hybridized with the TTAGGG probe as described [48]. To verify complete denaturation of the DNA, the other half of the gel was neutralized for 45 min, soaked in 2× SSC for 30 min at room temperature, dried for 30 min at room temperature on a gel drier, and hybridized with the same probe. A telomeric DNA marker ladder was prepared by digesting a TTAGGG repeat bearing pTH5 plasmid [44] with HindIII and religation. The procedure for in-gel detection of native and denatured telomeric DNA fragments was the same as that published in [46].

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