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Breaks at telomeres and TRF2-independent end fusions in Fanconi anemia

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Fanconi anemia (FA) is a rare genetic disease characterized by chromosome instability, progressive pancytopenia and cancer susceptibility. Telomeres are intimately related to chromosome stability and play an important role in organismal viability at the hematological level. Since previous works suggested an accelerated shortening of telomeres in FA, we have studied several markers of telomere integrity and function in FA patients and age-matched controls to get insights into the mechanisms and consequences of telomere erosion in FA. A higher frequency of extra-chromosomic TTAGGG signals and of chromosome ends with undetectable TTAGGG repeats was observed in FA cells by fluorescence *in situ* hybridization (FISH), suggesting intensive breakage at telomeric sequences. This was proven by measuring the frequency of excess of telomeric signals per cell, which was 2.8-fold higher in FA. Consistent with previous reports, quantitative FISH analysis showed an accelerated telomere shortening of 0.68 kb in FA, which occurred concurrently in both chromosome arms in a similar magnitude. Our data therefore suggest that the telomere erosion in FA is caused by a higher rate of breakage at TTAGGG sequences *in vivo* in differentiated cells, in addition to mere replicative shortening during lymphocyte proliferation. Consistent with impaired telomeres in FA patients, we observed a >10-fold increase in chromosome end fusions in FA compared to normal controls. This observation was independent of TRF2, a telomere binding factor that protects human telomeres from end fusions, since immunohistochemistry studies in FA cell lines and corrected counterparts by retrovirus-mediated transfer of *FANCA* and *FANCD2* cDNA showed that a functional FA pathway is not required for telomere binding of TRF2.

INTRODUCTION

Fanconi anemia (FA) is a rare autosomal recessive genetic disease characterized by increased spontaneous and induced chromosome instability, a diverse assortment of congenital malformations, progressive pancytopenia and cancer susceptibility, especially acute myelogenous leukemia, but also solid tumors. The genetics of FA is highly heterogeneous with at least eight different genes involved (*FANCA*, *B*, *C*, *D1*, *D2*, *E*, *F* and *G*); all of them except *FANCB* and *FANCD1* have been cloned and characterized (reviewed in 1). Most of the FA proteins (A, C, E, F and G) assemble in a nuclear complex that is required for the activation, via monoubiquitination, of FANCD2 in response to DNA damage (2). The recently cloned *FANCD2* gene is though to be a key player in the FA pathway since it is the only FA gene conserved in evolution (3). In addition, FANCD2 interacts with the DNA repair protein BRCA1 and the FA protein complex is assembled in the absence of FANCD2, indicating that FANCD2 is downstream in the FA pathway (2). Additional cytoplasmic roles of the FA proteins have also been reported, especially for FANCC (4,5).

Telomeres play important roles in genome stability and in maintaining the individuality of linear chromosomes (6–8). Telomeres are constituted by long arrays of TTAGGG sequences in association with a number of telomeric proteins. One of the major telomere-binding factors preventing chromosome end fusions is the TTAGGG repeat factor 2, TRF2 (9). Telomere integrity is also crucial for organismal viability, and in particular for the normal functionality of the hematopoietic system (10,11). Consistent with this, an apparent accelerated shortening of telomeres has been reported in FA patients (12–14) and in similar hematopoietic syndromes such as acquired aplastic anemia (12,15) and myelodysplastic syndromes (16). An accelerated telomere shortening has also been reported in the chromosome fragility syndrome ataxia telangiectasia (AT) (17). In addition, Hande *et al.* (18) recently reported the presence of extra-chromosomic telomeric DNA in AT patients and $ATM^{-/-}$ mice.

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The molecular mechanism/s leading to telomere shortening in FA cells, however, is/are still unknown. Leteurtre *et al.* (13) hypothesized that an excess of proliferation of surviving hematopoietic cells in FA would lead to telomere shortening and senescence. However, it must be kept in mind that, like AT cells, FA cells are breakage-prone, and direct breakage at telomeric sequences would dramatically shorten the telomere. Consistent with telomere breakage as an alternative mechanism of telomere shortening, recent investigations have shown accumulation of breaks at telomeres after oxidative stress in human fibroblasts *in vitro* (19,20) and DNA repair at telomeres is defective (21,22). It is known that FA cells are highly impaired in their response to oxidative stress (23; reviewed in 1).

The aim of the present study is to get insights into the mechanisms and biological consequences of telomere erosion in FA cells. In addition to the previously reported accelerated shortening in FA, we observed an excess of breakage at telomeric sequences leading to the presence of extra-chromosomic TTAGGG signals in blood lymphocyte metaphases. Consistent with impaired telomeres, an increase in chromosome end fusions was observed in FA cells. We also show that the increase in end fusions is independent of TRF2.

RESULTS

The results of the present investigation are summarized in Table 1. The presence of extra-telomeric TTAGGG repeats (extra- and intra-chromosomic) was first studied in blood lymphocyte samples of FA patients and age-matched controls. A high frequency of extra-chromosomic telomeric DNA signals was observed in FA patients (7.82 ± 1.0 per cell; mean ± SE) compared to age-matched controls (2.67 ± 0.5 per cell), the difference being statistically significant ($P < 0.001$; unpaired Student's *t*-test) (Fig. 1A). The same is true for extra-telomeric intra-chromosomic TTAGGG signals per cell with a frequency of 0.62 ± 0.1 in controls and 1.22 ± 0.2 in FA patients ($P = 0.014$; unpaired Student's *t*-test). The frequency of chromosome ends with undetectable TTAGGG repeats was measured in the same donors (Table 1). A percentage of 0.26 ± 0.04 of telomeres measured in the FA group, but only 0.15 ± 0.04% of telomeres in the control group, had undetectable TTAGGG repeats. This difference was statistically significant ($P < 0.05$; unpaired Student's *t*-test) although it is not known whether a missing signal represents a missing telomere or whether it is just too small or inaccessible to the probe.

Although our results are highly indicative of direct breakage at telomeres in FA cells, very terminal breaks at subtelomeric sequences leading to small terminal fragments could not be disregarded. To clarify this point we next analyzed the frequency of excess of telomeric signals per cell (ETSC) in the same metaphases. Breaks at telomeric sequences would split a single telomere in two signals leading to an excess of telomeric signals. However, a subtelomeric break would lead to a telomere end with an undetectable TTAGGG signal and an extra-telomeric TTAGGG signal without increasing the total yield of telomeric signals. The ETSC was calculated by applying the formula ETSC = NTS – 4NC, where NTS is the total number of telomeric signals per cell and NC is the total number of chromosomes per cell. The results of this analysis are shown in Table 1 and Figure 1B. An average of 6.60 ± 0.9 ETSC were found in FA cells compared to 2.36 ± 0.5 ETSC in age-matched controls, the difference being extremely significant ($P < 0.001$; unpaired Student's *t*-test). The excess of telomeric signals found in the control probably account for the technical background. Subtracting the frequency of ETSC in FA and in controls, an absolute increase of 4.2 ETSC was quantified in FA. A very high correlation ($r^2 = 0.99$; $P < 0.001$; Pearson's correlation test) was observed between extra-telomeric TTAGGG signals and excess of telomeric signals indicating that the two variables are directly and causally related. We therefore concluded that telomeres in FA cells are breakage prone. Considering the excess of telomeric signals found, and that the DNA content at telomeres represents less than 1/1000 of the whole genome, our data indicate that FA telomeres are extremely breakage prone, although it cannot be disregarded that breaks in other places in the genome lead to cell cycle arrest and so cells with interstitial breaks are under-represented at metaphase.

Results on telomere length by quantitative fluorescence *in situ* hybridization (Q-FISH) in FA patients and age-matched healthy controls are also summarized in Table 1. The correlation between the telomere length measured in the short arm and in the long arm is highly significant ($r^2 = 0.95$; $P < 0.001$; Pearson's correlation test) indicating concurrent shortening of the two telomeres. As indicated in Table 1, the mean telomere length in FA patients (3.95 ± 0.40 kb) is, on average, 0.68 kb shorter than in controls (4.63 ± 0.62 kb) and is consistently observed in both chromosome arms in a similar magnitude and with statistical significance ($P = 0.037$ and 0.030 for p and q arms, respectively; one-factor ANOVA with age as a co-variable). This concurrent shortening of telomeres at the two chromosome arms is indicative of replicative telomere shortening during lymphocyte proliferation.

We next examined the frequency of end fusions (dicentrics and centric rings without accompanying fragment as well as clearly terminal fusions) as a marker of telomere dysfunction in FA cells and controls. Only one end fusion in 427 metaphases (0.2%) was found in control samples, whereas 3.1% of the 512 FA metaphases presented end fusions (Table 1). Thus, the frequency of end fusions is >10-fold higher in FA patients ($P < 0.001$).

Telomeres are nucleoprotein complexes comprized of several telomere binding factors. Since the telomeric protein TRF2 is the major telomere binding protein protecting chromosomes from end fusions (9), we hypothesized that the high frequency of end fusions in FA could be related to a defect in telomere binding of TRF2. To answer this question we performed immunohistochemistry analysis of TRF2 binding in wild-type cells as well as in FA cell lines and corrected counterparts by retrovirus-mediated gene transfer of the corresponding wild-type FA gene cDNA. Metaphase spread from controls and FA patients as well as from deficient and corrected cell lines belonging to complementation groups A and D2 were used. FA cells transduced with the empty vector were also included in the experimental design as an internal control. As shown in Figure 2, TRF2 binds to telomeres in a FA cell line. The same results were obtained in the FAD2 cells and in wild-type or corrected FA cell lines, as well as in lymphocytes from FA patients and controls (data not shown), indicating that a functional FA pathway is not required for recruitment of TRF2 to the telomere. Thus, the high frequency of end fusions in FA is independent of TRF2.

Table 1. Telomere abnormalities in peripheral blood lymphocytes from FA patients and age-matched controls

Donor	FA	Age (years)	Cells scored	% CUT[a]	Extra-telomeric signals/cell			End fusions		Telomere length by Q-FISH (kb)			
					Extrachromosomic	Intrachromosomic	ETSC[b]	Cells scored	% cells with end fusions	Telomeres measured	All arms	P-arm	Q-arm
1	Yes	3	10	0.11	10.50 ± 0.7	1.60 ± 0.4	6.00 ± 1.2	50	0.0	1808	3.08 ± 0.02	3.00 ± 0.02	3.16 ± 0.02
2	Yes	14	12	0.31	10.30 ± 1.1	0.75 ± 0.1	9.70 ± 0.8	100	2.0	2164	2.20 ± 0.02	2.10 ± 0.01	2.30 ± 0.02
3	Yes	7	10	0.27	6.00 ± 1.1	1.00 ± 0.3	9.62 ± 1.2	36	11.1	1828	3.15 ± 0.02	2.90 ± 0.02	3.36 ± 0.02
4	Yes	9	11	0.26	4.00 ± 0.6	0.90 ± 0.2	9.00 ± 0.6	50	4.0	1920	2.60 ± 0.02	2.40 ± 0.02	2.79 ± 0.02
5	Yes	11	16	0.38	6.93 ± 0.6	1.18 ± 0.2	8.42 ± 0.9	100	1.0	2868	4.28 ± 0.00	4.08 ± 0.03	4.48 ± 0.00
6	Yes	10	10	0.27	3.36 ± 0.6	1.45 ± 0.2	5.00 ± 0.8	50	0.0	1816	4.70 ± 0.05	4.53 ± 0.05	4.80 ± 0.05
7	Yes	19	11	0.45	8.00 ± 1.4	0.81 ± 0.3	3.18 ± 0.5	26	3.8	1980	5.15 ± 0.04	4.82 ± 0.04	5.48 ± 0.04
8	Yes	10	13	0.17	11.80 ± 0.8	2.44 ± 0.3	5.69 ± 0.6	50	4.0	2328	5.49 ± 0.04	5.29 ± 0.04	5.69 ± 0.04
9	Yes	9	12	0.13	9.50 ± 1.0	0.84 ± 0.2	2.82 ± 0.6	50	2.0	2204	4.90 ± 0.04	4.59 ± 0.04	5.20 ± 0.04
mean ± SE		**10.2 ± 1.5**	**11.7 ± 0.6**	**0.26 ± 0.04**	**7.82 ± 1.0**	**1.22 ± 0.2**	**6.60 ± 0.9**	**56.9 ± 8.6**	**3.1 ± 1.1**	**2101 ± 114**	**3.95 ± 0.40**	**3.75 ± 0.39**	**4.14 ± 0.42**
10	No	6	11	0.15	1.83 ± 0.4	0.91 ± 0.2	3.27 ± 0.2	50	0.0	1960	2.47 ± 0.02	2.30 ± 0.02	2.56 ± 0.02
11	No	14	7	0.41	1.00 ± 0.3	0.62 ± 0.2	3.75 ± 0.6	50	0.0	1200	8.28 ± 0.11	7.34 ± 0.10	9.23 ± 0.13
12	No	7	11	0.14	1.14 ± 0.2	0.07 ± 0.0	4.54 ± 0.4	50	0.0	2016	3.90 ± 0.04	3.56 ± 0.03	4.24 ± 0.04
13	No	8	12	0.13	4.58 ± 0.4	0.58 ± 0.1	4.25 ± 0.4	27	0.0	2180	3.09 ± 0.03	2.87 ± 0.02	3.31 ± 0.03
14	No	11	10	0.17	0.90 ± 0.2	0.18 ± 0.1	1.64 ± 0.4	50	0.0	1716	3.52 ± 0.03	3.16 ± 0.03	3.88 ± 0.04
15	No	10	13	0.12	4.61 ± 0.4	0.92 ± 0.2	0.93 ± 0.2	50	0.0	2388	5.18 ± 0.06	4.83 ± 0.05	5.53 ± 0.05
16	No	19	14	0.15	3.72 ± 0.3	1.09 ± 0.2	0.67 ± 0.3	50	0.0	2632	6.12 ± 0.04	5.70 ± 0.04	6.54 ± 0.04
17	No	9	13	0.04	1.93 ± 0.1	0.60 ± 0.1	1.63 ± 0.2	50	2.0	2468	3.32 ± 0.03	3.14 ± 0.03	3.50 ± 0.03
18	No	9	11	0.00	4.30 ± 0.6	0.58 ± 0.1	0.53 ± 0.1	50	0.0	1988	5.80 ± 0.04	5.67 ± 0.04	5.94 ± 0.04
mean ± SE		**10.3 ± 1.3**	**11.3 ± 0.7**	**0.15 ± 0.04**	**2.67 ± 0.5**	**0.62 ± 0.1**	**2.36 ± 0.5**	**47.4 ± 2.6**	**0.2 ± 0.2**	**2060 ± 143**	**4.63 ± 0.62**	**4.29 ± 0.56**	**4.97 ± 0.69**

[a]CUT, chromosomes with undetectable TTAGGG signals.
[b]ETSC, excess of telomeric signals per cell.

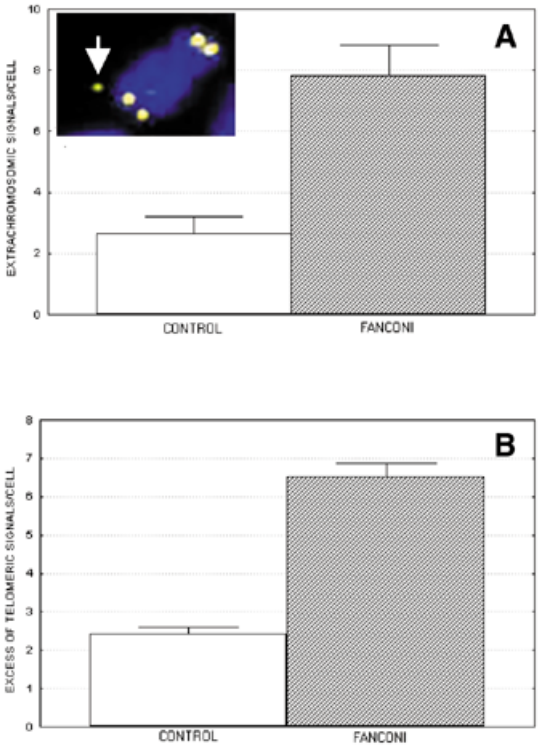

Figure 1. Frequency of extra-chromosomic telomeric TTAGGG signals per cell (**A**) and of excess of telomeric signals per cell (**B**) in PHA-stimulated peripheral lymphocytes from FA patients and age-matched controls. Means and SE are represented by histograms and error bars, respectively.

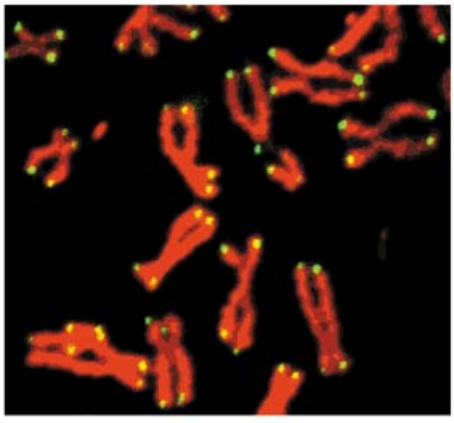

Figure 2. TRF2 binds to telomeres in an FAA cell line. The same result was obtained in an FAD2 cell line as well as in wild-type or corrected FA cell lines and in control and FA peripheral lymphocytes.

DISCUSSION

Our data clearly show that telomere integrity is impaired in FA peripheral lymphocytes *in vivo*. We observed high frequencies of chromosome ends with undetectable TTAGGG repeats and extra-chromosomal telomeric DNA signals leading to an excess in the total yield of telomeric signals in FA cells. This result is interpreted as an excess of breaks in telomeric repeat arrays in FA lymphocytes. In addition, and consistent with previous publications (12–14) an overall reduction of telomeric length in FA was also observed. Interestingly, this reduction was consistently observed in both chromosome arms in a similar magnitude and in a concurrent fashion indicating that, in addition to telomeric breakage, an accelerated replicative telomere shortening occurred during lymphocyte differentiation and proliferation. Thus, we conclude that both telomere breakage and replicative shortening account for the observed telomere shortening in FA.

Because of the important biological roles of telomeres, many efforts have been made to unravel the genetic factors involved in telomere length regulation (6,7). There is increasing evidence of an extensive interplay between DNA repair and telomere maintenance from yeast to mammalian cells. Interestingly, FANCD2 foci were observed in autosomal telomeres in mice diplotema, suggesting a dual role for this protein in telomere length regulation (2). In addition, the Nijmegen breakage syndrome protein, NBS1, was also found at meiotic chromosome telomeres, and a telomere phenotype has been reported in mice and human cells defective in a number of proteins involved in double-strand breaks (DSB) repair (reviewed in 22). These repair proteins include Ku80, an essential component of non-homologous end joining (NHEJ), and the NBS1/Mre11/Rad50 complex, mainly involved in DSB repair by homologous recombination (HR) but also by NHEJ (24,25). A role for the AT gene, ATM, in telomere maintenance has also been reported in mice and humans (17). In a recent study, Hande *et al.* (18) reported a high frequency of extra-chromosomal telomeric DNA in cells from $Atm^{-/-}$ mice and in fibroblasts from AT patients. Since both ATM and FANCD2 interact with BRCA1 (2,26), suggesting a role in DNA repair for both of these proteins, the similarity of telomere abnormalities between AT and FA is very interesting. Thus, one of the functional consequences of the increasingly evident molecular links between AT, FA and NBS would be a defect in telomere maintenance.

Telomeres are crucial to maintaining the individuality of eukaryotic chromosomes. The relationship between telomere length and chromosome fusions in mammalian cells became evident in telomerase-deficient mice with short telomeres where a high frequency of Robertsonian fusions was observed (8,27). Consistent with impaired telomeres in FA, the frequency of chromosome end fusion was much higher in FA lymphocytes when compared to controls. Since the TTAGGG repeat factor 2, TRF2, is one of the major telomere binding proteins protecting chromosomes from end fusions (9), we hypothesized that the high frequency of end fusions in FA could be related to a defect in telomere binding of TRF2. However, our immunohistochemistry data in wild-type and FA lymphocytes as well as in FANCA- and FANCD2-deficient cell lines and corrected counterparts indicate that TRF2 binds to telomeres independently of the FA pathway. The *FANCA* and *FANCD2* genes were chosen to cover the FA pathway upstream and downstream (see above). Thus, we concluded that the FA pathway is not required for telomere association of TRF2 and that the high frequency of end fusion is not explained by a defect in the telomere binding activity of this protein. A possible explanation is that higher DNA breakage at the FA telomeres could result in loss of the protective end-capping structures leading to end-to-end fusions, as shown here.

In summary, telomere integrity is highly impaired in FA peripheral T-cells *in vivo*. Our data suggest that the apparent telomere shortening observed in FA is not only related to replicative shortening as hypothesized previously but also caused by a higher rate of breakage at telomeric sequences *in vivo*. Consistent with impaired telomeres, we also observed a >10-fold increase in the frequency of end fusions in FA. This increase was not related to any defect in the end-binding activity of TRF2 in FA cells. Together with recent reports in other chromosome fragility syndromes, we suggest that one of the functional consequences of the increasingly evident molecular links between AT, FA and NBS would be a defect in telomere maintenance.

MATERIALS AND METHODS

Subjects

Blood samples were obtained, with informed consent, from nine unrelated FA patients (three males and six females, 10.2 ± 1.5 years old; mean ± SE) during routine clinic visits, and from nine healthy individuals (four males and five females, 10.3 ± 1.3 years old). All FA patients belong to the complementation group A, except two patients whose complementation group is not known for technical reasons (data not shown). Two patients underwent bone marrow transplantation a few days after extracting the blood sample used in this study so that only actual FA cells were analysed. This study was approved by the University Ethics Committee on Human Research.

Cell culturing and preparation of slides

Whole blood (500 µl) was incubated in 5 ml cultures and lymphocytes were stimulated to divide as described elsewhere (28) for 48 h. In order to obtain lymphocyte metaphases, colcemid (0.1 µg/ml; Gibco, Paisley, UK) was added 2 hours prior to harvesting, following standard cytogenetic procedures. Cells were dropped onto clean slides and air-dried overnight. Some of the slides were stained with Giemsa and the remaining slides were kept at –20°C until used fo FISH.

Detection and length quantification of telomeres

FISH was performed with a Cy3-labeled telomeric PNA probe essentially as described in detail elsewhere (28–30). Every Q-FISH experiment included slides from a FA patient and an age-matched control and the Q-FISH analysis was performed blind. Digital images were recorded using a COHU CCD camera on a Leica Leitz DMRB fluorescence microscope, and captured using Leica Q-FISH software at 2000 ms integration time. Separate DAPI and Cy3 images were subjected to telomere fluorescence analysis by using the dedicated computer program TFL-telo, kindly provided by Dr P.Lansdorp. The integrated fluorescence analysis intensity for each telomere was calculated after correction for image acquisition exposure time. A minimum of 10 lymphocyte metaphases per sample was analysed. The details of calibration for telomere fluorescence intensity are described elsewhere (31). In brief, two calibration levels were used to ensure a reliable measure of the telomeres. To correct for daily variations in lamp intensity and alignment, images of fluorescent beads were acquired and analyzed using the TFL-telo program. Secondly, telomere fluorescence units were extrapolated from telomere fluorescence of LY-R and LY-S lymphoma cell lines of known lengths of 80 and 10 kb, as described elsewhere (30). The calibration-corrected telomere fluorescence intensity was calculated as described (11). The same digitalized metaphases were used to calculate the frequency of excess telomeric signals, extra-telomeric TTAGGG signals and telomeres with undetectable TTAGGG signals per cell.

Chromosome end fusions

Replicates of the slides used for Q-FISH were stained with 10% Giemsa in phosphate buffer, pH 6.8. A total of 26–100 (50 in most cases) complete metaphases per blood sample were analysed. The presence of end fusions (dicentrics and centric rings without accompanying fragment as well as clearly terminal fusions) was studied in detail.

Immunodetection of TRF2 in lymphocytes and cell lines in metaphase

Immunohistochemistry analysis of TRF2 binding was performed in wild-type lymphocytes as well as in FA cell lines and corrected counterparts by retrovirus-mediated gene transfer. Deficient and corrected cell lines belonging to complementation groups A (lymphoblastoid cell lines) and D2 (PD20 transformed fibroblasts) were kindly provided by Dr Juan A.Bueren (Madrid, Spain), and Drs Alan D.D'Andrea and Irene García-Higuera (Boston, MA), respectively. FA cells infected with the empty vector were also included in the experimental design. Cells were grown following standard cell culturing procedures and immunodetection of TRF2 in cytospin-spread, protein-preserved acid-free fixed metaphases was performed as we reported previously for acetylated histone H4 (32), but using a primary mouse antibody against human TRF2 (BD-Transduction Laboratories, NJ) diluted 1:100, an FITC-conjugated sheep anti-mouse (Sigma, MO) diluted 1:20, and an FITC-conjugated donkey anti-sheep (Sigma) diluted 1:20. Chromosomes were counterstained in red with propidium iodine and images were captured with a laser confocal microscope (Leica TCS 4D) and edited using the program Adobe Photodeluxe 1.0.

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