Human chromosome 1 in mouse immortal cell background

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Abstract

Telomeres are specialized structures at the ends of linear chromosomes and are essential for normal cellular function. Telomeres prevent degradation and aberrant recombination of chromosome termini and facilitate appropriate replication of chromosome ends. In this work we followed telomere dynamics in immortal mouse cell strain A9 in comparison with A9+1. The later one is derived from A9 cells by introduction of human chromosome 1. In spite of telomerase presence, we noticed great decrease in telomere lengths in A9+1 in comparison with A9 cells. We also followed the behavior of individual human and mouse telomeres under conditions of observed gross telomere shortening. Human chromosome 1 followed the overall telomere length in hybrid cells. We suggest that telomere lengths are primarily determined by cell protein background.

Sažetak

Telomere su specijalizirane strukture na krajevima linearnih kromosoma i esencijalne su za normalnu stanicnu funkciju. One sprecavaju degradaciju i pogrešnu rekombinaciju krajeva kromosoma i olakšavaju replikaciju kromosomskih krajeva. U ovom radu pratili smo dinamiku telomera u imortalnim mišjim stanicnim linijama A9 i A9+1. A9+1 stanicna linija dobivena je unošenjem ljudskog kromosoma 1 u A9 stanice. Usprkos prisustvu telomeraze primijetili smo veliko skracenje telomera kod A9+1 u usporedbi s A9 stanicama. Pratili smo i ponašanje ljudskog kromosoma 1 u mišjim stanicama u uvjetima pod kojim je došlo do skracivanja telomera. Raspon duljina telomera ljudskog kromosoma 1 odgovaro je rasponu mišjih telomera stanica domacina. Ovi rezultati sugeriraju da na raspon duljina telomera najveci utjecaj ima ukupan sastav telomernih proteina stanice.

Graphic abstract

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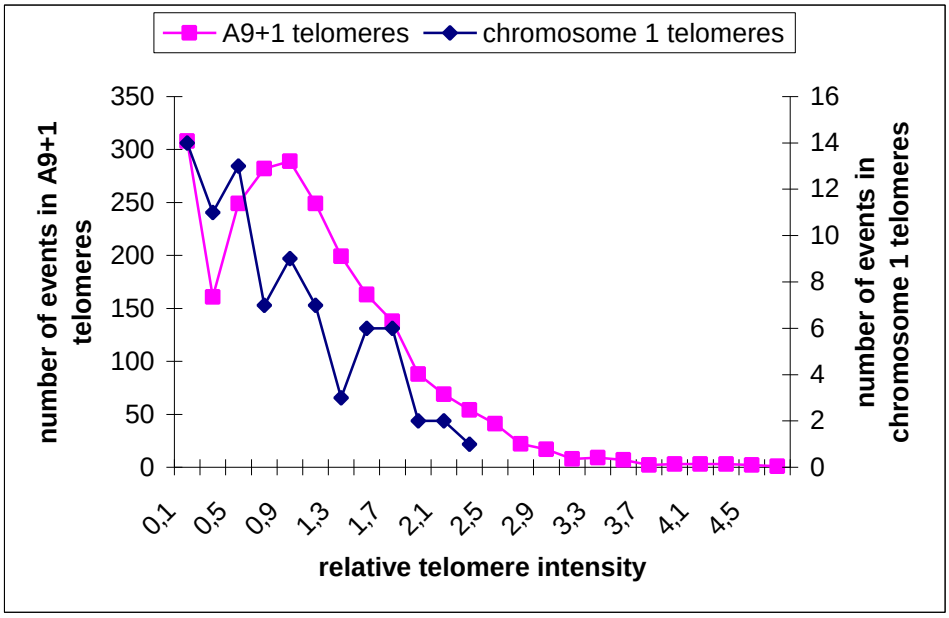

Introduction

Telomeres are specialized structures at the ends of linear chromosomes, composed of short repeat sequences, essential for normal cellular function. They prevent degradation and aberrant recombination of chromosome termini and facilitate proper replication of chromosome ends. In order to stabilize its conformation, tandem repeats of short G-rich sequences $(TTAGGG)_n$ form stable telomere loop (t-loop) structures in complex with telomere proteins[1,2].

Telomeres shorten at each cell division as a consequence of the property of conventional DNA polymerase that cannot replicate the very ends of linear DNA[3]. Cells unable to maintain constant telomere length stop dividing when at least one of their telomeres shorten to a critical length. This irreversible growth arrest state, called replicative senescence[4], is believed to be a mechanism that prevents cell immortalization[5]. Cells undergoing permanent divisions, such as tumor and stem cells, circumvent replicative senescence and maintain constant telomere length by constitutive telomerase expression[6,7,8]. Generally, significant telomerase activity is absent in most somatic cells[9], although recent data indicate that its presence in a low amounts could have role in maintaining stable telomere structure of some normal human cells[10].

In order to identify factors involved in mechanisms of cell senescence and immortalization, normal diploid fibroblasts were fused with various immortal human cells. Surprisingly, all obtained hybrids had limited life span, clearly indicating dominance of cellular senescence[11,12]. Similar experiments also enabled identification of individual chromosomes that carried genes involved in induction of senescence, one of which is human chromosome 1. Using microcell-mediated chromosome transfer (MMCT) of human chromosome 1 lead to induction of senescence in various human immortal cell lines assigned to complementation group C[13], as well as in various rodent cell lines[14,15,16]. Although mechanisms are still mostly unknown, it is found that, unlike chromosomes 3, 4, 7 and 10, senescence induced by chromosome 1 is telomerase independent[16,17,18,19,20]. In the present work, we followed changes in gross telomere length as well as telomeres of individual

mouse chromosomes and human chromosome 1 upon its introduction into immortal mouse cell line A9. Although introduction of this chromosome induces senescence in some other mouse cells such as melanoma B16-F10[21], A9+1 cells maintained immortal phenotype. Telomere repeats of immortal mouse A9 cells in the culture are around 10 kb long, but hybrid cells with introduced human chromosome 1 have significantly shorter telomeres, around 4 kb. This could be explained by the influence of certain human proteins expressed in these cells that influence telomere conformation and stability.

Experimental procedures

Cell culture

A9, A9+1 and HCA2 cell lines were gift from O. M. Pereira-Smith, Department of Cellular and Structural Biology, Sam and Ann Barshop Center for Longevity and Aging Studies, San Antonio, USA. A9+1 was created by microcell fusion of a single copy of a human chromosome 1 tagged with the selectable marker Neo, and A9 mouse fibroblasts[13,22]. A9, A9+1 and young human diploid HCA2 fibroblasts were grown at 37^{o}C in DMEM (Dulbecco's modified Eagle's medium, Sigma), supplemented with 10% fetal bovine serum (FBS) in the presence of 5% CO_2.

Metaphase preparation

Cells were treated with colcemid (0.1 ?g/ml) for 4 h and harvested with trypsin. Cells were incubated in hypotonic KCl buffer prior to fixation with methanol:acetic acid (3:1). Slides were prepared as reported previously[23].

Fluorescence in situ hybridization

Metaphases were prepared after colcemid treatment, fixed in methanol:acetic acid (3:1) and drained slides hybridized with telomeric PNA

probe labeled with rhodamine. After hybridization slides were stained with DAPI, and analyzed under UV light microscope. Telomere length was analyzed by fluorescence in situ hybridization (FISH) according to manufacturer's instructions (Applied Biosystems Framingham, MA, USA). Typically, slides were treated with pepsin (0.1 % in 0.01 M HCl), dehydrated in ethanol and, after denaturation at 70^{o}C for 6-8 min, hybridized using rhodamine-conjugated $(C_3TA_2)_3$ peptide nucleic acid (PNA) probe in hybridization solution (10 mM sodium phosphate buffer pH=7.4, 10 mM NaCl, 20 mM Tris pH=7.5, 70% formamide, 1x Denhart solution, 0.1 ?g/ml tRNA) for 2 h at room temperature. Slides were rinsed in PBS + 0.1% Tween 20 at 57 ^{o}C for 20 min. and counterstained with DAPI (4',6-diamidino-2-phenylindole, Sigma).

Slides were analyzed under Olympus fluorescence microscope BX51, 1000x magnification. Relative signal intensities were analyzed by densitometry on Image Master VSD Software (Pharmacia). Mean metaphase intensities were obtained by subtracting the mean pixel value of background from the mean pixel value for all telomeres in the metaphase. The relative intensities of individual telomeres were obtained by dividing the mean pixel value associated with that telomere by the mean pixel value of all telomeres in the metaphase[24].

DNA preparation and Southern blot analysis

High molecular weight genomic DNA was prepared using Qiagen DNeasy Tissue Kit according to the manufacturer's instructions. DNA was digested with restriction enzymes Rsa I and Hinf I (Roche). Telomere probe was digoxigenin labeled by PCR. Primers specific for telomere sequence (F: CCCTAA, R: TTAGGG) were amplified by non-template PCR (94 ^{o}C/1.5 min, (94 ^{o}C /45 sec, 52 ^{o}C /30 sec, 72 ^{o}C /1 min, 72 ^{o}C / 10 min, 30 cycles). Southern blot was performed by alkaline transfer[25], and membrane (Hybond N+, Amersham) hybridized with digoxigenin labeled telomeric probe. Signal detection was performed according to manufacturer's instructions (Dig DNA

labeling and detection kit, Roche). Mean molecular weight of DNA fragments was estimated by densitometric analysis using Aida 2.0 software.

Telomerase activity assay

Telomerase repeat amplification protocol (TRAP) assay was performed using TRAPEZE kit (Chemicon) under these PCR conditions (90 ^{o}C/90 sec, 94 ^{o}C /30 sec, 50 ^{o}C /30 sec, 72 ^{o}C /45 sec, 35 cycles). Products were separated on 12.5% polyacrylamide gel and visualised with ethidium bromide under UV light. Telomerase activity was calculated as TPG (Total Product Generated) according to the formula:

$$\text{TPG (units)} = \frac{(x-x^{\circ})/\, c}{(r-r^{\circ})/\, cR} \times 100$$

where x is activity of telomeric bands of the sample, x° is the background, c is the activity of the internal control of the sample, r is the activity of the telomeric bands of the quantitation control, r° is activity in primer-dimer/PCR contamination control and cR is the activity of the internal control of quantitation control. Densitometric analysis was performed on Image Master VSD Software (Amersham).

Results

TRF analysis of A9 and A9+1 cells

Introduction of a single normal chromosome 1 into several immortal human cell lines assigned to complementation group C lead to the growth arrest and appearance of senescent phenotype[13]. Chromosome 1 also induced senescence in several rodent cell lines: mouse melanoma hybrid cell line[15, 26] and immortal Syrian hamster cell line[14]. In contrast, mouse A9 cell line served as a "general recipient" and these cells retained immortal phenotype after introduction of all human chromosomes by microcell-mediated transfer, with the exception of chromosome 9[18, 22]. Indeed, A9+1 growth rate was similar to their parental A9 cells. Surprisingly, analyzing telomere restriction fragments obtained from A9 and A9+1 cells we found great differences in their telomere lengths: southern blot densitometric analysis showed that A9 cells had significantly longer telomeres (mean 9.3 kb) than A9+1 (mean 4 kb). As comparison, human young fibroblasts (HCA2) at PD ~25 showed mean telomere length of 8.3 kb (Fig.1). These results suggest possible involvement of proteins expressed from chromosome 1 in telomere maintenance and cellular senescence.

A9 and A9+1 telomere analysis by PNA FISH

Since A9+1 cells demonstrated drastic telomere shortening upon introduction of human chromosome 1, we examined individual telomere lengths of mouse chromosomes as well as human chromosome 1 and compare it to original A9 cell strain. Telomeres of A9+1 and parental A9 cells were analyzed by PNA fluorescence in situ hybridization. Results are shown in Fig. 2. Telomeres in A9+1 showed great variability in fluorescent signals, including sister chromatides, in comparison with A9. Also, in both cell lines some chromosomes completely lacked telomeric signals. The percentage of unlabeled telomeres in A9+1 cells was as high as 44.2 %, and in A9 cells only 7.3 % (Fig. 3 A). Thus, low percentage and intensity of labeled telomeres in

A9+1 cells, in contrast to A9, match dramatic telomere shortening observed by TRF analysis.

Human chromosome 1 telomere analysis

We further analyzed human chromosome 1 telomeres in contrast to mouse chromosome telomeres in the same cell. The percentage of unlabeled human chromosome 1 telomeres corresponded to mouse telomeres in A9+1 (46.3 % and 44.2 % respectively) (Fig. 3 A). Also, analysis of relative fluorescence intensities in A9+1 cells showed the same range of telomere lengths of human chromosome 1 as those of surrounding mouse chromosomes (Fig. 3 B). These results indicate crucial role of cell protein background in telomere length regulation rather than specific chromosomal DNA properties.

Telomerase activity analysis

It is known that the level of telomerase activity could influence telomere length[27, 28]. Therefore we compared telomerase activity in both A9 and A9+1 cells, using TRAP assay as described in experimental procedures. As shown in Fig. 4, there was no significant difference in telomerase activity between these cell strains which demonstrate that telomerase did not contribute to observed telomere length reduction upon introduction of human chromosome 1 in mouse A9 cells.

Discussion

Telomere length analysis in mouse cell line A9 containing human chromosome 1, in comparison with their parental cell line, revealed that they have significantly shorter telomeres, as shown by PNA-FISH (relative signal intensity and labeling percentage) and TRF analysis. Human chromosome 1 also showed the same range of telomere lengths as mouse chromosomes in the cell. There are several possible explanations for the observed differences in telomere lengths between hybrid and parental cell line. Although

recombination between mouse and human chromosome 1 have not been observed in A9+1 cells used in these studies[13], it has been demonstrated that they could lead to changes in mouse cell protein background including changes in expression of some telomere–regulating proteins[18]. There is also possibility that proteins expressed from human chromosome 1 affect the length of telomeres in these cells. This hypothesis is supported by experiments performed with some other A9 hybrid cell lines. It was found that total telomere lengths depended on human chromosome introduced in these cells so that A9+3 cells showed slight lengthening of telomeres, and chromosome 4 introduction showed similar profile as A9+1 (data not shown). In comparison, experiments made with artificial “telomere seeds” introduced in cell lines containing telomerase showed increase in their size with time in culture and followed telomere dynamics in cancer cell lines[29, 30, 31]. Differences in telomerase activity, as a cause of changes in the mean telomere length, could be excluded: A9 and A9+1 cells had nearly the same telomerase activity[27, 28].

There are several lines of data indicating the role of human chromosome 1 in cell senescence. Pereira-Smith[32] found four complementation groups of tumor cells indicating that there are four basic mechanisms responsible for cell immortalization. Introduction of a single normal chromosome 1 into immortal human cell lines assigned to complementation group C caused loss of proliferative potential and induction of senescent phenotype[13]. Possibly, human chromosome 1 did not induce senescence in A9 because these cells did not belong to appropriate complementation group, but contributed to the changes of protein expression. Some of these could affect telomere maintenance and their reduction to the shorter length. On the other hand, human chromosome 1 was able to induce senescence in an immortal Syrian hamster cell line[14], a mouse melanoma hybrid cell line[15] and a human uterine endometrial carcinoma cell line as well[16]. This suggests that this chromosome is a carrier of some senescence-related genes that are functionally conserved across evolutionary boundaries. Loss of q arm of chromosome 1 by spontaneous deletion was unable to induce senescence in some experimental cell lines, indicating the presence of senescence associated genes in this region[13,33]. Structural changes and

deletions of chromosome 1 have been documented in the cases of several clinical tumors[34]. Two putative senescence loci were localized on chromosome 1[35] and Yawata[21] identified region of ~600 kb in 1q42-3 position involved in induction of cellular senescence. It acted in a telomerase independent pathway, as these cells retained telomerase activity upon restoration of senescence phenotype.

Unlike chromosome 1, microcell-mediated chromosome transfer of human chromosomes 3 and 10 in the human tumor cell lines lead to telomerase repression and progressive shortening of telomeres[17,20,36,37]. Introduction of chromosome 2[38], 4[39], 6 and 7[20] in various immortal cell lines also induced senescence; most of them had suppressed telomerase activity[39,40,41]. Parallel experiments with several other chromosomes showed no changes in proliferation of various cell lines[15].

A9+1 cells showed shortened telomeres, but remained immortal and telomerase positive. They are phenotypically identical to their parental cell line as well. We did not detect significant increase in genome instability in hybrid cells nor increase in telomere associations observed under DAPI staining (data not shown). It is known that very short or missing telomeres could lead to chromosome rearrangements and fusions[42]. In spite of this, some cell lines manage to maintain stability and constant equilibrium of mean telomere length[8,31,43]. Although human chromosome 1 influenced the mean telomere length, A9+1 cells maintained their telomeres at constant length and avoided senescence.

As a conclusion, A9 mouse cell line showed telomere shortening after introduction of human chromosome 1 whose telomeres were also adapted to these changes. Telomere lengths are primarily determined by cell protein background and cause of telomere shortening in A9+1 cells could be explained by expression of some human proteins.

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Figure legends

Figure 1.

Telomere length analysis of A9, A9+1 and normal human fibroblasts. DNA was isolated from A9, A9+1, and normal human fibroblasts HCA2 as a control, digested with Rsa I/Hinf I restriction enzymes in subtelomeric regions and hybridized with digoxigenin labeled telomere probe. TRF signals were analyzed with Aida 2.0 program. NF - young diploid human fibroblasts HCA2. Bars represent average telomere lengths. BstE2 marker on the left.

Figure 2.

PNA FISH of A9 and A9+1 cell telomeres. Chromosomes were visualized with DAPI (blue) and telomere signals with Rho-labeled telomere PNA probes (red). A) A9 metaphase spread stained with DAPI, B) merged images of A9 metaphase spread and telomere signals, C) A9+1 metaphase spread stained with DAPI (1 labels human chromosome 1), D) merged images of A9+1 metaphase spread and telomere signals. Magnification 1000X.

Figure 3.

Telomere labeling statistics. A) Percentage of unlabeled telomeres in A9, A9+1 cells and human chromosome 1. B) Frequency of relative intensities of individual mouse telomeres in A9+1 cells compared with relative intensities of human chromosome 1 telomeres. Unmodified black and white PNA FISH images were used to estimate the mean pixel value for each telomeric signal. Images were analyzed using Image MasterVSD Software (Amersham).

Figure 4.

Telomerase activity in A9 and A9+1 cells determined by TRAP assay. Telomerase activity was calculated as TPG (total product generated) as described in Experimental procedures.

Figures

Figure 1

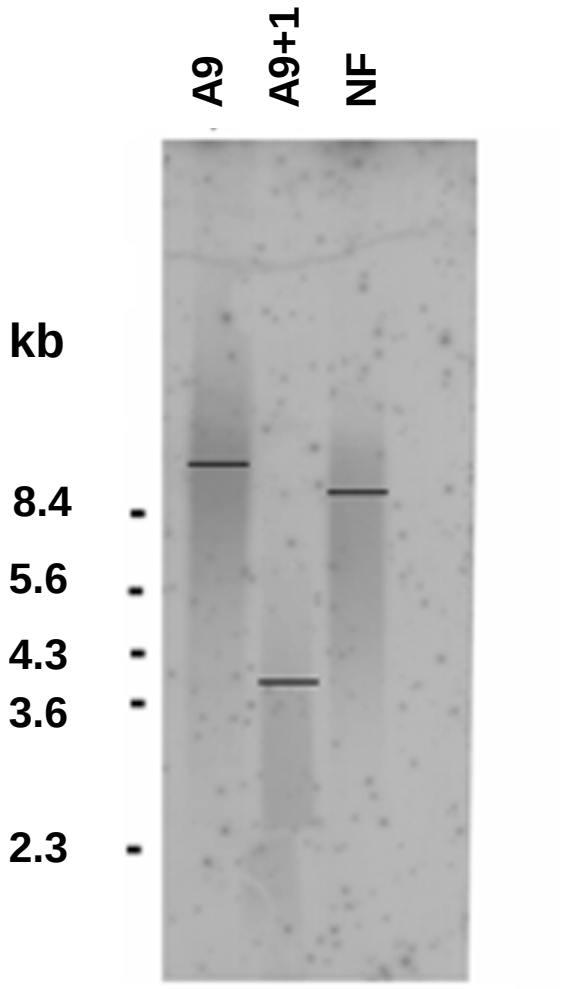


Figure 2

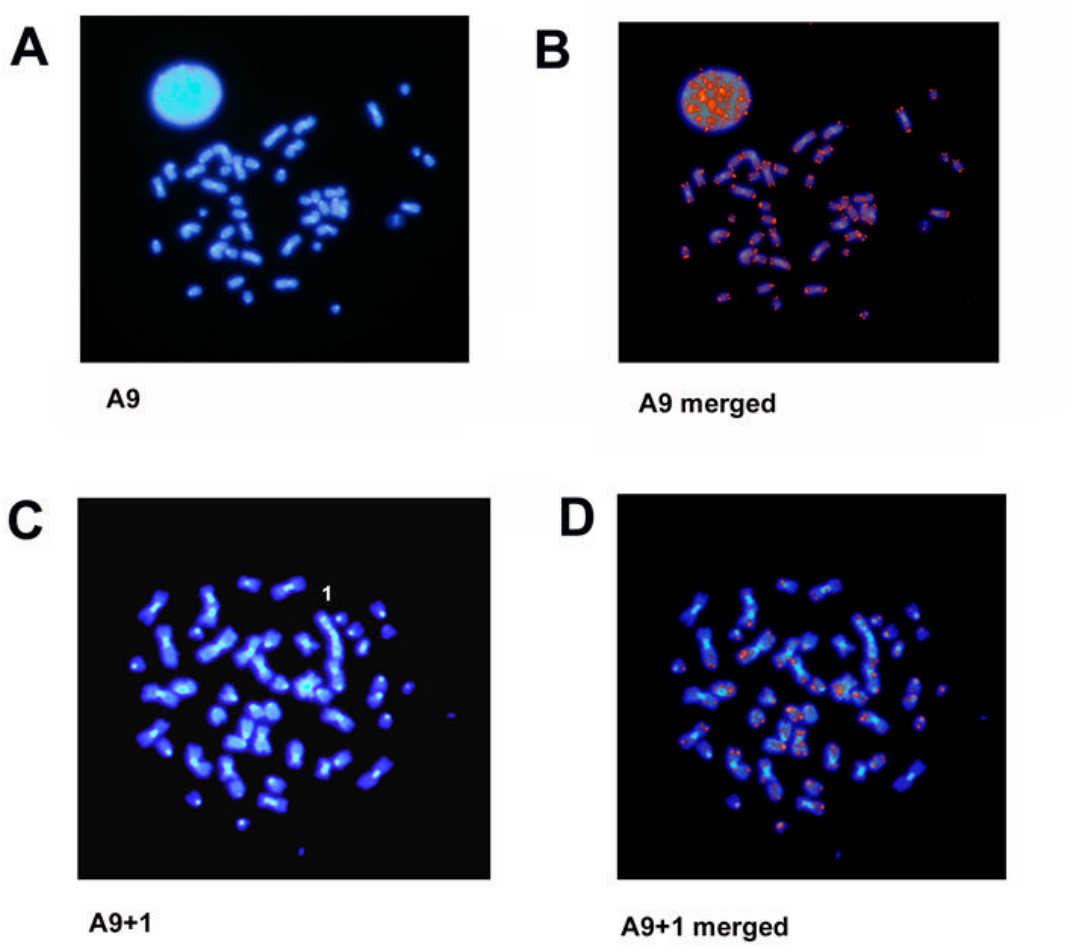
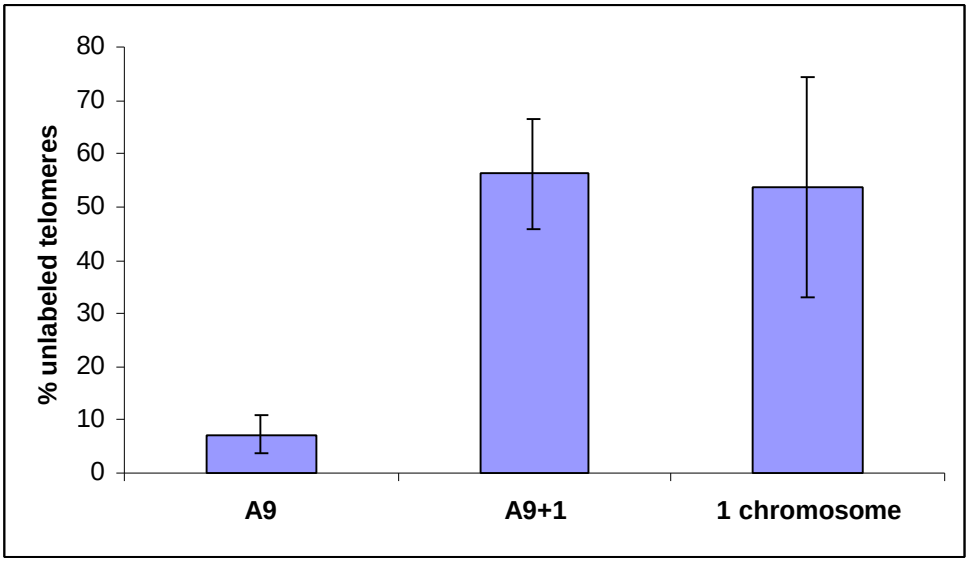


Figure 3

A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80
70
60
50
40
30
20
10
0
A9
A9+1
1 chromosome

B

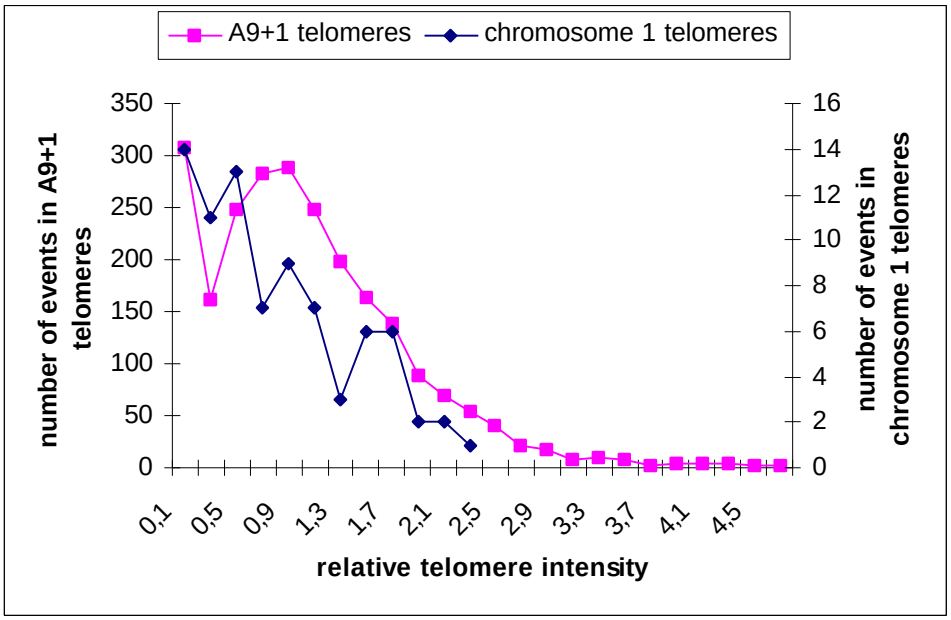
A9+1 telomeres
chromosome 1 telomeres
number of events in A9+1 telomeres
number of events in chromosome 1 telomeres
350
300
250
200
150
100
50
0
16
14
12
10
8
6
4
2
0
0,1
0,5
0,9
1,3
1,7
2,1
2,5
2,9
3,3
3,7
4,1
4,5
relative telomere intensity

Figure 4