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"Telomere alterations and chromosome segregation defects induced by oxidative stress"

"Alterazioni al telomero e difetti nella segregazione cromosomica indotti dallo stress ossidativo"

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Abbreviations

ALT	Alternative Lengthening of Telomeres
CIN	Chromosome Instability
SSB	Single Strand Breaks
DSB	Double Strand Breaks
NPBs	Nucleoplasmic Bridges
NBuds	Nuclear Buds
MN	Micronucleus
APBs	ALT associated PML Bodies
BER	Base Excision Repair
BFB	Break Fusion Bridge
CO-FISH	Chromosome Orientated FISH
DSB	Double Strand Break
FISH	Fluorescence *in situ* Hybridization
FITC	Fluorescein isothiocyanate
PML	Promyelocytic Leukaemia
Q-FISH	Quantitative FISH
ROS	Reactive Oxygen Species
RQ-TRAP	Real Time Quantitative TRAP
SCE	Sister Chromatid Exchange
SSB	Single Strand Break
T-loop	Telomeric loop
TRAP	Telomerase Repeat Amplification Protocol

Telomeres are nucleoprotein complexes that protect the ends of linear chromosomes and are required for a wide range of cellular processes, as apoptosis, aging, cancer and chromosome stability.
Telomere shortens every time a somatic human cell divides. Furthermore human cancers, avoid the progressive loss of telomeric DNA by the telomerase or the telomerase-independent mechanism, termed ALT (Alternative Telomere Lengthening), result into indefinite cell proliferation. However, it has been demonstrated, that several physical or chemical agents are able to modulate telomere length; in particular oxidative stress accelerates telomere shortening. Literature data showed that telomeres are the preferential target for oxidative damage because of ROS able to induce 8-oxodG from triplet GGG present in human telomeric sequence TTAGGG. Nevertheless, the effects of prolonged oxidative stress on telomere metabolism are still poorly investigated.
With the aim to investigate the effects of oxidative stress on telomere length and chromosome segregation and to test mitotic defects, human primary lung fibroblasts (MRC-5) were daily treated with 10μM H_2O_2 and analysed at regular intervals over period of 94 days. The telomere length analysed by Q-FISH (Quantitative-FISH), showed a telomere shortening at 9-21 days, and a telomere elongation between 21-48 days. Moreover we observed that this trend of "shortening-lengthening" was repeated on time up to 94 days.
Based on this repeated trend, we focused our attention to the first 45 days, because at longer time several mechanisms, as senescence, not straight related to oxidative stress effects, could interfere with the results. At this interval of time, we saw a significant telomere shortening after 5 days and a telomere elongation at 15 days of treatment. Based on these results, we confirm that prolonged oxidative stress is responsible of telomere shortening even at low daily doses.
Considering that telomere shortening could have an effect on cell viability, first of all, we analysed other endpoints:
a) Possible cell cycle perturbation by Cytofluorimetric analysis. We noticed any differences between treated and own control samples.
b) The entity of DNA damage induced by prolonged treatment with H_2O_2 by "Alkaline Comet Assay". Moreover we used Trypan Blue Assay to evaluate cell viability and this analysis has relieved in all cases that cell death was less than 30%, value above which the mortality is considered significant.

c) To understand if the DNA damage and telomere shortening observed could induce premature senescence, we assessed the number of β-galattosidase positive cells, typically expresses in senescence cells. As expected, we observed a light trend of senescence on time in treated and control samples, with a difference between the two only at 10-15 and 20-27 days. This led us to conclude that oxidative stress did not induce significant premature senescence in treated cells.

Considering that telomere shortening did not alter cell viability, we studied the mechanism responsible of telomere elongation observed at 15 days. To evaluate this mechanism the analysis of telomerase activity was performed by RTQ-TRAP assay that showed no telomerase activation for all fixation times. On the other hand, to verify ALT activation, we assessed two ALT markers: telomeric-sister chromatid exchanges (T-SCE) by CO-FISH analysis and colocalization of telomeres-PML proteins by FISH and immunostainig. We showed a higher frequency of both these ALT markers at 15 days of treatment, corresponding to the time of telomere elongation observed. This led us to hypothesize Human Primary Fibroblast could activate ALT mechanism, known in literature be present in a little percentage of tumor cells. However we cannot exclude the presence of a cellular selection system that promotes cells with longer telomeres due their major viability.

In literature is known that telomere shortening could alter chromosomes segregation by inducing chromosome bridges (end-to-end fusion). To analyse the relation between telomere length and chromosome segregation defects, like chromosome bridges, during my period at the Virginia Tech University we performed Time lapse on live cells and immunostainig (that detect Kinetochore and mitotic spindle) in mitotic cells in the range of 1-48 days. Data obtained were pulled and showed an increase of chromosome bridges in treated cells at 5, 27, 41 and 48 days of treatment. The induction of chromosome bridges at 5 days indicated a relation between telomere shortening and chromosome segregation defects. Moreover the reduction of chromosome bridges, observed at 15 days, was related to telomere lengthening saw at the same day.

Based on these results, we could observe that telomere shortening induced by oxidative stress at 5 days of treatment could triggers the increase of chromosome bridges induction. Subsequently, we observed a chromosome bridges decrease corresponding to the time of telomere elongation and based on previous results obtained, we hypothesized that ALT mechanism

restores telomere length inducing a reduction of chromosome segregation defects related to telomere shortening. In fact, according to literature data, the loss of chromosome ends could result in “sticky” chromosomes that will give rise to dicentric/ring chromosomes: when these dicentric/ring chromosomes will try to separate in anaphase, they will create a chromosome bridge that breaks before or in transition to telophase. Then, the breakage of bridge will create new sticky chromosome ends that could fuse with other sticky ends or that could determine the sister chromatid fusion at the following cell cycle, creating the so called “breakage/fusion/bridge cycle” (BFB). This BFB cycle will continue until the affected chromosome will acquire a new telomere, as in our case when ALT mechanism restores the telomere length.

To further assess the effects of telomere length changes on chromosome instability, we evaluated abnormal nuclear structures at 1-20 days. We estimated the quantity of NBPs (nucleoplasmic bridge) and NBuds (nuclear Buds), markers of chromosome segregation defects, measuring a significant increase of these biomarkers at 5 days of treatment and confirming previous result obtained for chromosome bridges.

These data have been supported by anaphase-lagging chromosomes, marker of chromosome loss. In both fixed- and live-cells. We observed an increase in anaphase lagging chromosomes frequencies over the control at 5, 20, 27 and 41 days of treatment.

Additionally, kinetochore positive micronuclei (MN) were analysed by immunostainig. Kinetochore positive MN represents a marker of chromosome loss and aneuploidy (Fenech, M. and Morley, A.A. 1985). Our analysis resulted in a significant increase of kinetochore positive MN in almost all-fixing time.

With the aim to evaluate if also other mitotic defects, in addition to chromosome segregation defects, were induced by prolonged oxidative stress, cells with tilted mitotic spindles, defined as the positioning of the spindle long axis at an angle instead of parallel to the substrate, were observed. We observed frequencies of tilted spindles above the control frequencies during the first 10 days, at 27, and 48 days of treatment. These results indicated that prolonged oxidative stress affected spindle structure and/or function.

Thus, in addition to the telomeric damage, which causes chromosome rearrangements and can result in chromosome bridges in mitosis, prolonged oxidative stress could also induce telomere-independent mitotic defects,

such as MN, lagging chromosomes, which can result in aneuploidy, and cells with tilted spindle. Taken together, these data indicated that prolonged oxidative stress could cause the two most common karyotype defects observed in cancer cells: chromosome rearrangements and aneuploidy.

I telomeri sono complessi nucleoproteici che proteggono le estremità dei cromosomi lineari e sono coinvolti in una grande molteplicità di processi: apoptosi, invecchiamento, cancro e instabilità cromosomica.
Comunemente, nelle cellule somatiche, i telomeri si accorciano a ogni divisione cellulare. Tuttavia le cellule tumorali, impediscono la perdita progressiva di DNA telomerico attivando la telomerasi o un meccanismo telomerasi-indipendente, definito ALT (allungamento alternativo del telomero), determinando un'illimitata di proliferazione cellulare.
È stato dimostrato che diversi agenti fisici e chimici possono modulare la lunghezza telomerica; in particolare, lo stress ossidativo è in grado di accelerare l'accorciamento telomerico. Dati pubblicati in precedenza hanno rivelato che i telomeri sono il bersaglio preferenziale dello stress ossidativo acuto, poiché quest'ultimo genera specie reattive dell'ossigeno (ROS) capaci di dare origine all'8-oxoG sulle triplette GGG presenti nella sequenza ripetuta del telomero (nell'uomo TTAGGG). Tuttavia, gli effetti dello stress ossidativo prolungato a livello telomerico sono ancora poco studiati.
Con l'obiettivo di indagare quali sono gli effetti dello stress ossidativo sulla lunghezza telomerica e sulla segregazione cromosomica, e testare se lo stress ossidativo induce difetti mitotici; fibroblasti primari umani (MRC-5) sono stati trattati quotidianamente con 10μM H_2O_2 e analizzati a intervalli regolari fino a 94 giorni.
L'analisi delle lunghezze telomeriche, eseguita con la Q-FISH (Quantitative-FISH), ha rilevato un accorciamento telomerico tra 9 e 21 giorni e un successivo allungamento telomerico nel periodo 21-48 giorni. Inoltre è stato osservato che tale "trend" di accorciamento-allungamento si ripeteva nel tempo fino a 94 giorni.
Tenendo conto della ripetizione nel tempo di tale "trend", la nostra attenzione è stata focalizzata nei primi 45 giorni, poiché a tempi più lunghi possono intervenire altri meccanismi confondenti, come la senescenza, non strettamente correlati agli effetti del trattamento prolungato con H_2O_2. In

questo intervallo più breve, è stato osservato un rilevante accorciamento telomerico a 5 giorni seguito dall' allungamento telomerico a 15 giorni di trattamento.
Sulla base di ciò, è stato confermato che anche lo stress ossidativo prolungato è in grado di indurre accorciamento telomerico anche a basse dosi quotidiane.
Considerando che l'accorciamento telomerico può influenzare la vitalità cellulare, per prima cosa sono stati valutati i seguenti end-points:
a) Possibili alterazioni del ciclo cellulare attraverso l'analisi cito-fluorimetrica e non è stata notata alcuna differenza tra i trattati e i rispettivi controlli.
b) Danno al DNA indotto dallo stress ossidativo prolungato con "l'Alkaline Comet Assay". Inoltre è stato utilizzato il Trypan Blue Assay per valutare la vitalità cellulare dei campioni, rivelando in tutti i casi mortalità cellulare inferiore al 30%, valore sopra la quale la mortalità è considerata significativa.
c) Per comprendere se il danno al DNA e l'accorciamento telomerico osservato potessero indurre senescenza prematura, è stato misurato il numero di cellule positive per la β-galattosidasi, tipicamente espressa in cellule senescenti. Come atteso, l'analisi ha rilevato un lieve "trend" di senescenza nel tempo sia nei trattati sia nei controlli, con una piccola differenza tra i due solo nei periodi 10-15 e 20-27 giorni. Questo ci ha permesso di ipotizzare che il trattamento non induce senescenza prematura nei trattati.
Costatato che l'accorciamento telomerico non alterava la capacità proliferativa delle cellule, abbiamo analizzato i campioni in cui avevamo misurato l'allungamento, telomerico per valutarne il meccanismo responsabile.
Per analizzare tale meccanismo è stata testata l'attività della telomerasi attraverso la RTQ-PCR, la quale non ha mostrato alcuna attività telomerasica in tutti i campioni fissati.
L'altro meccanismo responsabile dell'allungamento telomerico è l'ALT, e per verificarne l'attivazione, sono stati analizzati due "markers" di tale meccanismo alternativo: è stata eseguita un'analisi degli eventi di ricombinazione tra le sequenze telomeriche (T-SCE) utilizzando la CO-FISH, ed è stata valutata la co-localizzazione tra sequenze telomeriche e proteine del PML con FISH e immunofluorescenza. Entrambe le tecniche hanno evidenziato un incremento della frequenza di questi due marcatori

dell'ALT a 15 giorni, corrispondenti al tempo di allungamento telomerico osservato. Questo ci ha permesso di ipotizzare che in fibroblasti umani primari, può essere attivato il meccanismo ALT, noto in letteratura essere presente solo in una bassa percentuale di cellule tumorali. Tuttavia, non si esclude anche la presenza di un meccanismo cellulare di selezione, che promuova la sopravvivenza di cellule con telomeri più lunghi, per la loro maggiore capacità proliferativa.
In letteratura è noto che l'accorciamento telomerico può determinare alterazioni nel meccanismo di segregazione cromosomica attraverso l'induzione di ponti anafasici (end-to-end fusion). Per investigare la relazione tra lunghezza telomerica e difetti di segregazione cromosomica, come i ponti anafasici, è stata valutata la presenza di tali strutture fino a 48 giorni di trattamento. I dati ottenuti hanno mostrato un incremento nella frequenza di ponti anafasici a 5, 27, 41 e 48 giorni di trattamento. Quindi, è stato osservato che l'accorciamento telomerico indotto dallo stress ossidativo a 5 giorni di trattamento ha innescato un incremento dell'induzione di ponti anafasici. In seguito, è stata osservata una riduzione dei ponti anafasici in corrispondenza dell'allungamento telomerico, lasciandoci ipotizzare che il meccanismo ALT è responsabile del ripristino delle lunghezze telomeriche, e induce al contempo una riduzione dei difetti di segregazione correlati all'accorciamento del telomero. Infatti, in accordo con la letteratura, la perdita del telomero potrebbe generare estremità telomeriche appiccicose che diano origine a cromosomi dicentrici o ad anello. Quando questi cromosomi dicentrici/ad anello cercheranno di separarsi in anafase, creeranno nuovamente dei ponti anafasici che prima o in telofase si romperanno. La rottura del ponte creerà così nuovi cromosomi appiccicosi, che potranno fondere le estremità con altri cromosomi appiccicosi o determinare la fusione dei cromatidi fratelli al successivo ciclo cellulare, creando il cosiddetto ciclo di rottura/fusione/ponte. Tale ciclo continuerà finché i cromosomi affetti non acquisiranno nuovi telomeri e nel nostro caso, quando il meccanismo ALT riparerà la lunghezza telomerica.
Inoltre per valutare gli effetti dei cambiamenti nella lunghezza telomerica sull'instabilità cromosomica, è stata valutata la presenza di strutture nucleari anomale nel periodo 1-20 giorni. È stata stimata la quantità di ponti nucleoplasmatici (NPBs) e piccoli nuclei ancorati al citoplasma (NBuds), entrambi marcatori di segregazione cromosomica, misurando un

incremento rilevante di entrambi a 5 giorni di trattamento e confermando in tal modo i precedenti risultati ottenuti per i ponti anafasici.
Questi dati sono stati supportati dall'"l'anaphase lagging chromosome" e l'analisi ha rilevato un incremento di tali strutture a 5, 20, 27 e 41 giorni di trattamento. In aggiunta, sono stati analizzati i micronuclei con marcatura fluorescente del cinetocore, i quali sono un marker della perdita di cromosomi e quindi aneuploidia (Fenech, M. and Morley, A.A. 1985). Nei nostri esperimenti è stato evidenziato un incremento dell'induzione di micronuclei con cinetocore marcato in quasi tutti i tempi di fissaggio.
Con lo scopo di valutare se anche altri difetti mitotici, oltre a quelli di segregazione, fossero indotti dal trattamento prolungato con H_2O_2, cellule con fuso mitotico inclinato (tilted) sono state analizzate. Nelle cellule tilted l'asse di divisione cellulare descrive un angolo con il substrato, anziché essere parallelo a esso. Sono state osservate frequenze di cellule con fuso mitotico inclinato nei trattati superiori ai controlli nei primi 10 giorni e a 27 e 48 giorni di trattamento. Questi risultati hanno evidenziato che lo stress ossidativo altera la struttura/funzione del fuso mitotico.
Quindi in aggiunta al danno al telomero, che causa riarrangiamenti cromosomici e può risultare in ponti anafasici in mitosi, lo stresso ossidativo prolungato induce anche difetti mitotici telomero indipendenti, come MN, "lagging chromosomes", che possono risultare in aneuploidia, e cellule tilted. Complessivamente questi dati indicano che lo stress ossidativo prolungato può causare i due più comuni difetti del fenotipo osservati in cellule cancerogene: riarrangiamenti cromosomici e aneuploidia.

INTRODUCTION

1. Telomere

Understanding the physiological mechanisms that control life cell is of central importance to deduce what happens to body. One of cellular mechanisms particular significant is the maintaining of genome integrity, constantly threat by the tendency of DNA to engage in chemical reactions in its cellular environment. [1]. Nevertheless exist also other physiological mechanisms that damage DNA like the attrition of telomere.
Telomeres are highly conserved, noncoding, repetitive sequences of DNA that, together with a number of shelterin proteins (fig. 1), form caps at the ends of eukaryotic chromosomes [3] (fig. 2).

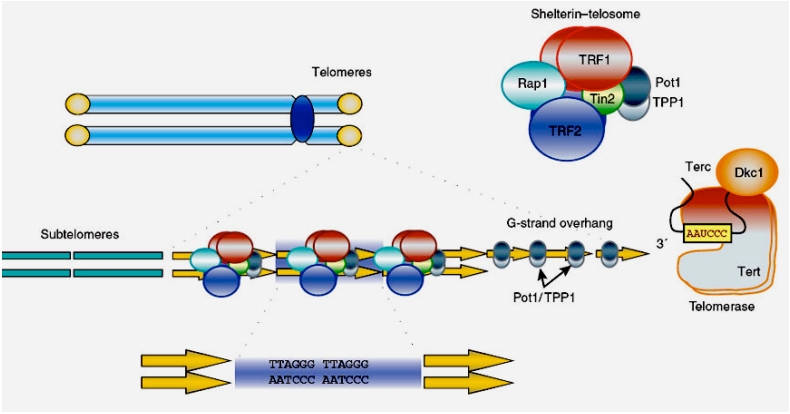


Fig. 1 Mammalian telomeres consist of tandem repeats of the TTAGGG sequence that are bound by the shelterin protein complex. Adjacent to telomeres are the subtelomeric regions, which are also rich in repetitive DNA [2].

Telomeres have been defined the first time in the 1930s as essential components that stabilize chromosome ends. In 1938 Muller found that he could not recover terminal deletions in the fruit fly *Drosophila Melanogaster* chromosome ends. So, he suggested that chromosome ends are specialized structures and coined the term telomeres, that means, from Gieek, telo = end and mere = part [4]. In the same time Barbara McClintock related phenotypic changes observed in maize plants with cytogenetic alterations in chromosomes and resolved that a natural chromosome must differ from broken chromosomes in possessing a structure that provides stability to the end [5-8]. Thus, telomeres had the function to distinguish natural chromosomes ands from DNA breaks.
Later, many studies have confirmed telomere importance for chromosome stability and have increased the knowledge of their molecular components [9, 10] and after thirty years (1962) James Watson have won the Nobel Prize for the DNA structure enlightening other telomere functions. Parallel he has discovered that DNA polymerases couldn't start DNA synthesis de novo (they need a primer, which is typically made of RNA) and synthesizes DNA only in the 5′ to 3′ direction. From these facts, Watson has reasoned that the complete replication of the ends of linear genomes presents a problem that is not realized by circular DNA molecules and have suggested that special structures at DNA ends might promote their replication by a nonstandard mechanism [11]. Elizabeth Blackburn and Carol Greider have discovered this, "nonstandard mechanism", in 1984. They, together with Jack Szostak, have been awarded the Physiology or Medicine Nobel Prize (2009) for discovering (1) the fundamental processes whereby chromosome ends are protected by telomeres and (2) the enzyme telomerase [12]. These findings further have corroborated the idea that telomeres are important for the stability of the genome and that their study would be of great significance in the context of human health and disease.

1.1 Structure

Telomeres are specialized structures at the ends of eukaryotic linear chromosomes, consisting of protein-bound tandemly repeated simple DNA sequences [13]. In eukaryotes telomeric DNA consist of tandem repeat of single non coding sequence and the number of these repeats varies among chromosomes and individual of the same species, usually within a species-

specific range [12], in addition to variances existing among different species.
The telomeric sequence is usually rich in guanine and is a repeat of six bases. The sequence is TTAGGG/CCCTAA in all vertebrates underlining that is highly conserved to protect genome. The orientation of telomere sequences is also conserved; in fact the G-rich strand runs 5' to 3' toward the end of the chromosome and thus makes up the molecular 3' end of the chromosome [14].
Telomeric DNA end is a single stranded "overhang" of the TTAGGG sequence and the length of this last vary from 50-300 nucleotides and it folds back on itself to form a "D-loop"(DNA displacement loop), or better defined "T-loop" because is a telomere loop; so a minimal number of repetition is necessary to occur this structure, even is it is not yet known (Fig.2).

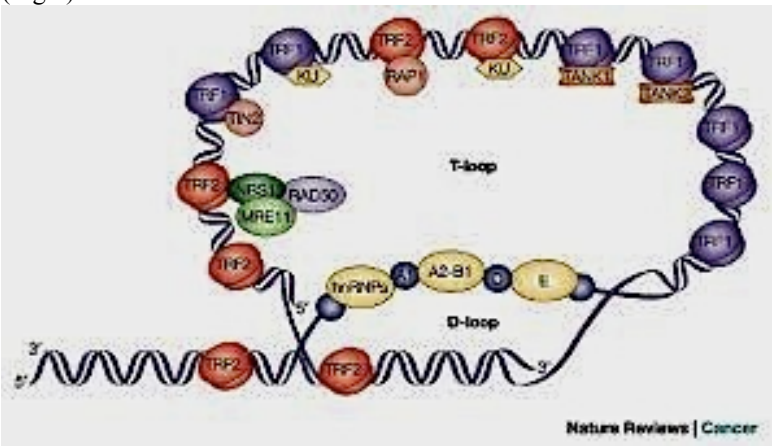


Fig. 2. Schematic representation of putative telomere T-loop structure (capping) with telomere-specific binding proteins. The single-stranded DNA at the end of the telomere is able to invade and anneal with part of the duplex DNA (thereby forming a displacement (D)-loop) in the same telomere, with the overall result being a telomere (T)-loop. Several proteins bind specifically to telomeric DNA, and these recruit other proteins to the chromosome end [15].
Telomere lengths are 0.5-15 kilobase pairs (Kb) in the human, whereas in the others model organisms the mean telomeric repeat length can be 36bp,

like in some ciliates, or 150kbp, like in mice [16]. Only in the birds there are some case in which telomere length reaches hundreds of kilobases [12]. Moreover the human telomeric sequences are adjoined by a subtelomeric sequence consisting of degenerated telomeric DNA sequences and unique repeats [17].
All chromosomes loss a small amount of telomeric DNA every time a somatic cell divides because of the noted "end replication problem" (Fig. 3).

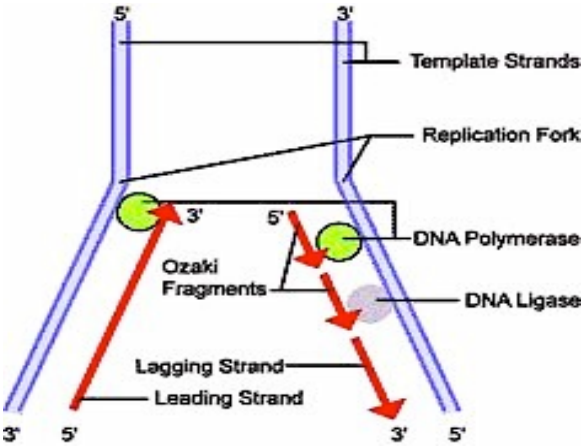


Fig. 3. This figure provides an overview of the DNA replication process. In the first step, a portion of the double helix (*blue*) is unwound by a helicase. Next, a molecule of DNA polymerase (*green*) binds to one strand of the DNA. It moves along the strand, using it as a template for assembling a leading strand (*red*) of nucleotides and reforming a double helix. Because DNA synthesis can only occur 5' to 3', a second DNA polymerase molecule (also *green*) is used to bind to the other template strand as the double helix opens. This molecule must synthesize discontinuous segments of polynucleotides (called *Okazaki Fragments*). Another enzyme, *DNA Ligase* (*yellow*), then stitches these together into the lagging strand (NCBI Home http://www.ncbi.nlm.nih.gov/About/primer/genetics_cell.html).

In fact every time a cell starts a replication cycle, it duplicates its DNA, and DNA polymerase replicates in 5' to 3' direction by extending

polynucleotides chains. The mechanism of DNA replication differs for the leading and the lagging DNA strands. The leading strand is replicated continually. To replicate the lagging strand, DNA polymerization starts from several RNA primers, which are elongated to create DNA fragments termed Okazaki fragments. These RNA primers are finally degraded and replaced by DNA sequences. Removal of the terminal RNA primer on the lagging strand leaves a gap that ordinarily is filled in by extension of the next Okazaki fragment. Because there is no template for the "last" Okazaki fragment beyond the 5' end of the chromosome, one strand cannot be synthesized to its very end. This so-called "end replication problem" predicts the progressive reduction of chromosomal DNA at the 3' ends during multiple cell cycles [18]. It is estimated that telomere loss consists of 100-200 bp per division in most human cells [19].

1.2 Telomere proteins

Telomere length regulation and structure (T-loop) are mediated at least in part by telomere binding proteins (TBPs) (Fig. 4). These proteins can be divided in two classes: those that bind the single stranded repeats at the extreme termini, and those that bind along the length of double-stranded telomere repeats [14].
Moreover some of these proteins are exclusively telomere-specific, while others are involved in double-strand breaks DNA repair pathways [20].
In mammals the T-loop is held together by seven known proteins, the most notable ones being TRF1, TRF2, POT1, TIN1, and TIN2, collectively referred to the Shelterin complex. The Shelterin complex consists of six proteins: TRF1 (Telomeric Repeat binding Factor 1), TRF2, RAP1 (Repressor/Activator Protein 1), TIN2 (TRF1 Interacting protein 1), TPP1 (TINT1/PIP1/PTOP 1) and POT 1 (Protection Of Telomeres 1). TRF1 [22], TRF2 [23] bind the double stranded repetitive telomeric DNA while POT1 [24] attaches to the 3' single strand overhang. These proteins recruit other proteins: TRF1 recruits TRF2; on the other hand TRF2 recruits RAP1 and its modulator TIN2 that links TPP1 to create an end-capping complex that protects telomere structure and regulates telomere length [2, 25, 26].
TRF1 and TRF2 are constitutively present to telomere and the quantity of these loaded to telomere is important for telomere length regulation. TRF1, in fact, is noted being a negative modulator of telomere length blocking

telomerase access to chromosome termini and its overexpression accelerate telomere shortening, as reported in literature [26].

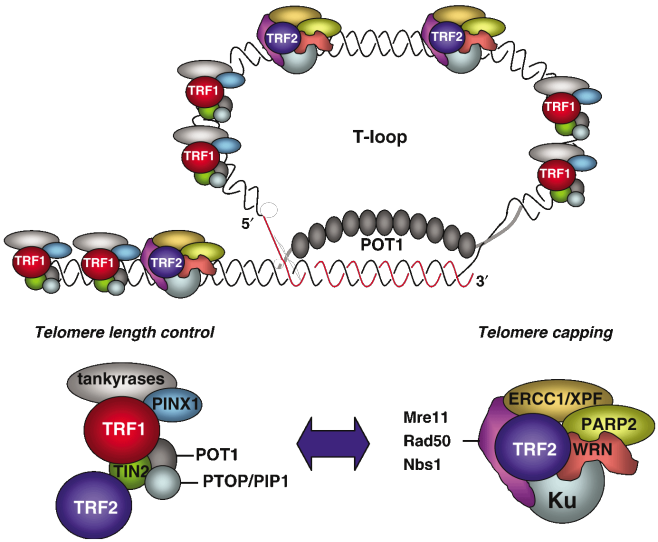


Fig. 4. Telomere-binding proteins. Scheme showing the telomere in a T-loop conformation, as well as with different protein complexes found at mammalian telomeres, some that regulate telomere length and other are involved in DNA repair pathways. The TRF1 complex has been shown to influence telomere length, while the TRF2 complex has been shown to influence both telomere length and telomere capping [21].

Telomere shortening is also reported by overexpression of TRF2 [27]. However, TRF1 and TRF2 have an essential role in telomere end protection and t-loop formation [28, 29] protecting telomere in every phase of cell cycle [30] and safeguard chromosomes from being recognized as double-strand breaks [31], inhibiting chromosome end-to-end fusion through a large duplex loop that protects telomeres shortened preserving the complete loss of telomeric DNA [28, 29].
Consistent with this role of TRF2, it forms a complex with RAP1, which protects telomeres from non-homologous end joining [32] and regulate

telomere length. Besides, RAP1 also seems playing a role in telomere length regulation; in fact its inhibition with RNA interference results in elongated telomeres [32]. POT1 also contributes to telomere protection by binding to the overhang, and its high specificity for single stranded telomeric DNA leaves the possibility open that it might bind to the displaced G-strand in the T-Loop and "lock-in" the closed configuration of this structure [33].
These observations suggest that RAP1, TIN2, POT1, and PTOP may function in the same pathway and are related to TRF1 and TRF2.
Nevertheless, other important proteins have been shown to be involved in DNA repair and replication and are believed to contribute to telomere length regulation.

1.3 Telomerase

Normally cells loss 50-100bp/cell divisions during which telomeres are slowly lost and when they become critically short most of cells die [34].
In 1980s, the discovery of a mechanism that could elongate specifically telomeres has represented the beginning to investigate how the cellular lifespan could be changed. Carol Greider and Elizabeth Blackburn, using Tetrahymena cells, observed this mechanism, the fist time in 1985 [35] and discovered that telomere elongation was performed by a ribonucleoprotein reverse transcriptase that consists of an enzymatic part (transcriptase, TERT) and a RNA component (TR). This last functions like a template for the de novo synthesis of telomeric DNA sequences (Fig. 5).
Moreover they shown that this activity was RNA dependent "in *vitro*".
Later, the Blackburn's laboratory mutated the putative template region of the *Tetrahymena* telomerase RNA and reintroduced it into cells, demonstrating that mutant repeats were incorporated into telomeric DNA. This result provided definitive proof that telomerase uses its integral RNA component as the template for making telomeric DNA [36].
Then, many laboratories focused their attention on telomerase and its role in telomere length regulation. In addition, using different ways, they found that telomerase knockout mice shown telomere shorted [37].
The human RNA component (hTR) is 445 nucleotides long with an 11 nucleotide putative template sequence (5'-CUAACCCUAAC-3') coding for the telomere repeat (TTAGGG)n [38].

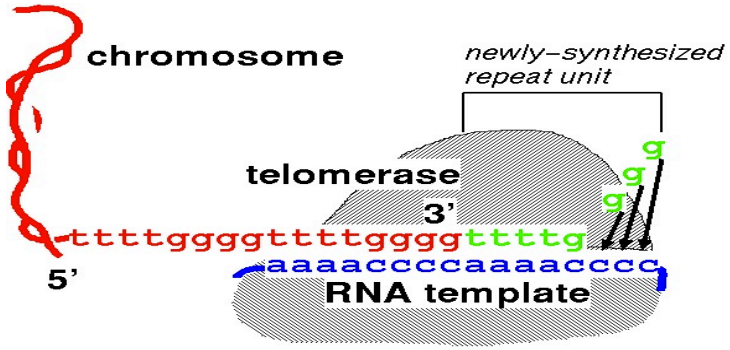


Fig. 5. Human telomerase is a cellular reverse transcriptase, composed of two essential components: telomerase reverse transcriptase catalytic subunit (hTERT) and functional telomerase RNA (hTR), which serves as a template for the addition of telomeric repeats.

hTERT consists of four protein domains, a conserved N-terminal domain (TEN), an RNA-binding domain (TRBD), a reverse transcriptase (RT) and a C-terminal extension (CTE) [39].
Telomerase links only to 3' overhang and adds several repeated DNA sequences, then releases and a second enzyme, DNA polymerase, attaches the opposite or complementary strand of DNA completing the double stranded extension of the chromosome ends.
However, telomerase activity varies across taxa [11] and differs in mortal and immortal cells [40]. It is repressed in the majority of normal somatic cells (with the exception of a transient S phase activity thought to maintain the single-stranded overhang [41]), while its activity is higher in immortal cell lines, germline cells, stem cells, activated lymphocytes, and most of the tumour cells analysed [42-47]. Telomerase is also variably present in cells of the immune system [48] to maintain stable their telomere length.
Is noted that loss of telomerase enzymatic function leads to progressive telomere shortening over time [38], eventually resulting in the disappearance of detectable telomeric DNA and the formation of end-to-end chromosome fusions, followed by growth arrest or cell death [49, 50]. Moreover inhibition of telomerase in human cancer cells by both genetic

[51, 52] and pharmacologic means [53, 54] leads to telomere shortening and cell death through apoptosis. Similarly, genetic deletion of telomerase in certain tumour susceptible murine strains, suppresses tumour initiation [55, 56]. These observations, in aggregate, support the view that telomerase participates in tumor transformation by facilitating cell immortalization.
Barbara McClintock in 1941 to describe the phenomenon that halted the chromosome instability in the embryo of plants [5], coined term "chromosome healing". This mechanism is based on capability of telomerase to add telomeric sequences directly on to non-telomeric DNA [57] to the ends of broken chromosomes. This phenomenon has been also observed in protozoans, yeast, plants, insects, and mammals [58-64]. Nevertheless this telomerase function could lead to chromosomal fragmentation and karyotype instability, because chromosome healing prevents repair of broken ends. Therefore, telomerase must be prevented from accessing internal DSBs. Data literature show that Ku, a DSB protein which has a high affinity for DNA ends, acts to prevent telomerase from accessing internal DSBs [65]. This model is supported by the fact that the efficiency of chromosome healing is extremely low, about 1% [66]. This can prevents increase of the occurrence of chromosomal instability and then cancer.

1.4 Alternative Telomere Lengthening (ALT)

More than 85-90% of all human cancers express telomerase activity [43]. However, 10-15% of mammalian cells without any telomerase activity are able to maintain the length of their telomeres for many population doublings (PDs) [67-70] indicating the presence of one or more mechanisms telomerase independent, that have been termed *Alternative Telomere Lengthening* (ALT) [71, 72].
Currently the molecular details of the ALT pathway have not been elucidated; however, this pathway is likely to involve homologous recombination mechanism [67, 73, 74] (Fig. 6).
ALT cells are characterized by presence of ALT-associated PML bodies (APBs), which contain the Promyelocytic leukemia bodies (PNBs), TRF1, TRF2 and different recombination proteins, including RAD51 and the Warner and Bloom syndrome proteins [75-77] all associated to telomeric DNA.

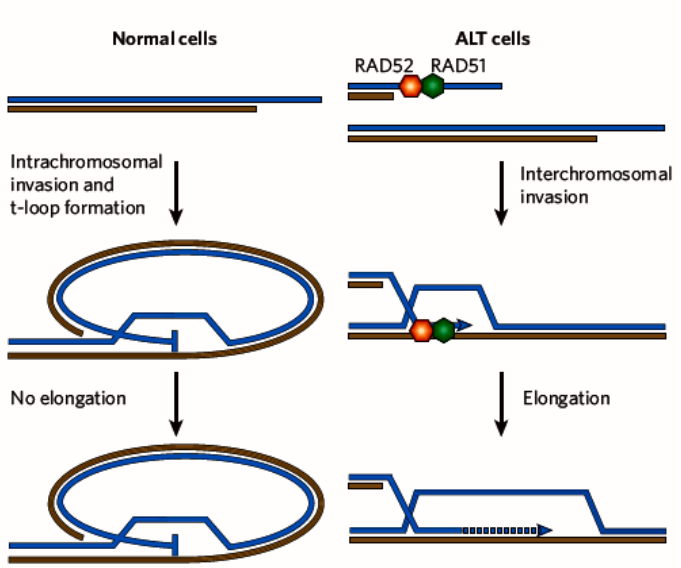


Fig. 6. ALT cells is based on a recombination between telomeres sister chromatid to restore telomere length [74].

Other experiments have shown that ALT mechanism is either suppressed or less efficient than telomerase in somatic cell hybrids create between ALT cell lines and telomerase-positive immortal cell lines or primary mortal cell lines [78, 79].
In ALT cells the global homologous recombination rates do not appear to change; at contrary ALT cells undergo post-replicative telomeric exchanges (T-SCEs: Telomere Sister Chromatid Exchanges) with higher frequency than telomerase-positive cells without increase of G-SCEs (genomic SCEs) at interstitial sites [80, 81]. Unequal T-SCEs might be responsible for the rapid lengthening and shortening of telomeres observed in ALT cells.
In mortal cells, APBs have not been reported; however, literature data have reported smaller co-localized PML and telomeric DNA signals in endothelial cells adjacent to the tumour [82]. The smaller size of the co-

localized signals in normal compared with tumour cells could explain why APBs have not previously been reported in non-neoplastic cells and in the same time it suggest that ALT mechanism could occur in normal cells, but without the generation of abundant heterogeneous telomere lengths.
Moreover, it is reported small co-localized signals of PML and telomere DNA are not only in a peritumoural tissue but also in a variety of adult and neonatal tissues [83] and there are evidences of telomere lengthening at different times after exposure of human primary fibroblasts to different types of radiation (i.e., low- and high- LET) [84] in which ALT represent the only mechanism involved [85]. These data underline the importance of ALT mechanism to modulate telomere length after DNA damage giving the cells the power to proliferate indefinitely and to increase cell file that is typical of tumour, but in the same time it can increase cell lifespan.

2. Telomere Functions

2.1 Senescence and aging

Senescence is defined as irreversible growth arrest due to reduced number of cellular divisions. Hayflick was the first to describe this limitative replicative potential [86]; later others hypothesized that this mechanism was genetically defined [19] and proposed that telomere shortening was correlated to cellular senescence [87, 88]. In fact, because of bidirectional DNA replication and DNA polymerases are unidirectional, 50-200 bp of 3' telomeric DNA aren't replicated at the end of each S phase, resulting in telomere shortening to each cell division. So, when telomeres reach a critical length, the cells arrest proliferation and acquire enlarged morphology expressing senescence-associated gene like the β-galattosidase and p16 [89]. This response has been called "replicative senescence" [90] and attribute to telomere function the role of "molecular clock" [91] that measure cell proliferative history. Many works report that some telomerase negative cells can be immortalized by introduction of hTERT, while someone require also inactivation of p16, calling in question telomere role in replicative senescence [92-94].
Cells that undergo replicative senescence fail to divide further but remain viable and are metabolically active displaying an array of activities that have profound consequences for the tissue microenvironment and the entire

organism [95]. In fact, cellular senescence causes an arrest growth in G1 with a repression of genes required for cell cycle progression and upregulation of growth inhibitory genes like *p21* and *p16*. In other words, p21 and p16 and other proteins inhibit several CDK (cyclin dependent kinases) that control cell division and CDK targets like Rb (retinoblastoma proteins) that allows indirectly transcription of genes required to entry in phase S and DNA synthesis [96, 97].
If cell cycle regulators are mutated or blocked, the cell continues to divide and shorts its telomeres up to a second block that represents a *crisis state* characterized by genomic instability and massive cell death [98].
Aging can be defined as the progressive functional decline of tissue function that eventually results in mortality [99].
The link between replicative senescence related to telomere and aging is unclear.
It remains unclear whether there is a single or multiple types of senescence and the precise role of telomere length in triggering senescence is also poorly resolved.

2.2 Cancer

It is known for more than a century that malignant tumours often display a wide variety of cell division anomalies. In 1891, the German pathologist David Hansemann published a systematic study of abnormal mitotic figures in tumours [99]. The theory of carcinogenesis suggests that an unlimited cell proliferation is necessary for envelopment of malignant disease, and cancer cells must reach immortality for progression to malignant states. It has been hypothesized that telomere shortening plays a central role to limit cell lifespan and avoid its malignant transformation [100]. Therefore the enzyme telomerase may be essential for cell unlimited proliferation. However, telomerase is absent in most human somatic cells with the exception of some tissues in which has been found a low quantity [101, 102], while this enzyme is detected in almost 85% of tumour [103-107].
In the 15% of tumour is activated an alternative mechanism of telomere lengthening (ALT) [108].
In this way, telomere length acquires much value contributing to cell proliferation and transformation. In fact the length of telomeres in cancer cells depends on a balance between the telomere shortening at each cell

cycle and the telomere elongation resulting from telomerase activity or ALT mechanism [108-110] and inducing indefinite cell proliferation and tumour progression.
Telomere shortening progresses with each cell cycle due to the "end replication problem" [see par. 1.1] up to reach a critically telomere length that induce cell cycle arrest and activation of senescence profile that contribute to tumour suppressor mechanism. Though, sometimes cells lose the ability to senesce because, for example, of mutation in p53 protein, responsible of cell predetermined death.
Despite many factors have been involved in cell transformation, up to today telomerase activity seems be the principal responsible of illimitate proliferation of tumour cells and recent works focus their attention to crate an anti-cancer therapy that inhibit telomerase activity [111].

2.3 Apoptosis

Cell life is based on a balance between mechanisms that promote indefinite proliferation and others that induce senescence and apoptosis.
Apoptosis is the process of programmed cell death characterized by cell morphological changes (blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation).
Recently, the linkage between telomere dysfunction and apoptosis has been recorded by TIFs (Telomere Dysfunction-Induced Foci) that represent signals of proteins and chromatin changes on telomeres due to apoptosis or senescence induction [19].
Telomere length variation and telomere protein mutations assume an important role in cell viability. This theory is supported by literature data that show an increase of apoptosis in highly proliferative cells of testis and of intestine of mice that have altered expression of telomeric proteins such as POT1 [112] and TRF2 [113, 114].
Telomerase reduces apoptosis due DNA damage prompting initiation of DNA repair mechanisms before a significant telomere shortening could take place [115], but in the same time its activation is a crucial event during cell transformation [116].

2.4 Chromosome instability (CIN)

Genomic instability it is well know to play an important role in cancer initiation by increasing cellular changes accumulation responsible of cancer cell evolution [117].
CIN can occur through a variety of mechanisms that include defects in DNA replication, defects in chromosome segregation and defective response to DNA damage [118] (Fig. 7).

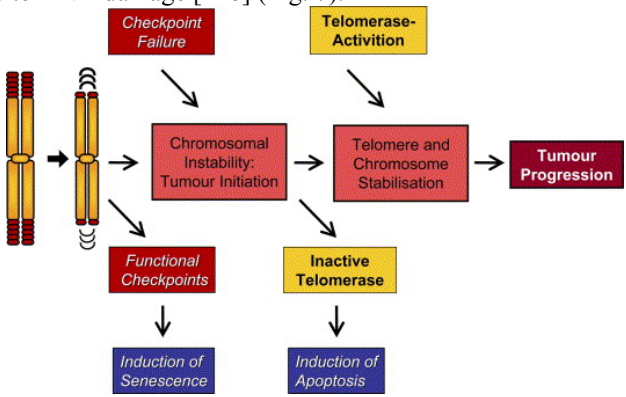


Fig. 7. Telomere shortening and telomerase activation have a dual role in cancer. Telomere shortening during ageing and chronic disease lead to telomere dysfunction and induction of senescence checkpoints. Checkpoint failure co-operates with telomere dysfunction to induce chromosomal instability and cancer initiation. Initiated cancer cells have to stabilize telomeres and on-going instability in order to survive and progress. Most human cancers achieve telomere stabilization by activating telomerase [119].
One mechanism for chromosome instability in cancer is thought being the loss of telomere [59, 120]
Nevertheless to understand the link between telomere attrition and CIN in necessary know how telomere protect chromosomes under physiological conditions, when Shelterin complex suppresses the action of non-homologous end joining (NHEJ) on free DNA ends towards a telomere capping structure [121]. Telomeres serve multiple functions in preserving chromosome stability; including protecting the ends of chromosomes from degradation and preventing chromosomal end fusion [117] (Fig 8).

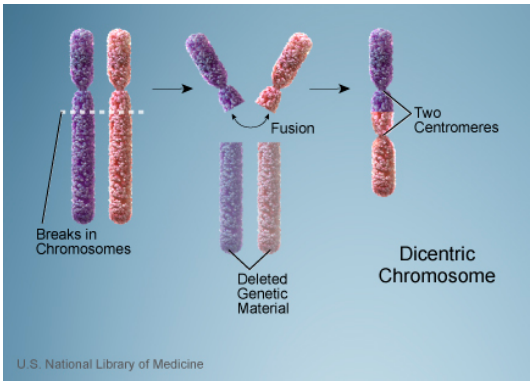


Fig. 8. Image of chromosome break and subsequent fusion between sticky ends generated. The fusion determine dicentric chromosome induction [122].

It is of interest to note that a 'free end' remains following a telomere–DSB fusion, thus providing a means of generating on-going instability. Then, telomere fusions could contribute significantly to the background level of chromosomal aberrations [117]. In fact, chromosome ends breaks can induce breakage-fusion-bridge (BFB) cycle, which was first described in maize by McClintock [7], and lead to genome remodelling [6], changing copy number of chromosome segments and also gene dosage.
Summarizing, telomere shortening and telomerase activation have a dual role in cancer initiation. Telomere shortening during ageing and chronic disease lead to telomere dysfunction and induction of senescence checkpoints. Checkpoint failure co-operates with telomere dysfunction to induce chromosomal instability and cancer initiation. Then, initiated cancer cells have to stabilize telomeres and on-going instability in order to survive and progress and as previously mentioned most human cancers achieve telomere stabilization by activating telomerase.

3. Chromosome instability

3.1 SSB and DSB

To protect its function, the structure at telomere is such that its DNA is not recognised as a double-strand breaks (DSBs).
DNA sequence is involved in a great variety of damages, all caused by three main factors: first, environmental agents such as the ultraviolet (UV) component of sunlight, ionizing radiation and numerous genotoxic chemicals cause alterations in DNA structure, which, if left unrepaired, may lead to mutations and enhancing cancer risk. Second, (by) products of normal cellular metabolism those constitute a permanent enemy to DNA integrity from within. These include reactive oxygen species (superoxide anions, hydroxyl radicals and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation. In this case evolution has invested significantly in reducing the price of its own metabolism by implementing an intricate antioxidant defence system composed of enzymatic (superoxide dismutase, catalase, glutathione peroxidase and peroxyredoxins) and low- molecular-mass scavengers (such as glutathione) [123]. Finally, some chemical bonds in DNA tend to spontaneously disintegrate under physiological conditions. Hydrolysis of nucleotide residues leaves non-instructive abasic sites [124].
These and many other forms of DNA base damage arise in the cell at least 10,000 times every days and only the action of specialized DNA repair systems can prevent the changing or loss of genetic information [1].
These lesions can occur in different moments of cell cycle, involving different repair pathways that depend by DNA damage type. At moment four main pathways are noted to operate in mammals: nucleotide-excision repair (NER), base-excision repair (BER), homologous recombination (HR) and non-homologous recombination end joining (NHEJ) [125, 126].
NER deals with the wide class of helix-distorting lesions that interfere with base repairing, obstructing transcription and replication and is involved to resolve exogenous sources lesions, except for some oxidative lesions in witch BER is involved together to resolution of damage of endogenous origin. However both these two repair processes affect only SSBs and simple base modifications that arise spontaneously and operate in a “cut and

patch" way that cut the injury and refill the single stranded gap using the intact complementary strand as template [127].
When not corrected, these types of lesions can induce disease. Many work report in fact a straight relation between NER defects and Xeroderma Pigmentosum, Cockayne syndrome and Trichothiodystrophy (TTD) all characterized by sun sensibility [127, 128].
At the contrary, more complex lesions like DSBs, which arise from ionizing radiation (for example X-rays), free radicals, chemicals and during replication of a SSB, can arise when both DNA strands are broken or mutated. In this case HR and NHEJ occur to resolve this DNA damage. HR seems to dominate in S and G2 when DNA is replicated and provide a new copy of the sequence damaged (sister chromatid) for aligning the breaks; while NHEJ works in the G1 phase and not use a template to repair DSBs [129].
Many study speculate that arrest in G1 prevent aberrant replication of damaged DNA and arrest in G2 allows cells to avoid segregation of defective chromosomes [130]. So, when the DNA damage is recognized the cell cycle is arrested by checkpoints system and resolved. Nevertheless if these checkpoints are compromised cell cycle continues despite DNA damage leading genomic instability. In fact DNA double-strand breaks (DSB) are considered to be critical primary lesions in the formation of chromosomal aberrations.

3.2 Chromosome segregation

The ultimate aim of the mitotic cell cycle is to produce two genetically identical cells from the parent one. The mother cell must replicate its chromosomes only one time before entering mitosis and each daughter cell must receive only one copy of each chromosome. So, to guarantee this, the cell has enveloped the spindle mitotic checkpoint [131].
Two checkpoints have been noted during mitosis, one in the G2-M phase and another in metaphase. The first monitors all microtubule events upon mitotic entry to permit the correct interaction between microtubules and each chromosome and the right distribution of all chromosomes into daughter cells. The cells in fact cannot enter in anaphase before to complete their congression to the metaphase plate in an amphitelic attachment, in which every sister kinetochore of a chromosome in associated to

microtubules of opposite pole. If it doesn't happen, we can have a monotelic (one kinetochore bound to microtubules and one not) or syntelic (both kinetochore linked to microtubules of the same pole) attachment. In this case the cell activates spindle checkpoint but in the same time chromosomes can progress through mitosis without their congression. So, the chromosomes could not separate correctly during anaphase producing trisomic and monosomic daughter cells [132, 133].
The second checks the attachment of the mitotic spindle to kinetochores, a multiprotein complex that assemble on each chromatid and is involved in their segregation during anaphase.
However, the cell has also DNA damage checkpoint and DNA replication checkpoints and their inactivation can cause genomic instability as previously mentioned [132].
Also telomere was shown be involved in the correct chromosome segregation; the loss of chromosome ends resulted in "sticky" ands that could give rise to dicentric chromosomes of ring chromosomes [5]. When these dicentric/ring chromosomes try to separate in anaphase, they create a chromosome bridge that breaks before or in transition to telophase [134]. However, the breakage of bridge created new chromosome ends sticky that could fuse with either sticky ends or with sister chromatid at the following cell cycle, creating a breakage/fusion/bridge cycle (BFB) (Fig. 9).
This BFB cycle will continue until the affected chromosome acquires a new telomere often with translocation of the ends of other chromosomes propagating the instability from one chromosome to another [135, 136] and increasing chromosome rearrangements that can induce genomic instability and cancer. Interesting, chromosome bridges were observed in cancer cell from many different organs, like ovary [137], colon [138], urethra [139], oral mucosa [140, 141], Wilm's tumour in kidney [142] and brain [143]. Moreover it has been observed that Chromosome Bridge can induce failure of cytokinesis [144]. This results in formation of tetraploid cell that will give rise aneuploid daughter cells [145] common in cancer [146].
So telomere integrity seems be important to chromosome segregation and stability, and cell cycle checkpoints are required to avoid telomere dysfunction and trigger carcinogenesis.

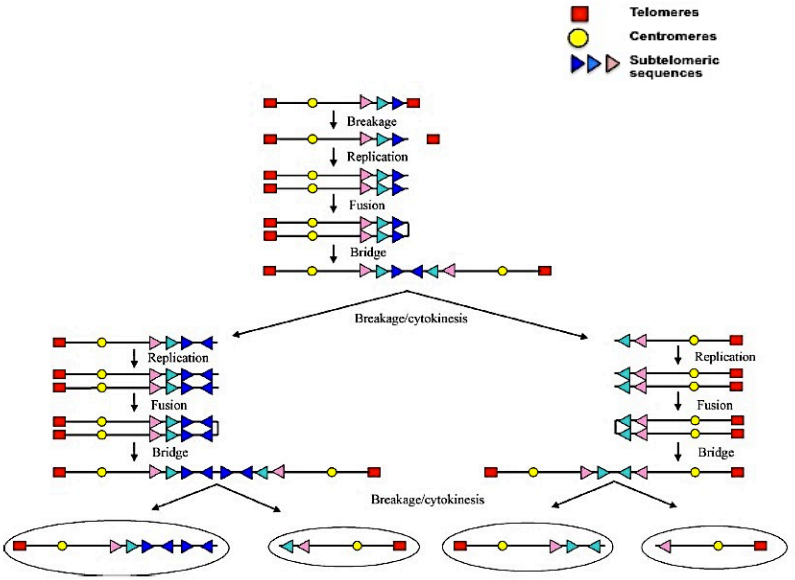


Fig. 9. B/F/B cycles, as a mechanism for chromosome instability, resulting from telomere loss. B/F/B cycles are initiated when sister chromatids fuse following the loss of a telomere. Owing to the presence of two centromeres, the fused sister chromatids break when the cell attempts to divide up its sister chromatids at anaphase. Because the break does not occur exactly at the site of the fusion, one daughter cell will receive a copy of the chromosome with an inverted repeat at its end, while the other daughter cell will have a copy of the chromosome with a terminal deletion. Owing to the lack of a telomere, these chromosomes will again undergo sister chromatid fusion after DNA replication, resulting in additional amplification and terminal deletions. The location of telomeres (squares), centromeres (circles) and orientation of the subtelomeric sequences (horizontal arrows) are shown [117].

3.3 NPBs, NBuds and MN

In the last 20 years, analysis of some cytogenetic biomarkers in the study of molecular epidemiology and cytogenetic has gained more and more to

valuate chromosome breakage, DNA misrepair, chromosome loss, non-disjunction, necrosis and apoptosis with cytokinesis-block micronucleus assay (CBMN) [147].
This method is based on the measure of some cytogenetic biomarkers specifically restricted on binucleated cells obtained by blocking of cytokinesis with Cytocalasin B.
CBMN is used to measure micronuclei (MN), nucleoplasmic bridges (NPBs) and nuclear buds (NBuds), cytogenetic biomarkers that characterize genomic instability [148].
MN were identified and described the first time in erythrocytes associated with deficiencies in vitamins [149]. Than they became an important biomarker to valuate genotoxic effects and chromosome instability induced by exogenous and endogenous factors [150].
MN originate from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division [151, 152] (Fig. 10); they are the result of chromosome breakage due to unrepaired or mis-repaired DNA lesions, or chromosome mal-segregation due to mitotic malfunction.
Nevertheless, based on analysis of fixed samples, it has been proposed many variety of mechanisms to originate MN [153]. One of these speculate MN origins by lagging chromosomes during anaphase or telophase of bipolar and multipolar divisions, when a few chromosomes move slowly than others or remain at the metaphase plate during anaphase, like observed in cancer cell and in human primary fibroblast (MRC-5) [154, 155].

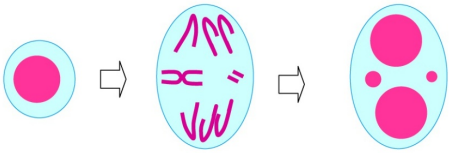


Fig. 10. A model for the formation of a micronucleus by a chromosome fragment or a whole chromosome [152].

On the other hand, MN could originate from broken chromosome bridge [156, 157]. Nevertheless, numerous studies have indicated an increase of MN formation associated also with abnormalities in microtubule assembly and disassembling, kinetochore structure and function, attachment of

microtubules to kinetochore, and other cytoskeletal abnormalities [156, 158, 159].
Other abnormal nuclear structures, recently considered biomarkers of genomic instability, are NBuds and NPBs, as previously mentioned.
NBuds are morphologically similar to MN, even if they are joined to the cell nucleus by a thin nucleoplasmic connection depending on the stage of the budding process [151] and can break to originate a MN.
However, literature data suggests a different mechanistic origin of NBuds and MN, where the first contain more interstitial DNA and the second centromeric and telomeric DNA [160].
Moreover Shimizu et al. [160], by *in vitro* experiments with mammalian cells, showed that amplified DNA is selectively localized to specific sites at the periphery of the nucleus and eliminated via nuclear budding to form MN during S phase of mitosis (Fig. 11).
Amplified DNA may be eliminated through recombination between homologous regions within amplified sequences forming mini-circles of acentric and not telomeric DNA (double minutes), which localize to distinct regions within the nucleus, or through the excision of amplified sequences after segregation to distinct regions of the nucleus [162, 163].

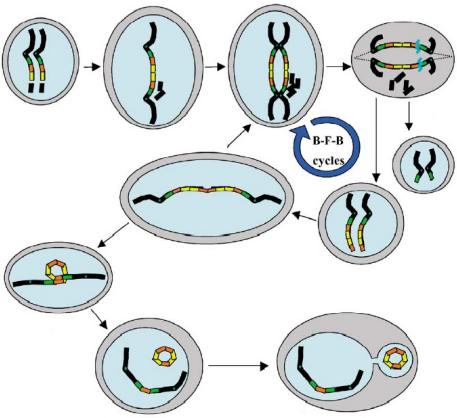


Fig. 11. Representative scheme of NBuds generation. Extrusion of amplified DNA throughout recombination between homologue sequences to create *double minute*. The double minute is joined to the cell nucleus by a thin nucleoplasmic connection. Modified by Fenech and Crott. 2002 [161].

The duration of nuclear budding process and its extrusion like MN remain still unknown.
At the contrary, nucleoplasmic bridges (NPBs) provide a measure of chromosome rearrangements that MN not supply [164].
NPBs can be considered a biomarker of DNA damage and literature data support this idea showing an increase of NPBs in a dose-related manner in cells treated with hydrogen peroxide or superoxide, known to induce DNA strand breaks [165]. NPBs occur when centromeres of dicentric chromosomes are pulled to opposite poles of the cell at anaphase. Rarely is it possible to observe dicentric anaphase bridges before the nuclear membrane is formed, because cells proceed through anaphase and telophase rapidly, completing cytokinesis, which ultimately results in breakage of the NPB when the daughter cells separate [166].
BN cells obtained by CBMN assay permit to accumulate NPBs because cytokinesis is inhibited and the nuclear membrane is eventually formed around the chromosomes allowing an anaphase bridge to be observed as an NPB (Fig. 12). strand breaks in DNA [166], either telomere-to-telomere fusion [157, 167] (Fig. 13).
The latter is caused by telomere shortening, loss of telomere capping proteins, or defects in telomere cohesion40; but in this case, an acentric chromosome fragment or an MN does not necessarily accompany the NPB [54,151, 168].

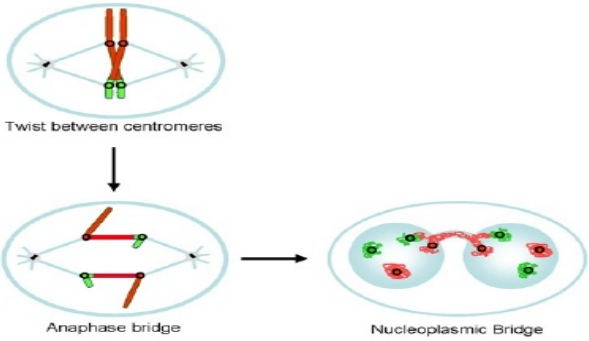


Fig. 12. Schematic representation of dicentric chromosome segregation. Dicentric chromosomes may encounter segregation problems at anaphase if a twist between the two centromeres occurs. The resulting anaphase bridge

and the subsequent nucleoplasmic bridge observed in bi-nucleated cells. Modified by Pampalona et al., 2010 [148].

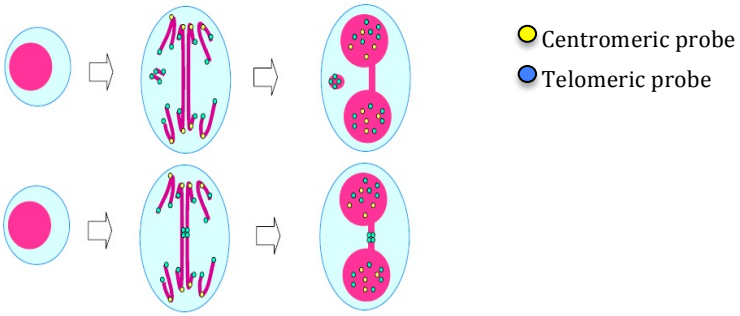


Fig. 13. NPBs from dicentric chromosomes resulting from dicentric chromosomes due to misrepair of DNA strand breaks (top) and caused by telomere end fusions (bottom) [152].
Various mechanisms could lead to dicentric chromosomes formation and then NPBs. Dicentric chromosomes originate either DNA misrepair of

3.4 NPBs and telomere

NBPS could raised also by telomere end fusions in which this last is caused telomere dysfunction, due to loss of telomere-binding proteins without telomere shortening.
Moreover the presence of NPBs in the cytokinesis-blocked cells provides support for the 'breakage–fusion–bridge (BFB) cycle model described by McClintock in maize [7]. According to this theory, different chromosome of sister chromatids, which have both undergone double-stranded breakage, fuse at a distal position (possibly telomeric) forming a dicentric chromosome. During anaphase these dicentric chromosomes are drawn towards both poles and form (nucleoplasmic) bridges. During cytokinesis these dicentric chromosomes, which span both daughter nuclei, are thought to break unevenly and may form a chromosome with two copies of one or more genes and a chromosome (fragment) with no copies of these genes. The chromatids that acquire or loss genomic DNA may fuse again during interphase forming a dicentric chromosome (doubling again the gene copy

number within the chromosome), which is then replicated during the next nuclear division leading to the next bridge–breakage–fusion cycle and further gene amplification. These mechanisms can be induced by DNA damage agents, as x-ray used by McClintock in maize [7] or from extensive telomere shortening, when telomere uncapping leads to chromosome end-to end fusion [21].
Moreover the formation of anaphase bridges and NPBs due to telomere fusion has been observed in models of rodent and human intestinal cancer in *vivo* that showed to correlate with telomere length, indicating that NPB formation may also be used as a surrogate measure of critically short telomeres [164].

4. Oxidative stress

4.1 Oxidative stress and DNA damage

The integrity of genetic information is under constant threat by the tendency of DNA to engage in chemical reactions in its cellular environment. These can damage the DNA in various ways, most frequently by oxidation, alkylation, or deamination of the coding bases [169].
Oxidative damage can act on different cellular components, such as lipids, proteins and DNA, and triggers signaling cascades leading alterations that can lead the cell in an oxidative stress state.
O_2 is involved in many chemical reactions important for cell viability. Normally molecular oxygen is not much reactive, but due to cellular metabolism or external agents, such as temperature, radiation of chemical agents, it can be transformed in reactive oxygen species (ROS) [170].
ROS produced from either external or internal sources are responsible to induce oxidative stress to the cell [171], and are widely recognized to damage biological molecules capable to induce cellular toxicity in all organisms [172].
The genotoxic effects of these molecules depend by their structure and by their cellular targets. Some substance can be metabolized to acquire mutagen capacity, either can cause directly genotoxic effects [173].
ROS include superoxide anion radical, hydroxyl radical, hydrogen peroxide and singlet oxygen that are generated in cells throughout metabolic

processes or also by ionizing radiation and oxidizing agents [174, 175](Fig. 14).
Hydrogen peroxide (H_2O_2) has 2 electrons in redox state. It is lip-soluble and can defund throughout cell membrane. Usually it is an intermediate of cellular metabolism and its activity is neutralized by catalase. Cells, in fact, are equipped with antioxidant and repair enzymes against all oxidative species. Superoxide dismutase (SOD), for example, reduces superoxide anion radicals to molecular oxygen and hydrogen peroxide. Catalase and glutathione peroxidase (GPx) reduce hydrogen peroxide or other hydroperoxides to water or corresponding hydroxyl compounds.
ROS can interact in different way with lipids of cell membrane, inducing breaks of membrane and consequent cell death and with proteins, producing alteration of aminoacids, denaturation and breaks of polypeptide chains. These molecules can also act on DNA throughout a molecular mechanism that may include activation of nucleases or direct reaction of hydroxyl radicals with the DNA to generate single and double strand breaks [176, DNA damage generated by ROS consists of SSBs, DSBs and oxidation of nucleotides [177] (Fig. 15).
If cell does not repair the SSB, it can induce DSB formation and can determine the loss of genomic information associated to chromosome aberration induction [180, 181].

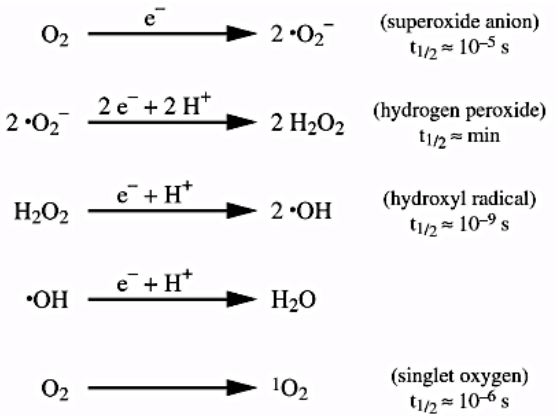


Fig. 14. The basic elements and reactions by which oxygen is converted to a variety of oxidants and free radicals are shown. Major oxygen derived

metabolites include superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen. Also shown is the approximate half-life (t1/2) for each metabolite [178].

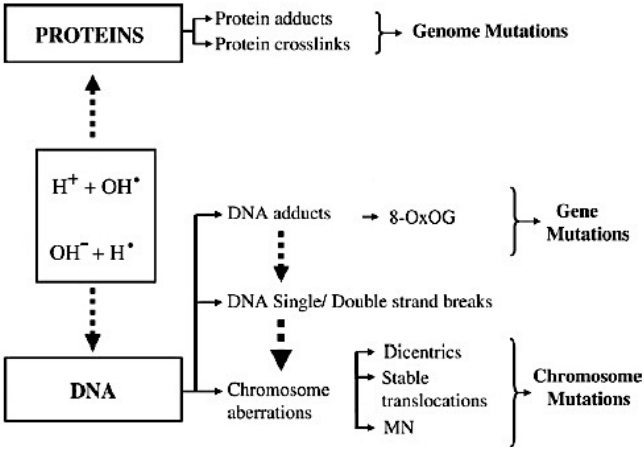
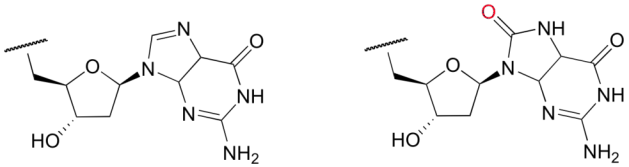


Fig. 15. Flow chart of DNA and proteins damage induced by ROS. DNA damage consists of chromosome aberrations induction, DNA adopts, SSBs and DSBs. These last are able to induce genomic instability. Modified by Mauteca et al. [179].

The main DNA damage, induced by oxidative stress is the modification of DNA bases to species such as 8-oxo-guanine (8-oxoGua), thymine glycol, and 5-hydroxy-methyluracil. The reaction of 2′ -deoxyguanosine in DNA with hydroxyl radicals produces 8-oxo-2′-deoxy- guanosine (8-oxodG), a species recognized as a typical biomarker of oxidative DNA damage [182] (Fig. 16).



DNA guanine base damaged 8-oxoguanine base

Fig. 16. Left image represents formula of guanine in DNA, while right image is an 8-oxo-2′-deoxy- guanosine (8-oxoG).

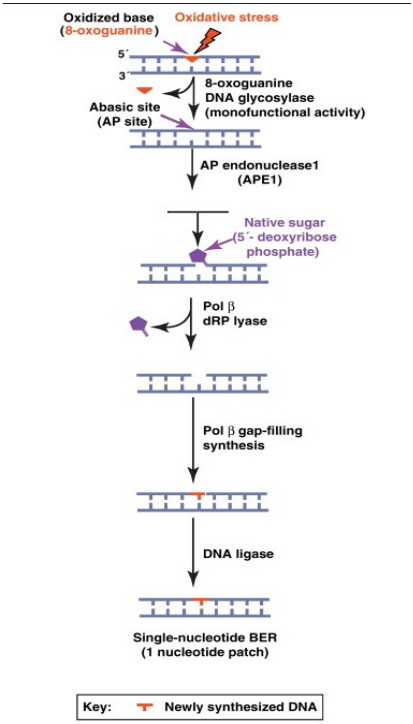


Fig. 17. Representative schema of BER mechanism [191].

Specifically, formation of 8-oxoG it has been found to be associated with such important biological processes as mutagenesis, carcinogenesis, aging, several age-related diseases [183, 184] and neurodegeneration [185].
This DNA lesion is usually repaired by glycosilases that remove the corresponding base, 8-oxoGua, from the DNA, or by endonucleases that excise the nucleoside 8-oxodG. Since the generation of DNA damage and its repair occur continuously, the steady-state level of oxidative damage to the DNA reflects the degree to which the damage incurred by oxidative stress has accumulated [186]. Usually, the 8-oxodG lesions lead to a GC → TA or AT → CG transversion in DNA. The first system involved in

excision of oxidized bases is BER, previous mentioned as a SSBs system repair. BER identifies the modified base and 8-oxoguanine glycosidase (OGG1) breaks the N-glycoside link. Another enzyme removes the damaged base creating an abasic site [188]. Successively a lyase removes the phosphate and the sugar and a polymerase synthetizes a new nucleotide using the other strand as a copy. Finally a ligase links the new nucleotide to the strand [187, 188, 189,190] (Fig. 17).

4.2 Oxidative stress and telomere

Eukaryotic telomeres assume an important role in cellular processes, including chromatin organization and control of cell life [192].
Despite the high efficiency of the DNA replication machinery, telomeric DNA is not fully replicated; about 50–200 base pairs are lost every time a somatic human cell divides [219].
Different mechanisms are currently thought to contribute to telomere shortening: the so-called end replication problem previously mentioned in par. 1.1 and the action of a C-strand–specific exonuclease [193]. The action of this exonuclease would shorten each telomere by half the overhang length per round of replication. Nevertheless, both the end replication problem and the C-strand degradation model of telomere shortening do not take into account the possibility that the shortening rate of telomeres depends on external influences, that include radiations and chemical agents, that promote the gradual or sudden loss of sufficient repeated sequences necessary to maintain proper telomere structure [194].
Among these agents, those induced by oxidative stress, produce reactive oxygen species ROS are able to induce 8-oxodG from triplet GGG present in human telomeric sequence TTAGGG [194].
In fact, literature data have shown that the telomere shortening rate in human fibroblasts in culture varies by more than one order of magnitude in dependence on oxidative stress [195, 196, 197].
Moreover the guanine expressed in sequences GG or GGG are more susceptible to oxidation than the single guanine [198, 199], and as known, telomeric sequence is reach in the triplet GGG, supporting the hypothesis of its major susceptibility [200] and that is why it is considered the preferential target of oxidative damage [201, 202]. Telomeric sequence is the first cause of higher oxidative damage on its sequence [203].

Literature data have shown that oxidative stress increases the frequency of S1 nuclease-sensitive sites, especially in telomeres [197, 197, 202, 201]. S1 nuclease detects a number of different lesions including abasic sites (because the assay is performed at low pH), single-strand breaks, ssDNA loops, gaps, and overhangs. (Fig. 18)
Moreover, ROS have shown to accelerate telomere shortening in replicating fibroblast "in vitro" [204]. This acceleration was attributed to enhanced induction of telomere single strand breaks by free radicals, leading to the loss of the distal fragments of telomeric DNA following replication [205]. Accelerated telomere shortening has been also detected in cells from patients with mutations in mitochondrial DNA that are characterized by an increased production of reactive oxygen species [206].
In addition it has been demonstrated that in normoxia condition, reactive oxygen species (ROS) accelerate telomere shortening and severely reduce proliferative lifespan of human somatic cells in *vitro*; while these phenotypes are delayed when cells are grown in hypoxia or in the presence of antioxidants [201].
Moreover, in strong contrast to all the rest of the genome, S1-sensitive sites induced in telomeres of human fibroblasts by an acute dose of hydrogen peroxide are never completely repaired [201, 205, 207].
This was also investigated by Petersen et al. [208], who exposed human fibroblasts to H_2O_2 and measured the frequency of single-stranded regions in telomeres and minisatellites. It was found that repeated exposure to oxidative stress increased the frequency of single-strand breaks in both minisatellites and telomeres. However, single-strand breaks in minisatellites were completely repaired after 1 day, whereas the repair in telomeres was much slower and incomplete [209]. It is well known that telomere regions, heterochromatic region, have less ability to repair than the genomic region. An explanation for the repair deficiency in telomeres might be that the binding of TRF2 to telomeres blocks the access of DNA repair enzymes to telomeric strand breaks [209, 210].
Furthermore, the t-loop structure suggests another possible explanation for the repair deficiency of telomeres: D-loops might block the access of repair complexes to all DNA within the t-loop. Only repair coupled to replication could be functional within the t-loop [193].
Since oxidative modification and shortening of telomeres are induced by ROS, it is expected that antioxidants may be preventive. This appears to be

supported by several studies that demonstrate antioxidant treatment able to reduce oxidative stress and the rate of telomere shortening [211].
Because telomere shortening increases the possibility to induce genomic instability, an event very frequent in tumour cells [212], it is useful to study the telomere length homeostasis after oxidative stress induction.

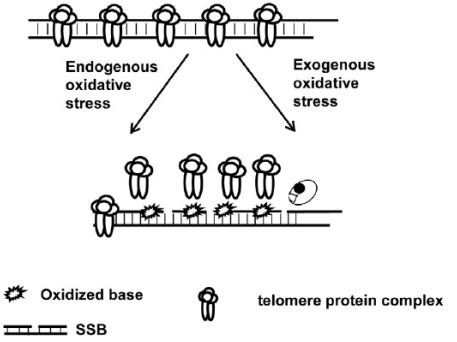


Fig. 18. Oxidative stress induced by exogenous and endogenous factors could determine telomere damage. This damage prevents telomeric proteins to bind. In this way the telomeric loop cannot be formed and the ends are unprotected and susceptible to erosion. Image modified by Wang et al., 2010 [213].

AIM OF THE PROJECT

Telomeres protect the ends of chromosomes and are considered to play an important role in maintaining chromosome and genomic stability [9, 10].
Telomere shortening is regarded as a biological clock of proliferating fibroblasts and, eventually, as an important trigger of replicative senescence [19,214].
Oxidative stress, involving production of reactive oxygen species (ROS), is believed to damage various cellular components that determine cell fate [215,216]. Moreover literature data have shown that acute oxidative stress accelerates telomere shortening [211, 217], even if the effects of prolonged oxidative stress on telomere metabolism are still poorly investigated.
Our aim was to investigate in human primary fibroblasts the effects of prolonged oxidative stress on telomere length and if this effect could modulate cellular division defects.
For this objective we performed the following analysis:
1) We observed if prolonged oxidative stress induced telomere shortening and we estimated the entity of DNA damage produced by daily treatment with hydrogen peroxide.
2) We evaluated possible cell cycle perturbation and premature senescence induction because it has been suggested that telomere shortening or telomere structure alteration can produce DNA-damage signal that activates replicative senescence mechanism and cell cycle block [90].
3) We tried to understand if cells activated some mechanisms involved in telomere length restored after DNA damage induced by oxidative stress.
4) Considering that the loss of telomere sequences is thought being involved in chromosome instability [56, 120], we evaluated chromosome segregation defects, like chromosome bridges.
5) Finally we focused our attention on relation between prolonged oxidative stress and possible telomere-independent mitotic defects, such as cytoskeletal damage and chromosome segregation defects not straight related to telomere shortening.

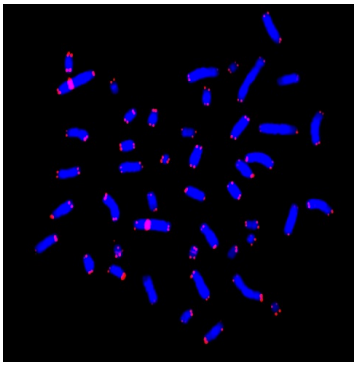
RESULTS

1. Prolonged oxidative stress induces telomere length alterations

We first measured telomere length by Q-FISH (Quantitative Fluorescent in Situ Hybridization; Fig. 1a) on metaphase spreads of MRC-5 at regular intervals over a period of about 3 months. We found that daily H_2O_2 treatment caused telomere shortening up to 21 days and a significant elongation at 37-45 days of treatment. Moreover, this fluctuating trend was repeated over time up to 94 days (telomere shortening after 52 days and telomere elongation after 80 days of treatment) (Fig.1b). So, considering this repeated fluctuating trend and that one of our aims is to understand the relation between prolonged oxidative stress and telomere length, we decided to focus our attention on the first 45 days. Moreover, at later times other phenomena not strictly related to the prolonged H_2O_2 treatment, such as senescence, could complicate the interpretation of the results.
Analysis of telomere length restricted to a 45-day time period confirmed the fluctuating trend. Specifically, we noticed a significant telomere shortening after 5 days of treatment and then a significant lengthening (doubling of the length) by 15 days (Fig.1c). A second fluctuation was observed between 39 (telomere shortening) and 45 days (telomere lengthening) of treatment.
To understand if prolonged oxidative stress has additional effects on cellular damage, cell growth, and cell viability, we performed a number of different assays described below.
To estimate the DNA damage, performed Alkaline Comet Assay to estimate DNA damage, and we used Trypan Blue Assay to evaluate cell viability in all samples and to avoid any form of cellular selection. Cell viability analysis has relieved no increment of death in treated samples versus controls and in all cases cell death resulted minor of 30%, value conventionally considered the limit threshold above which the mortality is considered significant (data not shown).
Alkaline analysis shown a significant number of DNA breaks at 5 days (3,5 fold increase in treated respect to the control) of treatment that decreased over time up to 20 days (Fig 2b).

To evaluate possible cell cycle perturbation, we performed Cytofluorimetric Assay. We did not notice any significant difference between treated and control samples (Fig. 2a).
To understand whether this observed DNA damage could have an effect on cellular senescence, we performed β-galattosidase assay at time points up to 48 days. As expected, our results indicated an increase of senescence for both treated and untreated samples over time, with a slight difference between the two only for the time-periods of 10-15 and 20-27 days (Fig. 2c).

a



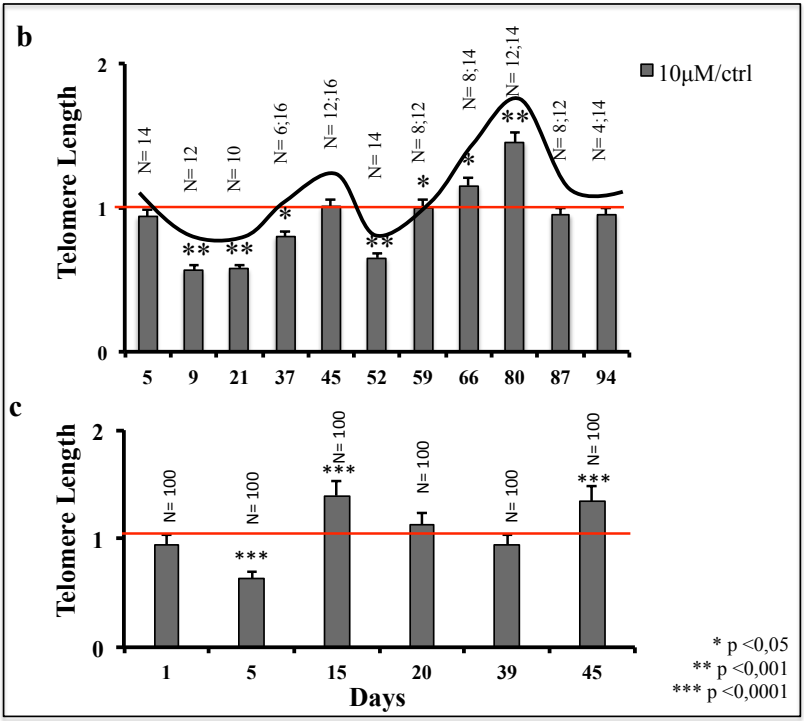


Fig. 1. *Prolonged oxidative stress produces fluctuating changes in telomere length.* **(a)** Representative image of metaphase spread with telomere and centromere of chromosome 2 labelled in by Cy3. **(b-c)** Mean telomere length expressed in T/C% (Telomere fluorescence/Centromere 2 fluorescence percentage) of values from treated samples normalized on own control at different days of treatment. The red line represents a treated/ctrl ratio of 1:1. Error bars represent standard error of the mean. N=number of cells analysed (where two numbers are present, the first refers to control cells and the second to treated cells analysed).

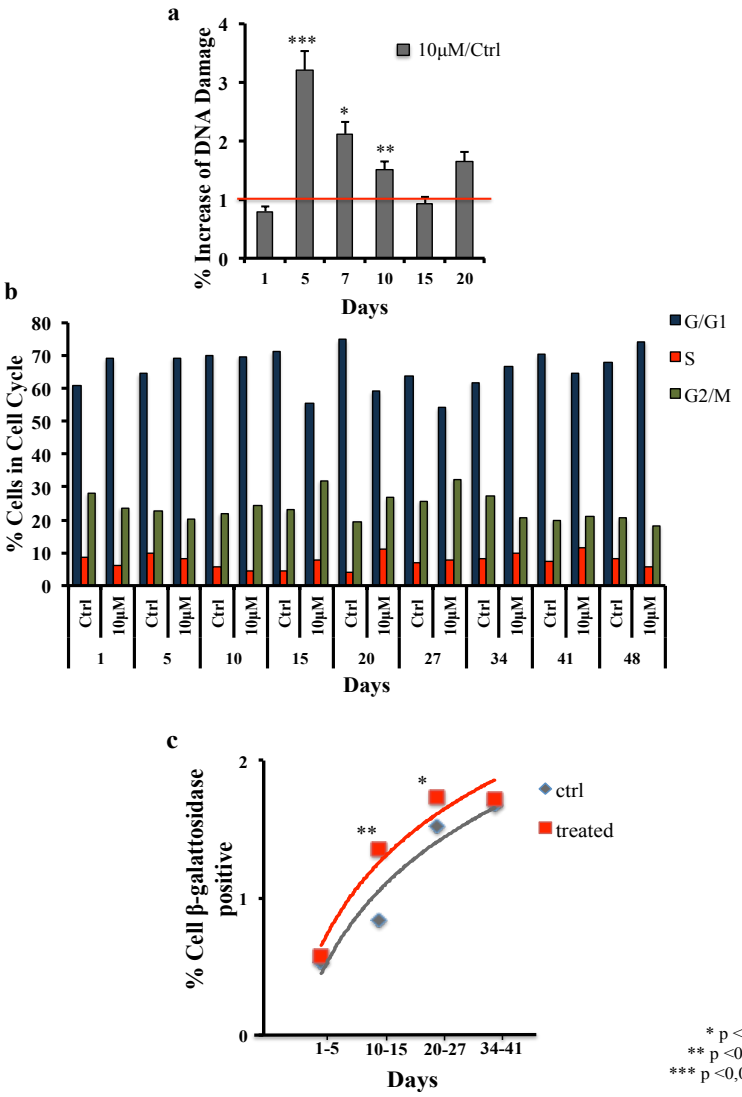

a
% Increase of DNA Damage
10µM/Ctrl
***
*
**
Days
b
% Cells in Cell Cycle
G/G1
S
G2/M
Ctrl
10µM
Days
c
% Cell β-galattosidase positive
ctrl
treated
1-5
10-15
20-27
34-41
Days
* p <0,05
** p <0,001
*** p <0,0001

Fig. 2. *Prolonged oxidative stress does not induce significant cell cycle modifications, but produces DNA damage and enhances cellular senescence.* **(a)** Relative increase in DNA damage induced by prolonged treatment with hydrogen peroxide (10μM). The values represent the percentage values for treated sample normalized on own controls at different fixation times. The red line represents a treated/control ratio of 1:1. Error bars represent standard deviations. **(b)** Representative cell cycle histogram for control and treated samples at different fixation times. **(c)** % of β-galattosidase positive cells in treated and control MRC-5 cells display as logarithmic regression fits. R^2 =0.9 for both control and treated values.

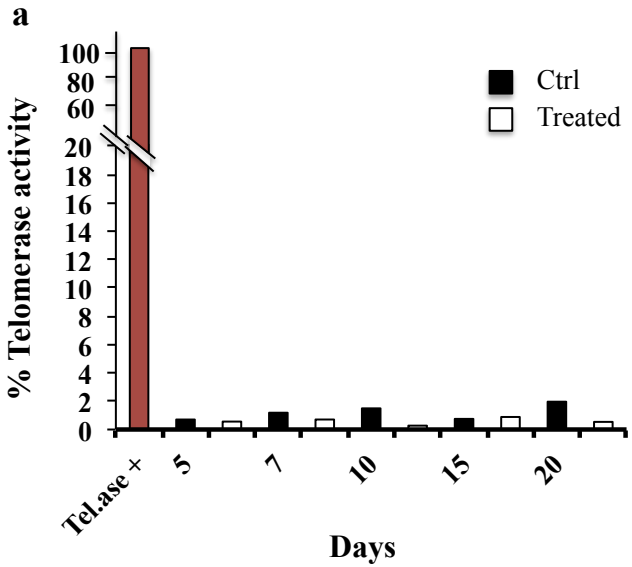
2. Evidence of ALT-dependent telomere lengthening in cells under oxidative stress

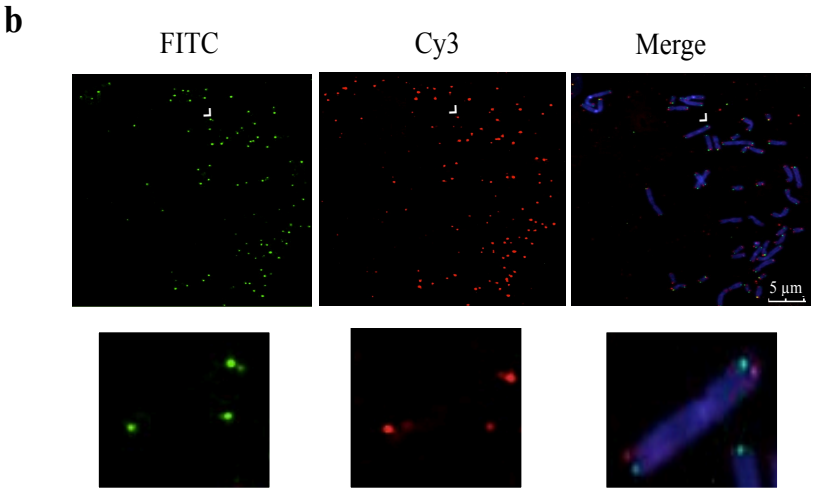
Understanding how telomeres can elongate after the initial shortening is important to understand how oxidative stress may affect genome stability and overall cell physiology. The telomere lengthening observed at 15 days of treatment suggested that cells could activate some mechanism to elongate telomeres and/or that cells with longer telomeres could be successfully selected. Given that we did not observe any cell cycle delay (Fig. 2a) or cell death, we tested whether cells under oxidative stress could activate a telomere elongation mechanism. It has been previously shown that cells can elongate telomeres by either telomerase dependent or telomerase-independent mechanisms [44]. The latter being based on recombination between telomere DNA sequences (Alternative Lengthening of Telomeres, ALT). Human primary fibroblasts have been previously shown to be able to activate the ALT pathway in response to exposure to High-LET radiation [84, 85], thus raising the possibility that this cell type may be able to activate the ALT pathway also in response to oxidative stress.

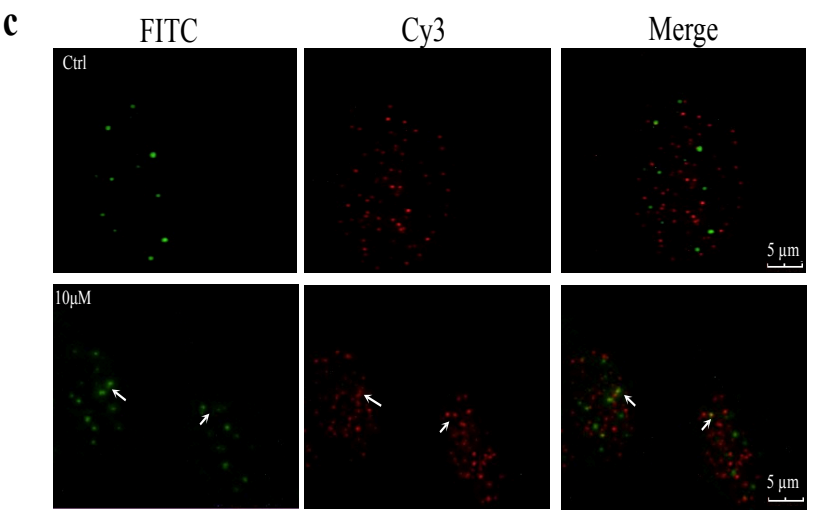
We first performed RTQ-TRAP assay to investigate whether any telomerase activity could be detected over the first 20 days of treatment, and we did not find any evidence of telomerase activation at any of the fixation times for either the controls or the treated samples (Fig. 3a).

Next, we evaluated possible ALT activation by (i) CO-FISH (Chromosome Orientation FISH), which can detect telomere recombination events between sister telomeres (Fig. 3b) and (ii) co-localization of telomere FISH

signals with PML protein immunostainig signals (Fig. 3c), as PML-protein is a key component of the promyelocytic leukemia bodies involved in telomerase-independent telomere lengthening mechanism [72, 76]. Our results indicated an increase of both ALT markers in treated cells over their respective controls (Fig. 3d-e). This increase was statistically significant for sister telomere exchange at 15 days of treatment (Fig. 3d), and for telomere-PML colocalization at both 15 and 20 days of treatment (Fig. 3e).




b
FITC
Cy3
Merge
5 μm


c
FITC
Cy3
Merge
Ctrl
10μM
5 μm
5 μm

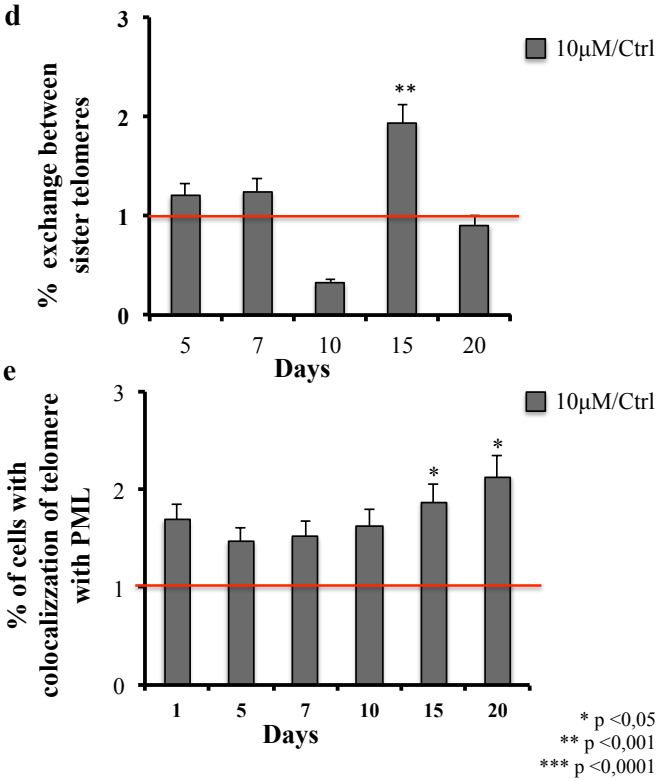
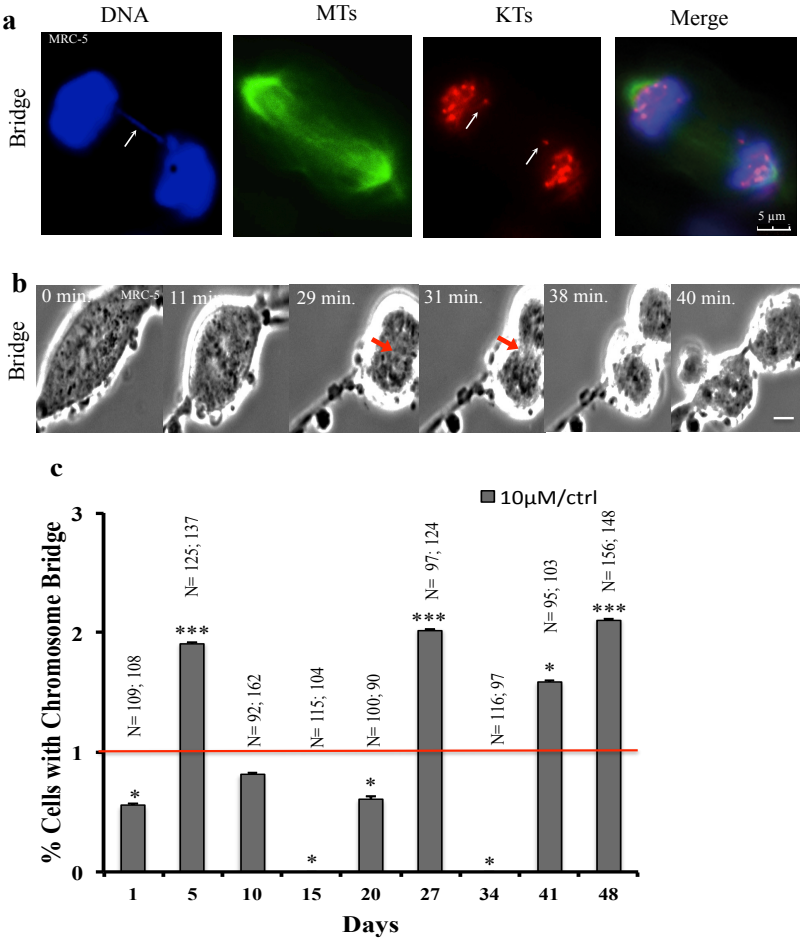


Fig. 3. *Oxidative stress induces activation of the ALT pathway of telomere lengthening.* **(a)** Telomerase activity in control and treated samples evaluated by RTQ-TRAP assay at different time points. The first column represents a telomerase positive control (H460). **(b)** Images of Co-FISH staining with C-rich (red) and G-rich (green) probes. An example of telomere-sister chromatid exchange (T-SCE, arrowhead) can be seen in this cell. An example of staining without T-SCE is indicated by the arrow. The inset shows an enlargement of the chromosome displaying T-SCE. **(c)** Images showing PML fluorescence (FITC-green) and telomere fluorescence (Cy3-red) or combined fluorescence (yellow, Merge) in control (top) and treated (bottom) MRC-5 cells. **(d)** Quantification of T-SCE in treated cells

normalized over control samples at different time points. **(e)** Percentage of cells with telomere-PML protein co-localization in treated cells normalized over control samples. The red line represents a treated/control ratio of 1:1. Error bars represent standard deviations.

3. Prolonged treatment with hydrogen peroxide promotes the formation of anaphase chromosome bridges and nuclear defects

Given the observed changes in telomere length (Fig. 1) and given the relation between telomere shortening and chromosome segregation defects described in the introduction, we next evaluated the frequencies of chromosome bridges in treated vs. control samples over period of 1-48 days of treatment. We performed our analysis in both fixed cells immunostained for kinetochores (using ACA antibodies) and microtubules (using anti-**α**-tubulin antibodies) (Fig. 4a) and live cells imaged by phase contrast time-lapse microscopy (Fig. 4b).
The combined fixed- and live-cell data (Fig. 4c) showed significant increases in the percentages of cells with chromosome bridges at 5, 27, 41 and 48 days of treatment. Interestingly, although the exact timing was not a perfect match, the increases in the frequencies of chromosome bridges in treated cells over control, displayed fluctuations similar to those observed for changes in telomere length (Fig.1).
To further assess the effects of telomere length changes, we evaluated the presence of abnormal nuclear structures in a cytokinesis-block assay, which allows the products of a single mitosis to be retained in the same cytoplasm. We determined the frequencies of nuclear buds (NBuds) and nucleoplasmic bridges (NPBs), as both of these defects can derive from anaphase chromosome bridges, and have been shown to directly correlate with telomere defects [148]. Analysis of NPBS and NBuds (Fig. 4d) over the first 20 days of treatment showed an increase in the frequencies of both of these biomarkers at 5 days of treatment (Fig. 4e), and a significant increase of NBuds at 15 days of treatment. Interestingly, the frequencies of NPBs (Fig. 4e) strictly correlate with the changes in telomere length (Fig. 1) and the frequencies of chromosome bridges (Fig. 4c).


a
DNA
MTs
KTs
Merge
MRC-5
Bridge
5 µm
b
Bridge
0 min.
MRC-5
11 min.
29 min.
31 min.
38 min.
40 min.
c
10µM/ctrl
% Cells with Chromosome Bridge
N= 109; 108
N= 125; 137
N= 92; 162
N= 115; 104
N= 100; 90
N= 97; 124
N= 116; 97
N= 95; 103
N= 156; 148
Days

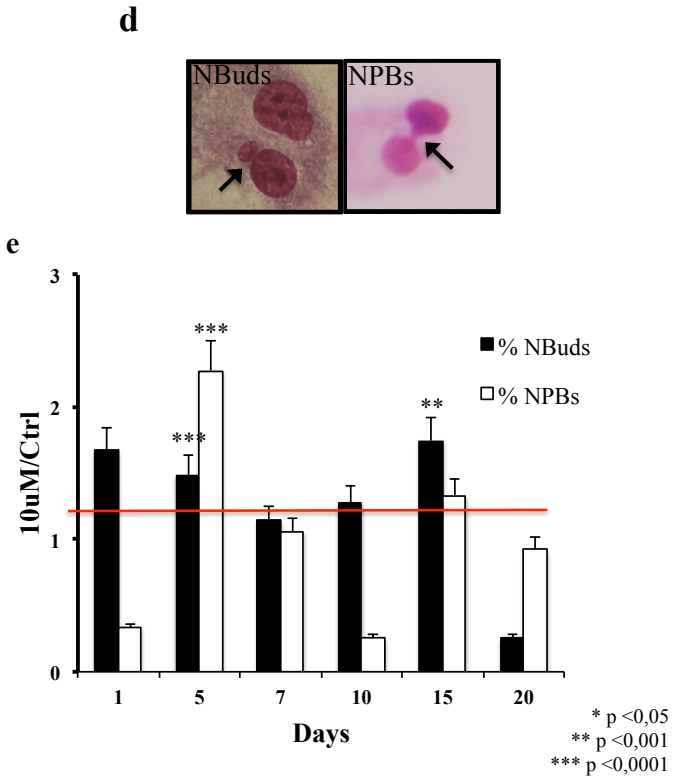


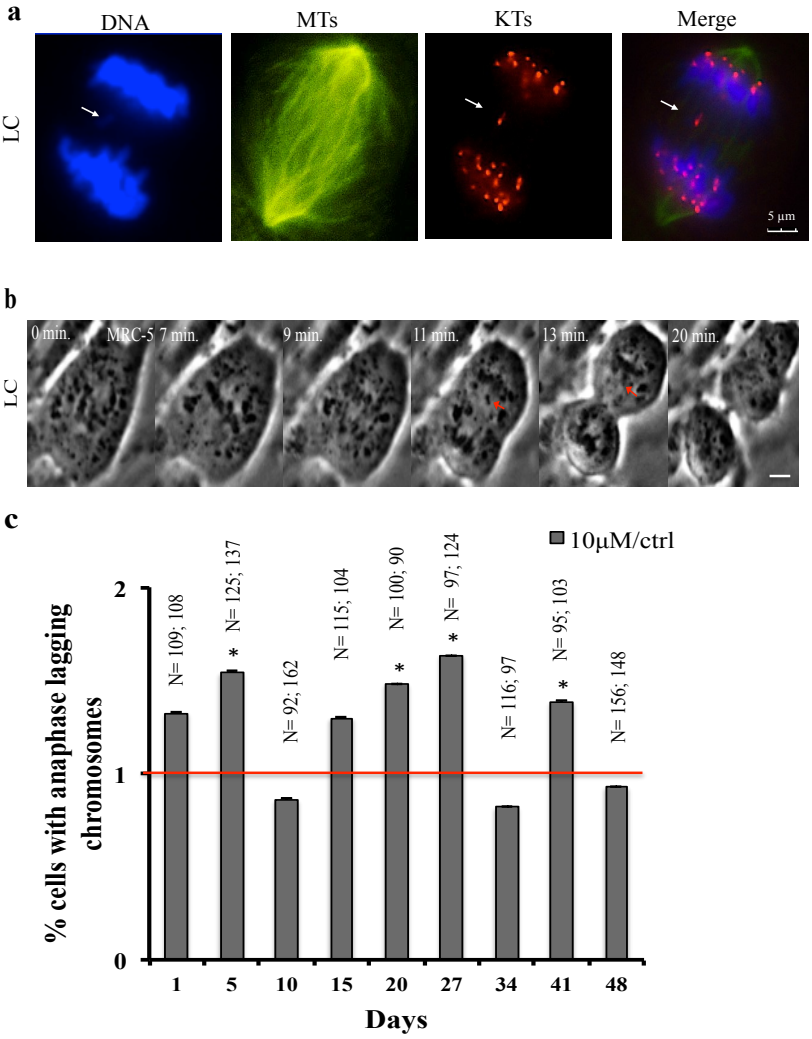
Fig. 4. *Oxidative stress induces chromosome bridges in mitosis and nuclear buds and nucleoplasmic bridges in interphase.* **(a)** Representative image of anaphase cell stained with DAPI for DNA (blue, first column), and immunostained for microtubules (MTs, green, second column) and kinetochores (KTs, red, third column). The images are maximum intensity projections of stacks of optical sections acquired at 0.6 mm intervals through the cell Z-axis. A merged image is shown in the fourth column. The image shows an example of chromosome bridge. Scale bar, 5 μm. **(b)** Still images from time-lapse movie of MRC-5 cell. Phase contrast images show a cell in which a chromosome bridge is visible in anaphase (29 and 31 min,

arrows). The inset in the 31 min frame is a 1.5-fold enlargement of the region showing the chromosome bridge (arrow). Scale bar, 10 μm. **(c)** % of cells with chromosome bridges obtained by combining data from fixed- **(a)** and live-cell **(b)** experiments. Values from treated samples are normalized on own controls. The red line represents a treated/control ratio of 1:1. The N values indicate the number of cells analysed for control and treated samples, respectively. **(d)** Representative images of nuclear buds (NBuds) and nucleoplasmic bridges (NPBs) obtained by bright-field microscopy. **(e)** Histograms showing the percentages of cells with NBuds and NPBs in treated cells normalized on own controls. The red line represents a treated/control ratio of 1:1. Error bars represent standard deviations.

4. Oxidative stress also causes telomere-independent mitotic defects

Combined analysis of fixed (Fig. 5a) and live (Fig. 5b) mitotic cells revealed the occurrence of other defects in addition to the anaphase chromosome bridges. Indeed, at several time points (. 5, 20, 27 and 41 days) we observed higher frequencies of anaphase lagging chromosomes (chromosomes that lag behind at the spindle equator as all the other chromosomes move to the spindle poles in anaphase) in the treated samples as compared to their respective controls (Fig. 5c). At the same time points, we also found an increase in micronuclei that immunostained positive for kinetochore proteins (Fig. 5d-e).

Finally, we observed an interesting mitotic phenotype, which consisted in the presence of tilted mitotic spindles (Fig. 6a-b), that is, spindles in which the long axis was oriented at an angle instead of being parallel to the substrate. In fixed cells immunostained for microtubules this was evident as the two spindle poles were positioned at different focal plane when viewed by fluorescence microscopy (see Fig. 6c, XZ view). In live cells imaged by phase contrast microscopy, this was evident as one of the two groups of segregating chromosomes moved away from the substrate during anaphase and the re-forming daughter cells were on different planes (Fig. 6b) instead of side-by-side (Fig. 6a). We observed frequencies of tilted spindle above the control frequencies during the first 10days, at 27 and 48 days of treatment (Fig. 6d).


a
DNA
MTs
KTs
Merge
LC
5 µm
b
LC
0 min.
MRC-5 7 min.
9 min.
11 min.
13 min.
20 min.
c
10µM/ctrl
% cells with anaphase lagging chromosomes
2
1
0
N= 109; 108
N= 125; 137
N= 92; 162
N= 115; 104
N= 100; 90
N= 97; 124
N= 116; 97
N= 95; 103
N= 156; 148
*
1
5
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Days

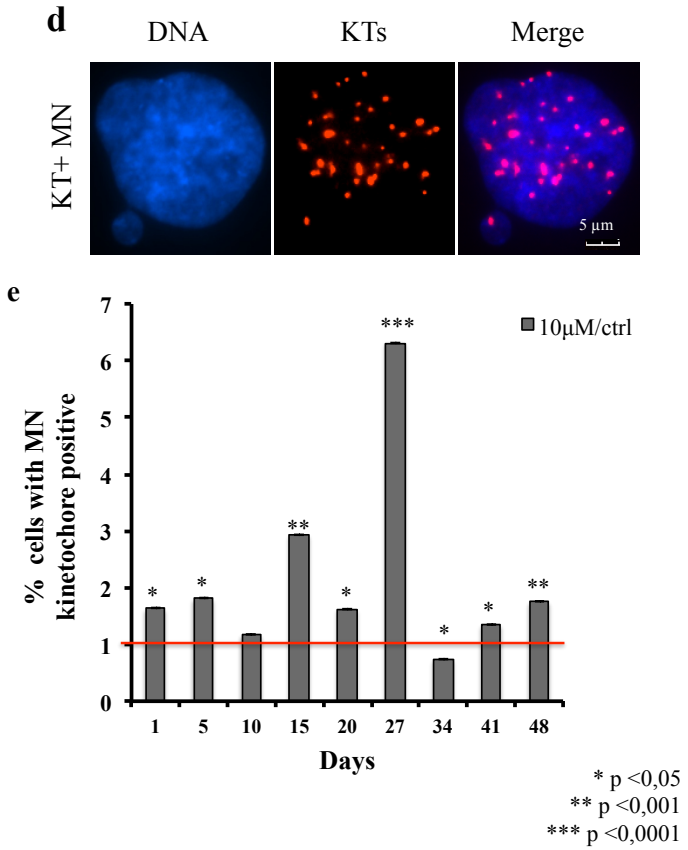
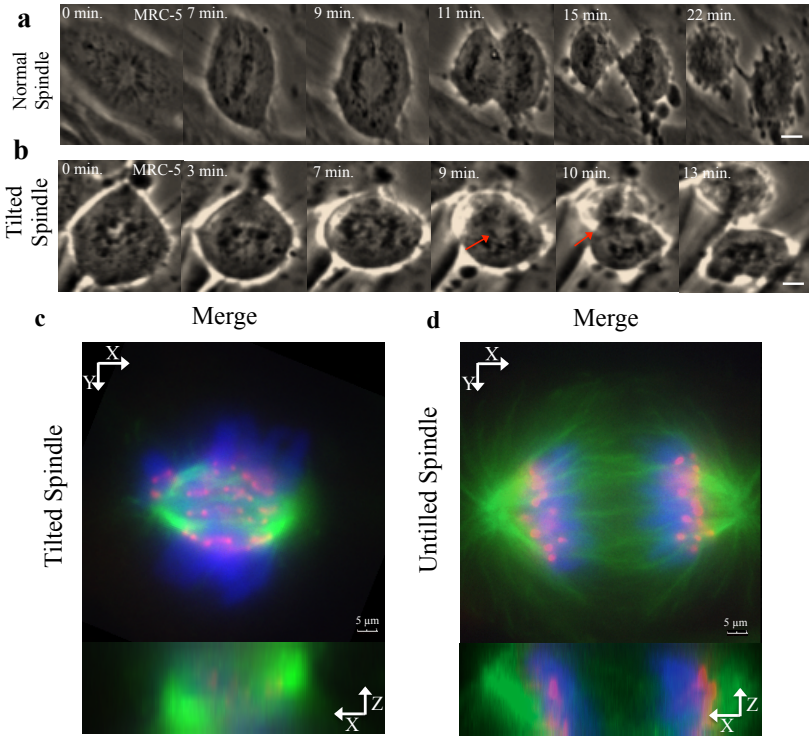


Fig. 5. *Oxidative stress induces anaphase lagging chromosomes and kinetochore-positive micronuclei.* **(a)** Representative image of anaphase cell stained with DAPI for DNA (blue, first column), and immunostained for microtubules (MTs, green, second column) and kinetochores (KTs, red, third column). The images are maximum intensity projections of stacks of optical sections acquired at 0.6 mm intervals through the cell Z-axis. Merged images are shown in the forth column. The image shows an example of anaphase lagging chromosome (LC, arrow). Scale bar, 5 µm.

(b) Still images from time-lapse movie of MRC-5 cell. Phase contrast images show a cell in which a LC in anaphase (9 and 11 min, arrows). Scale bar, 10 µm. **(c)** % of cells with LCs obtained by combining fixed- **(a)** and live-cell **(b)** data. Treated samples are normalized over own controls. The red line represents a treated/control ratio of 1:1. The N values indicate the number of cells analysed for control and treated samples, respectively. **(d)** Representative images of kinetochore-positive micronucleus (KT+ MN, arrows) in an interphase cell stained with DAPI for DNA (left), and immunostained for kinetochores (KTs, middle). The right panel shows a merged image. **(e)** Frequencies of KT+ MNi in treated MRC-5 cells normalized over own controls. The red line represents a treated/control ratio of 1:1. Error bars represent standard deviations.



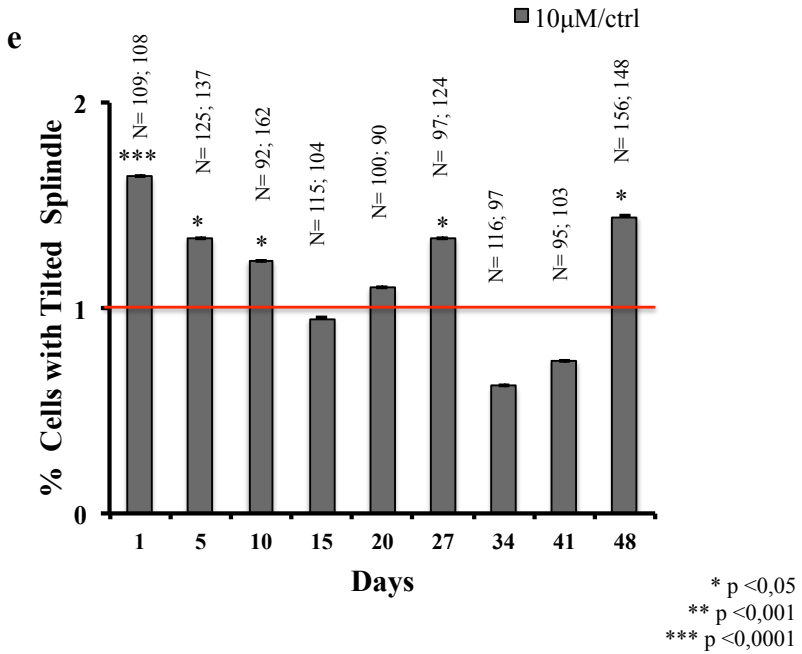


Fig. 6. *Oxidative stress induces changes in the orientation of the mitotic spindle axis.* **(a-b)** Still images from time-lapse movie of MRC-5 cell. Phase contrast images show a cell that divides normally **(a)** and a cell that divides with tilted spindle **(b)**. In anaphase, one edge of the cell can be seen moving away from the substrate and going out of focus (7 min, arrow). One of the re-forming daughter cells can also be seen significantly out of focus and laying in a focal plane above that of its sister (9 min, arrow). Scale bar, 10 μm**. (c-d)** Representative merged image of prometaphase MRC-5 cell stained with DAPI for DNA (blue), and immunostained for microtubules (green) and kinetochores (red), and displaying an untilled **(c)** and tilted spindle **(d)**. The orientation of the spindle can be appreciated in the XZ view (bottom), in which the two spindle poles can be seen at different heights (i.e., at different distances from the substrate). Scale bar, 5μm. **(e)**

% of cells with tilted spindle obtained by combining fixed- and live-cell data. The values for treated samples are normalized on respective control values. The red line represents a treated/control ratio of 1:1. The N values indicate the number of cells analysed for control and treated samples, respectively. Error bars represent standard deviations.

These results indicate that prolonged oxidative stress affects spindle structure and/or function. Interestingly, we also observed high frequencies of treated cells that exhibited mitotic delay compared to untreated cells (data not shown)

DISCUSSION

Genome integrity is critical for cell viability [218]. Impaired repair of damaged DNA can lead to the accumulation of mutations and genomic instability. One important structure for genomic instability is the telomere. Telomeres serve multiple functions in preserving chromosome stability, protecting the ends of chromosomes from degradation and preventing chromosomal end fusion [116].

Telomeres are nucleoprotein complexes that protect the ends of linear chromosomes and are required for a wide range of cellular processes, including apoptosis, aging, cancer and chromosome stability [9].

Telomeric DNA is composed by tandemly repeats short fragments sequences that vary among species ((TTAGGG)n in human).

Telomere shortening occurs every time a somatic human cell divides [219].

Telomere shortening is regarded as a biological clock of proliferating fibroblasts and is an important trigger of replicative senescence [148, 173].

Environmental factors such as radiation and various chemicals can accelerate telomere shortening. Oxidative stress has been shown to damage various cellular components that determine cell fate as well [214, 215]. In particular telomere has been shown to be the preferential target for acute oxidative damage because of ROS able to induce 8-oxodG from triplet GGG present in human telomeric sequence TTAGGG [201].

In this work, we sought to understand the effects of prolonged oxidative stress at low doses on telomere length and we analysed telomere function, specifically its role in chromosome instability.

We treated human primary fibroblasts daily for up to 94 days with 10μM of hydrogen peroxide and measured telomere length by Q-FISH. We observed a telomere shortening at 9-21 days of treatment. At longer 21-48 days of treatment we observed a telomere elongation.

In addition, telomere length appeared to fluctuate repeatedly up to 94 days.

This fluctuating trend led us to hypothesize that oxidative stress could modulate telomere length after prolonged low doses. We decided to focus our attention on cell treated for 45 days, corresponding to the first cycle of telomere shortening and elongation and analysed the effects of prolonged oxidative stress on telomere length. We exclude longer treatment teams, because several mechanisms, as senescence, not directly related to oxidative

stress effects, could interfere with the results. Indeed, natural telomere shortening is correlated to cellular senescence [87,88].
In the 45 days period, we again observed a fluctuating trend of telomere length over time with a telomere shortening at 5 days and a subsequent telomere lengthening at 15 days of treatment. This result confirmed that prolonged oxidative stress is responsible telomere shortening observed at 5 days. Such telomere shortening, accord to literature data, has previously been observed after acute treatment [218] and led us to hypothesize that DNA damage produced by oxidative stress, induced telomere shortening. We also observed telomere elongation at 15 days suggesting that DNA damage accumulation could trigger some mechanism to restore telomere length.
Concurrent, with telomere shortening, we evaluated the entity of DNA damage induced by oxidative stress and we observed the highest DNA damage at 5 days of treatment, with the highest frequency of DNA damage corresponding to the shortest telomeres. Subsequently, we observed a decrease in DNA breaks at 15 days to level similar to the control, when telomere length was restored. These results support the idea that DNA breaks and telomere length modulation are coupled and that the major part of DNA damage induced by oxidative stress could be localized on telomeric DNA, rich in G bases.
Because, it has been suggested that telomere shortening or telomere structure alterations can produce DNA-damage signals that activate replicative senescence mechanism and cell cycle block [97], we tested if the observed telomere shortening corresponded to cell cycle delay and activated premature senescence mechanism.
Cell cycle analysis showed that prolonged oxidative stress did not induce any cell cycle perturbation.
Moreover, when telomeres reach a critical length, the cells arrest proliferation and acquire enlarged morphology expressing senescence-associated gene like the β-galattosidase [89].
Then, to determine if telomere shortening could lead to cellular senescence previously seen, we analysed β-galattosidase expression, a marker of cellular senescence [89]. This analysis showed, as expected, a trend of senescence for both treated and control samples with passaging, indicating that prolonged treatment did not induce premature senescence.

Thus, DNA damage and subsequent telomere shortening did not appear to be enough to induce a significant cell cycle arrest or premature senescence.
We also sought to understand what was mechanism involved in telomere elongation observed at 15 days of treatment.
Previous reports show that only cancer cells and stem cells could avoid the progressive loss of telomeric DNA by the telomerase activation [104-108] or by the telomerase-independent mechanism, termed Alternative Telomere Lengthening (ALT) [109].
DNA damage usually results in telomere shortening [209, 220, 221] and thus genomic instability [204] and cell death [222]. Only when telomeres reach a critical length cells might activate some telomere lengthening mechanisms, such as telomerase reactivation [223], favouring the acquisition of unlimited replicative potential. Previous studies have found that the upregulation of telomerase activity occurred in *vitro* in X-irradiated mouse or human cells [224-226], indicating that telomere elongation occurred as a result of chromosome healing mediated by radiation-activated telomerase. Since another mechanism for telomere length maintenance, the so-called alternative mechanism of telomere lengthening or ALT, has been described in tumor cells [44], our goal was to demonstrate the existence of ALT pathway in normal cells. To exclude the possibility that telomerase activation is responsible for the telomere elongation observed, telomerase activity was evaluated. The data obtained with the RTQ-TRAP assay demonstrated that the enzyme was not induced by low dose of hydrogen peroxide at all fixing times, suggesting that the observed telomere elongation was the result of a telomerase-independent mechanism.
Recently, Morrish and Greider demonstrated telomere maintenance by a non-telomerase mechanism occurring in telomerase knockout primary mouse cells [227]. Similarly, Berardinelli et al. showed telomere elongation by ALT in human fibroblasts [85] treated wit high-LET radiation [84, 85].
To evaluate the possible activation of the ALT pathway in human primary fibroblasts, first we determined whether the telomere elongation observed after oxidative stress resulted in increased telomere recombination frequencies, since it has been proposed that increased T-SCE is a hallmark of ALT cells [228]. We used CO-FISH analysis to detect telomere recombination events between sister telomeres [229]. Our results showed an increase of telomere sister chromatid exchanges and colocalization between telomere sequences and PML-proteins at time in which we had telomere elongation. Since the colocalization of telomeres and PML protein is

another landmark of ALT cells [230], we investigated whether the observed telomere elongation could occur through an ALT mechanism. Immuno-FISH experiments have shown a significant increase in the frequency of cells with APBs and in the number of colocalization events per cell compared to untreated controls at 15 days, when telomeres were elongated. We speculated that the telomere lengthening we observed after exposure of cells to a low dose of hydrogen peroxide could be explained by recombination between telomeres and we proposed the activation of an ALT mechanism in human primary cells.

In addition, our data underscores the importance of the ALT mechanism in modulating telomere length after DNA damage, giving the cells the power to proliferate indefinitely that is typical of tumour.

However, we cannot exclude that the telomere elongation observed in our experiments, is not that results of selection for cells with longer telomeres due their major viability and could operate together to ALT mechanism.

Telomere shortening has been also shown to affect mitotic chromosome segregation by inducing chromosome bridges [175]. Then, we hypothesized an increase of chromosome segregation defects at time of telomere shortening.

According to literature, the loss of chromosome ends or shortening of telomeres can result in “sticky” chromosomes that will give rise to dicentric/ring chromosomes. These dicentric/ring chromosomes generate chromosome bridges during anaphase that may break in telophase [7]. The breakage of the bridge will create new sticky chromosome ends that could fuse with other sticky ends or that could determine the sister chromatid fusion at the following cell cycle, creating the so called “breakage/fusion/bridge cycle” (BFB) [6].

This BFB cycle will continue until the affected chromosome will acquire a new telomere, end in our case when ALT mechanism will restore telomere length.

Furthermore, the analysis of bi-nucleated cells has demonstrated an increase of nucleoplasmic bridge (NPBs) and nuclear buds (NBuds) biomarkers, which mediated telomere-dysfunction dependent chromosomal instability (CIN), according to Pampalona et al. [230].

The increase of these two biomarkers at time of telomere shortening, allowed us to hypothesize that telomere shortening may affect chromosome instability.

The strict correlation between NPBs and both changes in telomere length and frequencies of anaphase chromosome bridges indicates that this biomarker represents a good readout for telomere defects and consequent chromosome segregation error [148, 175]. On the other hand, the lack of a strict correlation between NBuds and telomere length/chromosome bridges supports previous findings [148, 231 and page 30-31 of introduction] and suggests that NBuds may form as a result of both broken anaphase chromosome bridges and anaphase lagging chromosomes incorporated in the main nucleus during nuclear envelope reassembly [152].
Since telomere shortening generated fusions of broken chromosome ends [148, 175], we think that this could cause an increase of chromosome bridges.
Our results demonstrated an increase of bridges in treated samples at 5 days, corresponding to telomere shortening and a decrease of bridges at 15 days, when telomere length was restored.
Then, we hypothesized that ALT mechanism restored telomere length inducing a reduction of chromosome segregation defects related to telomere structure. The fluctuating trend indicated that there were periods in which telomeres shortened excessively in treated cells, thus causing an increase in the rates of anaphase chromosome bridges, and periods in which telomere length recovered, thus resulting in a decrease in the frequencies of chromosome bridges (Fig. 7).
Considering the importance of the correct mitosis, we focused our attention to observe if there were other mitotic defects, and we studied anaphase lagging chromosome and chromosome loss.
Our results have shown an increase of anaphase lagging chromosomes in treated samples.
Anaphase-lagging chromosome was generated by chromosome mis-segregation that compromised correct cell proliferation and could generate aneuploidy cells.
At the same time, the increase of micronuclei kinetochore positive in almost all-fixing time was consistent with the data on anaphase lagging chromosomes, and these two phenotypes were expected to correlate. Indeed, it is widely acknowledged that anaphase lagging chromosomes form micronuclei upon mitotic exit [159, 232] and that the presence of kinetochore proteins in a micronucleus is indicative of its origin from an anaphase-lagging chromosome. Moreover, according to Fenech M. and

Morley A., MN kinetochore positive could represent an increase of chromosome loss and aneuploidy [147].

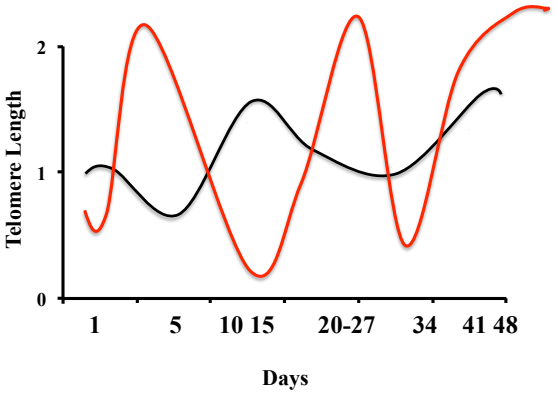


Fig. 7. Demonstrative histogram of relation between telomere length (black line) and chromosome bridge induction (red line).

Also defects of spindle apparatus could induce chromosome lagging, and the increase of cells with tilted spindle observed in treated samples indicated that prolonged oxidative stress affects spindle structure and/or function.
In cell with tilted spindles the long axis was oriented at an angle instead of being parallel to the substrate.
Thus, in addition to the telomeric damage, which causes chromosome bridges in mitosis and can result in chromosome rearrangements, prolonged oxidative stress can also induce telomere-independent mitotic defects, such as lagging chromosomes, which can result in aneuploidy, and tilted spindle. Taken together, these data indicate that prolonged oxidative stress can cause the two most common karyotypic defects observed in cancer cells: chromosome rearrangements and aneuploidy, and increase lifespan.

CONCLUSIONS

Based on our results we can sustain the hypothesis that prolonged oxidative stress at low dose induced DNA damage that was responsible of telomere shortening and chromosome instability observed. Chromosome instability was mainly represented by chromosome bridge, Nuclear Buds and Nucleoplasmic bridges related to telomere shortening, but also telomere independent damage was induced, as demonstrated by the anaphase lagging chromosome, micronuclei and mitotic spindle increase in treated samples.
At the same time, oxidative stress could induce a DNA damage such as to trigger on one hand a telomere shortening and on the other hand the activation of a mechanism able to restore telomere length and in our case it was ALT mechanism, inhibiting cell death, allowed the surviving of cells characterized by chromosome instability and favoured the acquisition of unlimited replicative potential.
Considering that chromosome instability and unlimited replicative potential are both involved in tumor progression, we could attribute to prolonged oxidative stress an important role to induce cell transformation throughout DNA and cytoskeletal structures damage (Fig. 8).

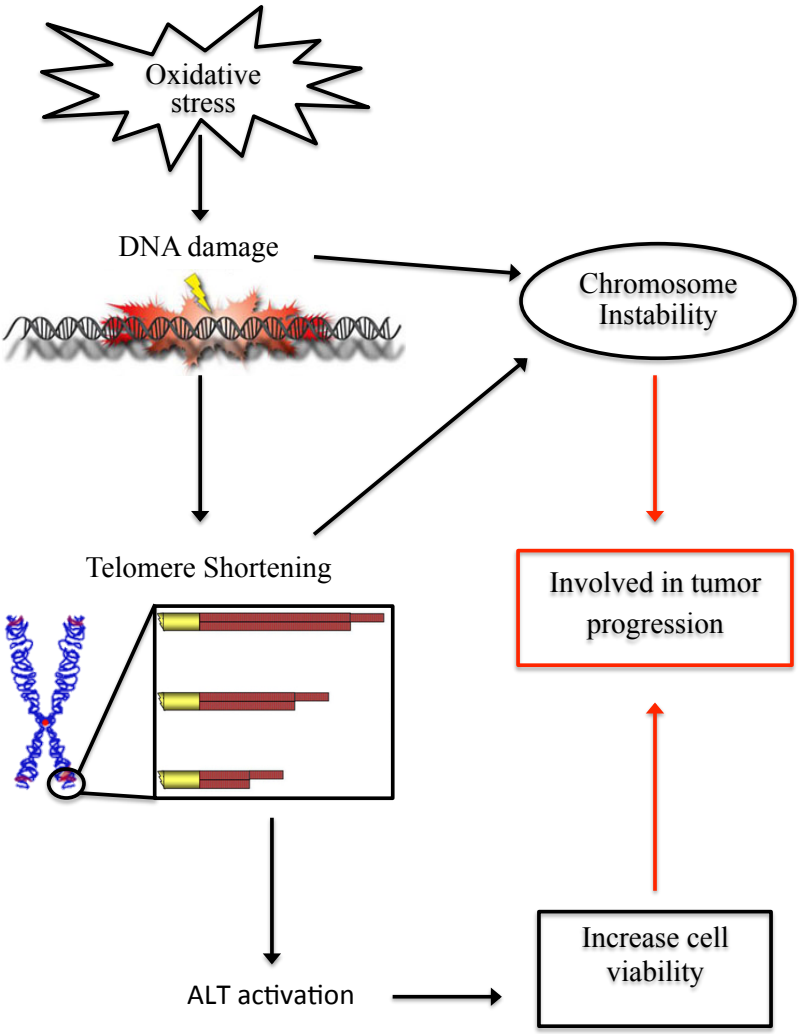


Fig. 8. Flow chart of oxidative stress action on DNA, and consequences of DNA damage on telomere length and chromosome instability.

1. Lindahl T. and Wood R.D. (1999). Quality control by DNA repair. Science, **286** (5446): 1897-905.
2. Blasco M.A. (2005) Telomeres and human disease: ageing, cancer and beyond Nature Reviews Genetics **6**: 611-622.
3. Blackburn E. H. (2004). Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. Febs letter, **579** (4): 859-862.
4. Muller, H.J. (1938). The remaking of chromosomes. *Collecting Net*, **8**: 181-195.
5. McClintock B. (1938). Mo. Agric. Exp. Stn. Res. Bull. **290**:1- 48.
6. McClintock B. (1939). The behaviour in successive nuclear divisions of a chromosome broken at meiosis. Proc. Natl. Acad. Sci. USA **25**:405-16.
7. McClintock B. (1941). The stability of broken ends of chromosomes in *Zea mays*. Genetics **26**:234- 82
8. McClintock B. (1942). The Fusion of Broken Ends of Chromosomes Following Nuclear Fusion. Proc. Natl. Acad. Sci. USA **28**:458-63
9. Blackburn E.H. (1991). Structure and function of telomeres. Nature **350**: 569- 73
10. Blackburn E.H. and Greider C.W. (1995). Telomeres. Cold Spring Harbor, NY: 93 Cold Spring Harbor Lab. Press.
11. Watson J.D. (1972). Origin of concatemeric T7 DNA. Nat New Biol. **239**(94): 197-201.
12. Monaghan P. (2010). Telomeres and life histories: the long and the short of it. Ann. N.Y. Acad. Sci. **1206**:130–14.
13. Blackburn EH. (1991). Telomeres. Nature. 16 (10): 378-81.
14. Greider C.W. (1996). Telomere length regulation. Annu. Rev. Biochem. **65**: 337-65.
15. Neumann A.A. and Reddel R.R. (2002) Telomere maintenance and cancer — look, no telomerase Nature Reviews Cancer **2**: 879-884.
16. Kalluri R. (1996). Telomere (telomerase) hypothesis of aging and immortalization. Indian J Biochem Biophys. **33**: 88-92.
17. Brown W., MacKinnon P.J., Villasanté A., Spurr N., Buckle V.J. and Dobson M.J. (1990). Structure and polymorphism of human telomere-associated DNA. Cell. **63**: 119-32.
18. Regine D. et al. (1997). Telomeres and telomerase: biological and clinical. Clinical Chemistry. **43**: 5708-714.

19. Harley C.B., Futcher A.B. and Greider C.W. (1990) Telomeres shorten during ageing of human fibroblasts. *Nature*, **345**: 458–460.
20. Lansdorp P.M. (2005). Major cutbacks at chromosome ends. Trends Biochem. Sci. **30**: 388–395.
21. Blasco M.A. (2005) Mice with bad ends: mouse models for the study of telomeres and telomerase in cancer and aging. EMBO J. **24** (6): 1095–1103.
22. Chong L., van Steensel B., Broccoli D., Erdjument-Bromage H., Hanish J., Tempst P. and de Lange T. (1995). A human telomeric protein. Science **270**:1663–1667.
23. Van Steensel B., Smogorzewska A. and de Lange T. (1998). TRF2 protects human telomeres from end-to-end fusions. Cell. **92**:401–413.
24. Baumann P. and Cech T.R. (2001). Pot1, the putative telomere end-binding protein in fission yeast and humans. Science. **292**:1171–1175.
25. De Lange T. (2005). Shelterin: the protein complex that shapes and safeguards human telomeres. Genes Dev. **19**:2100–2110.
26. Smogorzewska A., van Steensel B., Bianchi A., Oelmann S., Schaefer M.R., Schnapp G. and de Lange T. (2000). Control of human telomere length by TRF1 and TRF2. Mol. Cell Biol. **20**: 1659-1668.
27. Richter T., Saretzki G., Nelson G., Melcher M., Olijslagers S., von Zglinicki T. (2007) TRF2 overexpression diminishes repair of telomeric single-strand breaks and accelerates telomere shortening in human fibroblasts. Mechanisms of Ageing and Development **128**: 340–345.
28. Griffith J.D., Comeau L., Rosenfiel S., Stansel R.M., Bianchi A., Moss H. and de Lange T. (1999) Mammalian telomeres end in a large duplex loop. Cell: **97**: 503-514.
29. Wei C. and Price M. (2003) Protecting the terminus: t-loops and telomere end-binding proteins. Cell Mol Life Sci, **60**: 2283–2294.
30. Nishiyama A. and Ishikawa F. (2006). Cell-cycle-dependent regulation of telomere binding proteins: roles of Polo-like kinase. Cell Cycle **5**: 1403-1406.
31. Karlseder J., Broccoli D., Dai Y., Hardy S. and de Lange T. (1999) p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. Science **283**: 1321-1325.
32. Pardo B. and Marcand S. (2005) Rap1 prevents telomere fusions by non-homologous end joining. Embo J. **24**: 3117-3127.

33. O'Sullivan R.J. and Karlseder J. (2010). Telomeres: protecting chromosomes against genome instability. Nat Rev Mol Cell Biol. **11**(3): 171–181.
34. Bekaert S. (2005) Telomere length: the biological clock reviewed. Cell Cycle.
35. Greider C.W. and Blackburn E.H. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. (1985) Cell. **43**:405–413.
36. Zakian V.A. (2009) The Ends Have Arrived Cell **139**(6): 1038–1040.
37. Blasco M.A., Lee H.W., Hande M.P., Samper E., Lansdorp P.M., DePinho R.A. and Greider C.W. (1997) Telomere shortening and tumour formation by mouse cells lacking telomerase RNA. Cell **91**: 25– 34.
38. Morin G.B. (1989) The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. Cell **59**: 521-529.
39. Steczkiewicz K, Zimmermann MT, Kurcinski M, Lewis BA, Dobbs D, Kloczkowski A, Jernigan RL, Kolinski A, Ginalski K. (2011) Human telomerase model shows the role of the TEN domain in advancing the double helix for the next polymerization step. Proc Natl Acad Sci U.S.A. **108**(23):9443-8.
40. Harley C.B., Kim N.W., Prowse K.R., et al. (1994) Telomerase, cell immortality, and cancer. Cold Spring Harb. Symp. Quant. Biol. **59**: 307-15.
41. Masutomi K., Yu E.Y., Khurts S., Ben-Porath I., Currier J.L., Metz G.B., Brooks M.W., Kaneko S., Murakami S., DeCaprio J.A., Weinberg R.A., Stewart S.A., and Hahn W.C. (2003) Telomerase maintains telomere structure in normal human cells. Cell. **114**: 241–253.
42. Schmitt H., Blin N., Zankl H., and Scherthan H. (1994) Telomere length variation in normal and malignant human tissues. Genes Chrom Cancer **11**: 171-177.
43. Bolzán A.D., Páez G.L., Bianchi M.S. and Bianchi N.O. (2000) Analysis of telomeric repeats and telomerase activity in human colon carcinoma cells with gene amplification. Cancer Genet Cytogenet, **120**: 166-170.
44. Kim N.W., Piatyszek M.A., Prowse K.R., Harley C.B., West M.D., Ho P.L., Coviello G.M., Wright W.E., Weinrich S.L. and Shay J.W. (1994)

Specific association of human telomerase activity with immortal cells and cancer. Science **266**: 2011-2015.

45. Harle-Bachor C., and Boukamp P. (1996) Telomerase activity in the regenerative basal layer of the epidermis inhuman skin and in immortal and carcinoma-derived skin keratinocytes. Proc Natl Acad Sci USA **93**: 6476–6481.
46. Kolquist K.A., Ellisen L.W., Counter C.M., Meyerson M., Tan L.K., Weinberg R.A., Haber D.A., and Gerald W.L. (1998) Expression of TERT in early premalignant lesions and a subset of cells in normal tissues. Nat Genet **19**:182–186.
47. Hines W.C., Fajardo A.M., Joste N.E., Bisoffi M., and Griffith J.K. (2005) Quantitative and spatial measurements of telomerase reverse transcriptase expression within normal and malignant human breast tissues. Mol Cancer Res. **3**: 503–509.
48. Weng N.P. (2008) Telomere and adaptive immunity. Mech. Ageing Dev. **129**: 60–66.
49. Harrington L. (2005) Making the most of a little: dosage effects in eukaryotic telomere length maintenance. Chromosome Res **13**: 493–504.
50. Blackburn E.H. (2005) Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. FEBS Lett. **579**: 859-862.
51. Hahn W.C., Stewart S.A., Brooks M.W., York S.G., Eaton E., Kurachi A., Beijersbergen R.L., Knoll J.H., Meyerson M. and Weinberg R.A. (1999) Inhibition of telomerase limits the growth of human cancer cells. Nat Med **5**:1164-70.
52. Zhang X., Mar V.,Zhou W., Harrington L., Robinson M.O. (1999) Telomere shortening and apoptosis in telomerase-inhibited human tumour cells. Gene Dev **13**: 2388-99.
53. Herbert B., Pitts A.E., Baker S.I., Hamilton S.E., Wright W.E., Shay J.W. and Corey D.R. (1999) Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. Proc Natl Acad Sci USA **96**:14276-81.
54. Damm K., Hemmann U., Garin-Chesa P., Hauel N., Kauffmann I., Priepke H., Niestroj C., Daiber C., Enenkel B., Guilliard B., Lauritsch I., Müller E., Pascolo E., Sauter G., Pantic M., Martens U.M., Wenz C., Lingner J., Kraut N., Rettig W.J. and Schnapp A. (2001) A highly selective telomerase inhibitor limiting human cancer cell proliferation.

Embo J **20**: 6958-68.
55. Gonzalez-Suarez E., Samper E., Flores J. M. and Blasco M.A. (2000) Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis. Nat Genet **26**: 114-7.
56. Greenberg R.A., Chin L., Femino A., Lee K.H., Gottlieb G.J., Singer R.H., Greider C.W. and DePinho R.A. (1999) Short dysfunctional telomeres impair tumorigenesis in the INK4a (delta2/3) cancer-prone mouse. Cell **97**: 515-25.
57. Melek M., and Shippen D.E. (1996) Chromosome healing: spontaneous and programmed de novo telomere formation by telomerase. BioEssays 18: 301–308.
58. Yu G., and Blackburn E.H. (1991) Developmentally programmed healing of chromosomes by telomerase in Tetrahymena. Cell **67**: 823–832.
59. Diede S.J., and Gottschling D.E. (1999) Telomerase-mediated telomere addition in vivo requires DNA primase and DNA polymerase alpha and delta. Cell **99**: 723–733.
60. Wong A.C., Ning Y., Flint J., Clark K., Dumanski J.P., Ledbetter D.H., and McDermid H.E. (1997) Molecular characterization of a 130-kb terminal microdeletion at 22q in a child with mild mental retardation. Am J Hum Genet **60**: 113–120.
61. Flint J., Thomas K., Micklem G., Raynham H., Clark K., Doggett N.A., King A., and Higgs D.R. (1997) The relationship between chromosome structure and function at a human telomeric region. Nat Genet **15**: 252–257.
62. Sprung C.N., Reynolds G.E., Jasin M., and Murnane J.P. (1999) Chromosome healing in mouse embryonic stem cells. Proc Natl Acad Sci USA **96**: 6781–6786.
63. Fouladi B., Miller D., Sabatier L., and Murnane J.P. (2000) The relationship between spontaneous telomere loss and chromosome instability in a human tumor cell line. Neoplasia **2**: 540–554.
64. Slijepcevic P., and Bryant P.E. (1998) Chromosome healing, telomere capture and mechanisms of radiation-induced chromosome breakage. Int J Radiat Biol **73**: 1–13.
65. Slijepcevic P., and Al-Wahiby S. (2005) Telomere biology: integrating chromosomal end protection with DNA damage response. Chromosoma 114: 275–285.

66. Kramer K.M., and Haber J.E. (1993) New telomeres in yeast are initiated with a highly selected subset of TG1-3 repeats. Genes Dev **7**: 2345–2356.
67. Bryan T.M., Englezou A., Gupta J., Bacchetti S., and Reddel R.R. (1995) Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J. **14**: 4240 - 4248.
68. Hande M.P., Samper E., Lansdorp P. and Blasco M.A. (1999) Telomere length dynamics and chromosomal instability in cells derived from telomerase null mice. J. Cell Biol. 144: 589 - 601.
69. Niida H., Shinkai Y., Hande M.P., Matsumoto T., Takehara S., Tachibana M., Oshimura M., Lansdorp P.M. and Furuichi Y. (2000) Telomere maintenance in telomerase-deficient mouse embryonic stem cells: characterization of an amplified telomeric DNA. Mol. Cell. Biol., **20**: 4115 - 4127.
70. Rogan E.M., Bryan T.M., Hukku B., Maclean K., Chang A.C.M., Moy E.L., Englezou A., Warneford S.G., Dalla-Pozza L. and Reddel R.R. (1995) Alterations in p53 and p16INK4 expression and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts. Mol. Cell. Biol. **15**: 4745 - 4753.
71. Bryan T.M. and Reddel R.R. (1997) Telomere dynamics and telomerase activity in vitro immortalised human cells. Eur. J. Cancer, **33**: 767 - 773.
72. Reddel RR. (2003) Alternative lengthening of telomeres, telomerase, and cancer. Cancer Lett. **194**:155–62.
73. Murnane J.P., Sabatier L, Marder BA and Morgan WF. (1994) Telomere dynamics in an immortal human cell line. EMBO J., **13**: 4953 - 4962.
74. Dunham M.A., Neumann A.A., Fasching C.L., Reddel R.R. (2000) Telomere maintenance by recombination in human cells, Nat. Genet. **26**: 447-450.
75. Ramiro E.V. and Karlseder J. (2007) Replication and protection of telomeres. Nature **447**: 924-931.
76. Yeager T.R., Neumann A.A., Englezou A., Huschtscha L.I., Noble J.R., Reddel RR. (1999) Telomerase-negative immortalized human cells contain a novel type of promyelocytic leukemia (PML) body. Cancer Res. **59**: 4175–9.

77. Yankiwski V., Marciniak R.A., Guarente L., Neff N.F. (2000) Nuclear structure in normal and bloom syndrome cells. Proc Natl Acad Sci U S A **97**: 5214–9.
78. Fasching CL., Bower K. And Reddel RR. (2005) Telomerase-Independent Telomere Length Maintenance in the Absence of Alternative Lengthening of Telomeres–Associated Promyelocytic Leukemia Bodies. Cancer Res **65** (7): 2722-9.
79. Perrem K, Bryan TM, Englezou A, Hackl T, Moy EL and Reddel RR. (1999). Repression of an alternative mechanism for lengthening of telomeres in somatic cell hybrids. Oncogene **18**: 3383 - 3390.
80. Londono-Vallejo J.A., Der-Sarkissian H., Cazes L., Bacchetti S., Reddel R.R. (2004) Alternative lengthening of telomeres is characterized by high rates of telomeric exchange. Cancer Res **64**: 2324–2327.
81. Bechter O.E., Zou Y., Shay J.W., and Wright W.E. (2003) Homologous recombination in human telomerase-positive and ALT cells occurs with the same frequency. EMBO Rep **4**: 1138-1143.
82. Jeyapalan J.N., Mendez-Bermudez A., Zaffaroni N., Dubrova Y.E. and Royle N.J. (2008) Evidence for alternative lengthening of telomeres in liposarcomas in the absence of ALT-associated PML bodies. Int J Cancer **122**: 2414 – 2421.
83. Slatter T.L., Tan X., Yuen Y.C., Gunningham S., Ma S.S., Daly E., Packer S., Devenish C., Royds J.A. and Hung N.A. (2012) The alternative lengthening of telomeres pathway may operate in non-neoplastic human cells J Pathol **226**: 509–518.
84. Sgura A., Antoccia A., Berardinelli F., Cherubini R., Gerardi S., Zilio C. and Tanzarella C. (2006) Telomere length in mammalian cells exposed to low- and high-LET radiations. Radiat. Prot. Dosimetry **122**: 176–179
85. Berardinelli F., Antoccia A., Cherubini R., De Nadal V., Gerardi S., Cirrone GAP, Tanzarella C. and Sgura A. Transient Activation of the ALT Pathway in Human Primary Fibroblasts Exposed to High-LET Radiation (2010) Radiation Research **174**:000-000.
86. Hayfick L. and Moorhead P.S. (1961). The serial cultivation of human diploid cell strains. Exp. Cell. Res., **25**: 585 - 621.
87. Hastie N.D., Dempster M., Dunlop M.G., Thompson A.M., Green D.K., and Allshire R.C. (1990) Telomere reduction in human colorectal carcinoma and with ageing. Nature **346**: 866-868.

88. Rodier F. and Campisi J. (2011). Four faces of cellular senescence. J Cell Biol, **192**: 547-56.
89. Kim S., Kaminker P. and Campisi J. (2002) Telomeres, aging and cancer: In search of a happy ending. Oncogene **21**: 503-511.
90. Harley C.B., Vaziri H., Counter C.M. and Allsopp R.C. (1992) The telomere hypothesis of cellular aging. Exp. Gerontol. **27**: 375 - 382.
91. Kirkwood T.B.L. (2011) Systems biology of ageing and longevity. Phil. Trans. R. Soc. B **366**: 64–70.
92. Calado R. and Young N. (2012) Telomeres in disease Medicine Reports. **4**:8.
93. Kiyono T., Foster S.A., Koop J.I., McDougall J.K., Galloway D.A. and Klingelhutz A.J. (1998). Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. Nature **396**: 84 -88.
94. Dickson M.A., Hahn W.C., Ino Y., Ronfard V., Wu J.Y., Weinberg R.A., Louis D.N., Li F.P. and Rheinwald J.G. (2000). Human keratinocytes that express hTERT and also bypass a p16 (INK4a)-enforced mechanism that limits life span become immortal yet retain normal growth and differentiation characteristics**.** Mol. Cell. Biol. **20**: 1436 - 1447.
95. Sherr C.J. and Roberts J.M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. **13**: 1501–1512.
96. Vidal A. and Koff A. (2000). Cell-cycle inhibitors: three families united by a common cause. Gene **247**: 1–15.
97. Wright W., Shay J. (1995). Time, telomeres and tumours. Is cellular senescence more than an anticancer mechanism? Trends Cell Biol. **5**: 293–7.
98. Aubert G. And Lansdorp P.M. (2008) Telomeres and Aging. Physiol Rev **88**: 557–579.
99. Hansemann D. (1891) Ueber patologische Mitosen. Arch Pathol Anat Phys Klin Med **119**: 299-326.
100. 101Satyanarayana A., Manns M.P. and Rudolph K.L. (2004) Telomeres, telomerase and cancer. Cell Cycle **3**(9): 1138-1150 (Review).
101. Hiyama K., Hirai Y., Kyoizumi S., Akiyama M., Hiyama E., Piatyszek M.A, Shay J.W., Ishioka S. and Yamakido M. (1995) Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. J

Immunol. **155**: 3711–5.
102. Wright W., Piatyszek M., Rainey W., Byrd W. and Shay J. (1996) Telomerase activity in human germline and embryonic tissues and cells. Dev Genet **18**:173–9.
103. Yasumoto S., Kunimura C., Kikuchi K., Tahara H., Ohji H., Yamamoto H., Ide T. and Utakoji T. (1996) Telomerase activity in normal human epithelial cells. Oncogene **13**: 433–9.
104. Hiyama E., Gollahon L., Kataoka T., Kuroi K., Yokoyama T., Gazdar A.F., Hiyama K., Pietyszek M.A. and Shay J.M. (1996) Telomerase activity in human breast tumours. J Natl Cancer Inst **17**: 116–22.
105. Sommerfeld H.J., Meeker A., Piatyszek M., Bova S., Shay J., and Coffey D. (1996) Telomerase activity. A prevalent marker of malignant human prostate tissue. Cancer Res **56**: 218–22.
106. Langford L., Piatyszek M., Xu R., Schold S. and Shay J. (1995) Telomerase activity in human brain tumours. Lancet **346**:1267–8.
107. Chadeneau C., Hay K., Hirte H., Gallinger S., Bacchetti S. (1995) Telomerase activity associated with acquisition of malignancy in human colorectal cancer. Cancer Res **55**: 2533– 6.
108. Chang S. Khoo C.M., Naylor M.L., Maser R.S. and DePinho R.A. (2003) Telomere-based crisis: functional differences be- tween telomerase activation and ALT in tumour progression. Genes Dev., **17**: 88–100.
109. Gupta J., Han L.P., Wang P., Gallie B.L. and Bacchetti S. (1996) Development of retinoblastoma in the absence of telomerase activity. J Natl. Cancer Inst. **88**: 1152-7.
110. Bacchetti S. and Counter C. (1995) Telomeres and telomerase in human cancer [Review]. Int J Oncol **7**: 423–32.
111. Norton J., Piatyszek M., Wright W., Shay J. and Corey D. (1996) Inhibition of human telomerase activity by peptide nucleic acids. Nature Bio- technol. **14**:615–9.
112. He H., Wang Y., Guo X., Ramchandani S., Ma J., Shen M.F., Garcia D.A., Deng Y., Multani A.S., You M.J. and Chang S. (2009) Pot1b depletion and telomerase haploinsufficiency in mice initiate an ATR-dependent DNA damage response and elicit phenotypes resembling dyskeratosis congenita. Mol Cell Biol. **29**: 229-40.
113. Lechel A., Satyanarayana A., Ju Z., Plentz R.R., Schaetzlein S., Rudolph C., Wilkens L., Wiemann S.U., Saretzki G., Malek N.P., Manns M.P., Buer J. and Rudolph K.L. (2005) The cellular level of

telomere dysfunction determines induction of senescence or apoptosis in vivo. EMBO **6** (3): 275-81.
114. Biroccio A., Rizzo A., Elli R., Koering C.E., Belleville A., Benassi B., Leonetti C., Stevens M.F., D'Incalci M., Zupi G. and Gilson E. (2006) TRF2 inhibition triggers apoptosis and reduces tumourigenicity of human melanoma cells. Eur J Cancer. **42**(12): 1881-8.
115. Sharma G.G., Gupta A., Wang H., Scherthan H., Dhar S., Gandhi V., Iliakis G., Shay J.W., Young C.S. and Pandita T.K. (2003) hTERT associates with human telomeres and enhances genomic stability and DNA repair. Oncogene **22**(1): 131-46.
116. Bertuch A.A., Buckley K. and Lundblad V. (2003) The way to the end matters--the role of telomerase in tumor progression. Cell Cycle **2**(1): 36-8.
117. Bailey S.M. and Murnane J.P. (2006) Telomeres, chromosome instability and cancer.Nucleic Acids Research. **34** (8): 2408–2417.
118. Taylor A.M. (2001) Chromosome instability syndromes. Best Pract Res Clin Haematol. **14**(3): 631-44.
119. Ju Z. and Rudolph K.L. Telomeres and telomerase in cancer stem cells. Eur J Cancer. (2006) **42** (9):1197-203.
120. Rudolph K.L., Millard M., Bosenberg M.W. and DePinho RA. (2001) Telomere dysfunction and evolution of intestinal carcinoma in mice and humans. Nat Genet. **28**(2): 155-9.
121. Alonso Guerrero A. Gamero M.C., Trachana V., Fütterer A., Pacios-Bras C., Díaz-Concha N.P., Cigudosa J.C., Martínez-A C., van Wely K.H. (2010) Centromere-localized breaks indicate the generation of DNA damage by the mitotic spindle. Proc. Natl Acad. Sci. **107**: 4159–4164.
122. Image by Genetic Home Reference. (http://ghr.nlm.nih.gov/handbook/illustrations/dicentric) Published January 2013.
123. Jacob A.L. and Schar P. (2012) DNA glycosilases: in DNA repair and beyond. Chromosoma **121**(1): 1-20.
124. Friedberg, E. C., Walker, G. C. & Siede, W. DNA Repair and Mutagenesis (ASM Press, Washington DC, 1995).
125. Bootsma D., Kraemer K. H., Cleaver,J. and Hoeijmakers J. H. J. in The Metabolic and Molecular Basis of Inherited Disease Vol.1 (eds Scriver, C.R.Beaudet, A.L.Sly, W.S. and Valle, D.) 677–703 (McGraw- Hill, New York, 2001).

126. Lehmann A. R. (2001) The Xeroderma Pigmentosum group D(XPD) gene: one gene, two functions, three diseases. Genes Dev. **15**: 15–23.
127. Wilson D. M. and Thompson L. H. (1997) Life without DNA repair. Proc. Natl Acad. Sci. **94**: 12754–12757.
128. Takata M. Sasaki M.S., Sonoda E., Morrison C., Hashimoto M., Utsumi H., Yamaguchi-Iwai Y., Shinohara A. and Takeda S. (1998) Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. EMBO J. **17**: 5497–5508
129. McIntosh J.R. (1984) Mechanisms of mitosis. Trends Biochem Sci **9**: 195–8.
130. Rudner A.D. and Murray A.W. (1996) The spindle assembly checkpoint. Curr Opin Cell Biol. **8**: 773–80.
131. Wells W.A.E. (1996) The spindle-assembly checkpoint: aiming for a perfect mitosis, every time. Trends Cell Biol **6**: 228–34.
132. Rieder C.L. and Maiato H. (2004) What happens when cells cannot satisfy the spindle assembly checkpoint. Dev. Cell **7**: 637-651.
133. Pathak S., Multani A.S., Furlong C.L., Sohn S.H. (2002) Telomere dynamics, aneuploidy, stem cells, and cancer [review]. Int J Oncol **20**: 637–41.
134. McClintock B. (1938) The Production of Homozygous Deficient Tissues with Mutant Characteristics by Means of the Aberrant Mitotic Behaviour of Ring-Shaped Chromosomes. Genetics. **23**(4): 315-76.
135. Murnane J.P. and Sabatier L. (2004) Chromosome rearrangements resulting from telomere dysfunction and their role in cancer. Bioassays **26**(11): 1164-74.
136. Sabatier L., Ricoul M., Pottier G. and Murnane J.P. (2005) The loss of a single telomere can result in instability of multiple chromosomes in a human tumor cell line. Mol Cancer Res. **3**(3): 139-50.
137. Gisselsson D., Lv M., Tsao S.W., Man C., Jin C., Höglund M., Kwong Y.L., Jin Y. (2005) Telomere-mediated mitotic disturbances in immortalized ovarian epithelial cells reproduce chromosomal losses and breakpoints from ovarian carcinoma. Genes Chromosomes Cancer. **42**(1): 22-33.
138. Stewénius Y., Gorunova L., Jonson T., Larsson N., Höglund M., Mandahl N., Mertens F., Mitelman F., Gisselsson D. (2005) Structural and numerical chromosome changes in colon cancer develop through

telomere-mediated anaphase bridges, not through mitotic multipolarity. Proc Natl Acad Sci. **102**(15): 5541-6.
139. Jin Y., Stewénius Y., Lindgren D., Frigyesi A., Calcagnile O., Jonson T., Edqvist A., Larsson N., Lundberg L.M., Chebil G., Liedberg F., Gudjonsson S., Månsson W., Höglund M., Gisselsson D. (2007) Distinct mitotic segregation errors mediate chromosomal instability in aggressive urothelial cancers. Clin Cancer Res. **13**(6): 1703-12.
140. Reing J.E., Gollin S.M., Saunders W.S. (2004) The occurrence of chromosome segregational defects is an intrinsic and heritable property of oral squamous cell carcinoma cell lines. Cancer Genet Cytogenet. **150** (1): 57-61.
141. Saunders W.S., Shuster M., Huang X., Gharaibeh B., Enyenihi A.H., Petersen I., Gollin S.M. (2000) Chromosomal instability and cytoskeletal defects in oral cancer cells. Proc Natl Acad Sci **97**(1): 303-8.
142. Stewénius Y., Jin Y., Ora I., de Kraker J., Bras J., Frigyesi A., Alumets J., Sandstedt B., Meeker A.K., Gisselsson D. (2007) Defective chromosome segregation and telomere dysfunction in aggressive Wilms' tumors. Clin Cancer Res. **13** (22 Pt 1): 6593-602.
143. Glanz C., Rebetz J., Stewénius Y., Persson A., Englund E., Mandahl N., Mertens F., Salford L.G., Widegren B., Fan X., Gisselsson D. (2007) Genetic intratumour heterogeneity in high-grade brain tumours is associated with telomere-dependent mitotic instability. Neuropathol Appl Neurobiol. **33**(4): 440-54.
144. Mullins J.M., Biesele J.J. (1977) Terminal phase of cytokinesis in D-98s cells. J Cell Biol. **73** (3): 672-84.
145. Storchova Z., Pellman D. (2004) From polyploidy to aneuploidy, genome instability and cancer. Nat Rev Mol Cell Biol. **5** (1): 45-54.
146. Weaver B.A. and Cleveland D.W. (2006) Does aneuploidy cause cancer? Curr Opin Cell Biol. **18** (6): 658-67.
147. Fenech M. and Morley A.A. (1985) Measurement of micronuclei in lymphocytes. Mutat. Res. **147**: 29–36.
148. Pampalona J., Soler D., Genescà A., Tusell L. (2010) Telomere dysfunction and chromosome structure modulate the contribution of individual chromosomes in abnormal nuclear morphologies. Mutat Res. **683** (1-2): 16-22.
149. Dawson D.W., Bury H.P. (1961) The significance of Howell-Jolly bodies and giant metamyelocytes in marrow smears. J Clin Pathol. **14**:

374-80.
150. Mateuca R., Lombaert N., Aka P.V., Decordier I., Kirsch-Volders M. (2006) Chromosomal changes: induction, detection methods and applicability in human biomonitoring. Biochimie. **88** (11): 1515-31.
151. Fenech M. The in vitro micronucleus technique. Mutat Res. 2000 Nov 20; 455(1-2):81
152. Fenech M., Kirsch-Volders M., Natarajan A.T., Surralles J., Crott J.W., Parry J., Norppa H., Eastmond D.A., Tucker J.D., Thomas P. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis. 2011 Jan; **26** (1): 125-32.
153. Huang Y., Fenech M., Shi Q. (2011) Micronucleus formation detected by live-cell imaging. Mutagenesis. **26** (1): 133-8.
154. Yang A.H., Kaushal D., Rehen S.K., Kriedt K., Kingsbury M.A., McConnell M.J., Chun J. (2003) Chromosome segregation defects contribute to aneuploidy in normal neural progenitor cells. J Neurosci. **23** (32): 10454-62.
155. Cimini D., Fioravanti D., Salmon E.D., Degrassi F. (2002) Merotelic kinetochore orientation versus chromosome mono-orientation in the origin of lagging chromosomes in human primary cells. J Cell Sci. **115** (Pt 3): 507-15.
156. Huang Y., Jiang L., Yi Q., Lv L., Wang Z., Zhao X., Zhong L., Jiang H., Rasool S., Hao Q., Guo Z., Cooke H.J., Fenech M., Shi Q. (2012) Lagging chromosomes entrapped in micronuclei are not 'lost' by cells. Cell Res. **22**(5): 932-5.
157. Kimura M., Umegaki K., Higuchi M., Thomas P. and Fenech M. (2004) Methylenetetrahydrofolate reductase C677T polymorphism, folic acid and riboflavin are important determinants of genome stability in cultured human lymphocytes. J. Nutr. **134**: 48–56.
158. Amato A., Lentini L., Schillaci T., Iovino F., Di Leonardo A. (2009) RNAi mediated acute depletion of retinoblastoma protein (pRb) promotes aneuploidy in human primary cells via micronuclei formation. BMC Cell Biol. **10**:79.
159. Lindberg H.K., Wang X., Järventaus H., Falck G.C., Norppa H., Fenech M. (2007) Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. Mutat Res. **617**(1-2): 33-45.
160. Shimizu N., Itoh N., Utiyama H. and Wahl G.M. (1998) Selective entrapment of extrachromosomally amplified DNA by nuclear budding

and micronucleation during S phase. J. Cell Biol. **140**: 1307–1320.
161. Fenech M., Crott J.M. (2002) Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes—evidence for breakage–fusion-bridge cycles in the cytokinesis-block micronucleus assay Mut. Res. **504**:131–136.
162. Shimizu N., Shimuara T. and Tanaka T. (2000) Selective elimination of acentric double minutes from cancer cells through the extrusion of micronuclei. Mutat. Res. **448**: 81–90.
163. Hoffelder D.R., Luo L., Burke N.A., Watkins S.C., Gollin S.M., Saunders W.S. (2004) Resolution of anaphase bridges in cancer cells. Chromosoma. **112** (8): 389-97.
164. Thomas P., Umegaki K. and Fenech M. (2003) Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay. Mutagenesis **18**: 187–194.
165. MacGregor J. T. (1990) Dietary factors affecting spontaneous chromosomal damage in man. Prog. Clin. Biol. Res. **347**: 139–153.
166. Fenech M. (2010) The lymphocyte cytokinesis-block micronucleus cytome assay and its application in radiation biodosimetry. Health Phys. **98** (2): 234-43.
167. Murnane JP. (2006) Telomeres and chromosome instability. DNA Repair (Amst). **5** (9-10): 1082-92.
168. Shimizu N., Shingaki K., Kaneko-Sasaguri Y., Hashizume T. and Kanda T. ((2005) When, where and how the bridge breaks: anaphase bridge breakage plays a crucial role in gene amplification and HSR generation. Exp. Cell Res. 302: 233–243.
169. Chen Q. and Ames B.N., (1994) Senescence-like growth arrest induced by hydrogen peroxide in human diploid fibroblasts F65 cells, Proc. Natl. Acad. Sci. 91:4130–4134.
170. Elstner, E.F. (1987) Metabolism of activated oxygen species. In D.D. Davies, (ed.), Biochemistry of Metabolism (The Biochemistry of Plants: a Comprehensive Treatise, Vol. 11), Academic Press, San Diego, pp. 253-315.
171. Halliwell B., Gutteridge J.M. (1990) Role of free radicals and catalytic metal ions in human disease: an overview, Methods Enzymol. **186**:1–85.
172. Scandalios J.G. (2005) Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Braz J Med Biol Res, **38**: 995-1014.

173. Kirsch-Volders M., Vanhauwaert A., Eichenlaub-Ritter U., and Decordier I. (2003) Indirect mechanisms of genotoxicity. Toxicol Lett. **140-141**: 63-74
174. Halliwell B., Gutteridge J.M.C., (1989) Free Radicals in Biology and Medicine, Clarendon Press, Oxford.
175. Halliwell B., Aruoma O.I. (1991) DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett. **281**(1-2): 9-19.
176. Shigenaga M.K. and Ames, B.N., (1991) and Assays for 8-hydroxy-2' -deoxy-guanosine: a biomarker of in vivo oxidative DNA damage, Free Radic. Biol. Med. **10**: 211–216.
177. Halliwel B. and Aruoma O. I. (1991) DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett. **281**: 9-19.
178. 178Winkler B.S., Boulton M.E., Gottsch J.D., Sternberg P. (1999) Oxidative damage and age-related macular degeneration Molecular vision **5**: 32.
179. 179Mateuca R., Lombaert N., Aka P. V., Decordier I., and Kirsch-Volders M. (2006) Chromosomal changes: induction, detection methods and applicability in human biomonitoring. Biochimie **88**: 1515-31.
180. Natarajan A.T. (1993) Mechanisms for induction of mutations and chromosome alterations. Environ Health Perspect **101** (3): 225-9.
181. Friedberg E.C. (1995) Out of the shadows and into the light: the emergence of DNA repair. Trends Biochem Sci **20**: 381.
182. Kaneko T., Tahara S., Taguchi T., Kondo H. (2001) Accumulation of oxidative DNA damage, 8-oxo-2'-deoxyguanosine, and change of repair systems during in vitro cellular aging of cultured human skin fibroblasts. Mutat Res. **487** (1-2): 19-30.
183. Grollman A.P., Moriya M. (1993) Mutagenesis by 8-oxoguanine: an enemy within, Trends Genet. **9**: 246–249.
184. Kamiya H., Miura K., Ishikawa H., Inoue H., Nishimura S., Ohtsuka E. (1992) c-Ha-ras containing 8-hydroxyguanine at codon 12 induces point mutations at the modified and adjacent positions, Cancer Res. **52**: 3483–3485.
185. Blackburn E.H. (2001) Switching and signaling at the telomere. Cell **106**: 661–673.
186. Cooke H.J. and Smith B.A. (1986). Variability at the telomeres of the

human X/Y pseudoautosomal region. Cold Spring Harbor Symp. Quant. Biol. LI, 213–219.
187. Grollman A.P., Moriya M. (1993) Mutagenesis by 8-oxoguanine: an enemy within, Trends Genet. **9**: 246–249.
188. Memisoglu A. and Samson L. (2000) Base excision repair in yeast and mammals. Mutat Res, **451**: 39-51.
189. Robertson A.B., Klungland A., Rognes T. and Leiros I. (2009) DNA repair in mammalian cells: Base excision repair: the long and short of it. Cell Mol Life Sci, **66**: 981-93.
190. Dodson M.L., Lloyd R.S. (2002) Mechanistic comparisons among base excision repair glycosylases, Free Radic. Biol. Med. **32**: 678-682.
191. Liu Y. and S.H. Wilson (2012) DNA base excision repair: a mechanism of trinucleotide repeat expansion. Trends Biochem Sci. **37**: 162-72.
192. Kawanishi S. and Oikawa S. (2004) Mechanism of telomere shortening by oxidative stress. Ann. N.Y.Acad. Sci. **1019**: 278-284.
193. Makarov V.L. Hirose Y., Langmore J.P. (1997) Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. Cell **88**: 657–666.
194. Von Zglinicki T. (1999). The role of oxidative stress in telomere length regulation and replicative senescence. Ann. N.Y. Acad. Sci
195. Von Zglinicki T., Pilger R., Sitte N. (2000) Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. Free Radic Biol Med **28**: 64–74.
196. von Zglinicki, T. Saretzki G., Döcke W., Lotze C. (1995) Mild hyperoxia shortens telomeres and inhibits proliferation of human fibroblasts: a model for senescence? Exp. Cell Res. **220**: 186–193.
197. Vaziri H. West M.D., Allsopp R.C., Davison T.S., Wu Y.S., Arrowsmith C.H., Poirier G.G., Benchimol S. (1997) ATM-dependent telomere loss in aging human diploid fibroblasts and DNA damage lead to the post-translational activation of p53 protein involving poly(ADP-ribose)polymerase. EMBO J. **16**: 6018–6033.
198. Saito M., Hisatome I., Nakajima S. and Sato R. (1995) Possible mechanism of oxygen radical production by human eosinophils mediated by K+ channel activation. Eur J Pharmacol. **291**: 217-9.
199. Sugiyama H., Saito I., (1996) Theoretical studies of GG-specific photo-cleavage of DNA vie electron transfer: significant lowering of ionization potential and 5'-localization of HOMO of stacked GG bases

in B-form DNA, J. Am. Chem. Soc. **118**: 7063-7068.
200. Saito I., Nakamura K., Nakatani K. (1998) Mapping of the hot spots for DNA damage by one-electron oxidation: efficacy of GG doublets and GGG triplets as a trap in long-range hole migration, J. Am. Chem. Soc. **120**: 12686-12687.
201. Oikawa S., Kawanishi S. (1999) Site-specific DNA damage at GGG sequence by oxidative stress may accelerate telomere shortening. FEBS Lett **453**: 365–368.
202. Henle E.S., Han Z., Tang N., Rai P., Luo Y., Linn S. (1999) Sequence-specific DNA cleavage by Fe2+-mediated fenton reactions has possible biological implications. J Biol Chem **274**: 962–971.
203. Rhee D. B., Ghosh A., Lu J., Bohr V.A., and Liu Y. (2011) Factors that influence telomeric oxidative base damage and repair by DNA glycosylase OGG1. DNA Repair (Amst) **10**: 34-44.
204. Serra V, Grune T, Sitte N, Saretzki G, von Zglinicki T. (2000) Telomere length as a marker of oxidative stress in primary human fibroblast cultures. Ann N Y Acad Sci. **908**:327-30.
205. Sitte N., Saretzki G. and von Zglinicki T. (1998) Accelerated telomere shortening in fibroblasts after extended periods of confluency. Free Radic. Biol. Med. **24**: 885–893.
206. Oexle K, Zwirner A. (1997) Advanced telomere shortening in respiratory chain disorders. Hum Mol Genet. **6**(6): 905-8.
207. Opresko P.L., Fan J., Danzy S., Wilson III D.M. and Bohr V.A. (2005) Oxidative damage in telomeric DNA disrupts recognition by TRF1 and TRF2. Nucleic Acids Research **33**(4): 1230–1239
208. Petersen S. Saretzki G., von Zglinicki T. (1998) Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. Exp. Cell Res. **239**: 152–160.
209. Ohki R. and Ishikawa F. (2004) Telomere-bound TRF1 and TRF2 stall the replication fork at telomeric repeats. Nucleic Acids Res. **32**: 1627–1637.
210. Richter T., Saretzki G., Nelson G., Melcher M., Olijslagers S., von Zglinicki T. (2007) TRF2 overexpression diminishes repair of telomeric single- strand breaks and accelerates telomere shortening in human fibroblasts. Mech. Ageing Dev. **128**: 340–345.
211. Furumoto K., Inoue E., Nagao N., Hiyama E., Miwa N. (1998) Age-dependent telomere shortening is slowed down by enrichment of

intracellular vitamin C via suppression of oxidative stress. Life Sci. **3**: 935–948.
212. Griffin C.S. (2002) Aneuploidy, centrosome activity and chromosome instability in cells deficient in homologous recombination repair. Mutat Res. **504**(1-2): 149-55.
213. Wang Z., Rhee D. B., Lu J., Bohr C. T., Zhou F., Vallabhaneni H., de Souza-Pinto N. C. and Liu Y. (2010) Characterization of oxidative guanine damage.
214. Bodnar A.G., Ouellette M., Frolkis M., Holt S.E., Chiu C.P., Morin G. B., Harley C. B., Shay J. W., Lichtsteiner S., Wright W. E. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* **279:** 349–352.
215. Fiers W., Beyaert R., Declercq W., Vandenabeele P. (1999) More than one way to die: apoptosis, necrosis and reactive oxygen damage, Oncogene **18**: 7719– 7730.
216. Guachalla L.M. and Rudolph K.L. (2010) ROS induced DNA damage and checkpoint responses: influences on aging?, Cell Cycle **9**: 4058–4060.
217. Von Zglinicki T. et al. (1995) Mild hyperoxia shortens telomeres and inhibits proliferation of human fibroblasts: a model for senescence? Exp. Cell Res. **220**: 186–193.
218. Von Zglinicki T. (2002) Oxidative stress shortens telomeres. Trends Biochem Sci. 27(7): 339-44.
219. Raynaud C.M., Sabatier L., Philipot O., Olaussen K.A., Soria J.C. (2008) Telomere length, telomeric proteins and genomic instability during the multistep carcinogenic process. Crit Rev Oncol Hematol. **66** (2): 99-117.
220. Ducray C. and Sabatier L. (1998) Role of chromosome instability in long term effect of manned-space missions. Adv. Space Res. **22**: 597–602.
221. Der-Sarkissian H., Bacchetti S., Cazes L. and Londono- Vallejo J. A. (2004) The shortest telomeres drive karyotype evolution in transformed cells. Oncogene **23**: 1221–1228.
222. Lantuejoul S., Salon C., Soriaand J.C. and Brambilla E. (2007) Telomerase expression in lung preneoplasia and neoplasia. Int. J. Cancer **120**: 1835–1841.
223. Liu D. Y., Peng Z. H., Qiu G. Q. and Zhou C. Z. (2003) Expression of

telomerase activity and oxidative stress in human hepatocellular carcinoma with cirrhosis. World J. Gastroenterol. **9**: 1859–1862.
224. Hande M. P., Lansdorp P. M. and Natarajan A. T. (1998) Induction of telomerase activity by in vivo X-irradiation of mouse splenocytes and its possible role in chromosome healing. Mutat. Res. **404**: 205–214.
225. Leteurtre F., Li X., Gluckman E. and Carosella E. D. (1997) Telomerase activity during the cell cycle and in gamma- irradiated hematopoietic cells. Leukemia **11**: 1681–1689.
226. Xing J., Zhu Y., Zhao H., Yang H., Chen M., Spitz M.R. andWu X. (2007) Differential induction in telomerase activity among bladder cancer patients and controls on gamma-radiation. Cancer Epidemiol. Biomarkers Prev. **16**: 606–609.
227. Morrish T.A. and Greider C. W. (2009) Short telomeres initiate telomere recombination in primary and tumor cells. PLoS Genet. **5**: e1000357.
228. Bechter O.E., Shay J.W. and Wright W.E. (2004) The frequency of homologous recombination in human ALT cells. Cell Cycle 3: 547-549
229. Bailey S.M., Goodwin E. H. and Cornforth M. N. (2004) Strand-specific fluorescence in situ hybridization: the CO-FISH family. Cytogenet. Genome Res. **107**: 14–17.
230. Pampalona J., Frías C., Genescà A., Tusell L. (2012) Progressive telomere dysfunction causes cytokinesis failure and leads to the accumulation of polyploid cells. PLoS Genet. **8**(4):e1002679.
231. Gonzalo S., Jaco I., Fraga M.F., Chen T., Li E., Estellerand M. and Blasco M. A. (2006). DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat. Cell Biol. **8**: 416 – 424.
232. Cimini D. (2008) Merotelic kinetochore orientation, aneuploidy, and cancer. Biochim Biophys Acta. **1786**: 32-40.