ROMA TRE
UNIVERSITÀ DEGLI STUDI

DOTTORATO IN BIOLOGIA MOLECOLARE, CELLULARE E AMBIENTALE

XXX CICLO

ALLUNGAMENTO ALTERNATIVO DEL TELOMERO (ALT) COINVOLTO NELLA MODULAZIONE DELLA LUNGHEZZA TELOMERICA INDOTTA DA RAGGI-X IN FIBROBLASTI UMANI PRIMARI

ALTERNATIVE LENGTHENING OF TELOMERE (ALT) IMPLICATED IN TELOMERE LENGTH MODULATION INDUCED BY X-RAYS IN HUMAN PRIMARY FIBROBLASTS

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RIASSUNTO

I telomeri sono strutture nucleoproteiche poste alle estremità cromosomiche, organizzati in domini eterocromatici aventi un ruolo fondamentale nella stabilità genomica e cromosomica.
Nell'uomo consistono di 2-30 Kbp di ripetizioni TTAGGG e di proteine telomeriche.
A ogni divisione cellulare e replicazione del DNA i telomeri tendono ad accorciarsi e per questo rappresentano una sorta di "orologio biologico" per la cellula stessa. Una cellula normale avente un telomero corto, infatti, entrerà in senescenza e attiverà meccanismi di apopotosi.
Tale meccanismo è utile, poiché evita l'attivazione di sistemi di riparazione al telomero in grado di indurre instabilità cromosomica e genomica con conseguente possibile trasformazione neoplastica della cellula.
Alcune cellule, però, possono riuscire a evadere tale controllo, attivando una serie di meccanismi che, essendo capaci di riallungare il telomero, controbilanciano questo naturale e fisiologico accorciamento. Per questo motivo tali meccanismi sono definiti "meccanismi di mantenimento telomerico".
Questi sono principalmente due: la telomerasi, e *l'alternative lengthening of telomere* (ALT).

La telomerasi è il meccanismo di mantenimento telomerico che si ritrova maggiormente in natura ed è quello più conosciuto dal punto di vista molecolare. Si tratta di un complesso ribonucleoproteico che utilizza uno stampo a RNA per aggiungere ripetizioni telomeriche alle estremità 3' dei cromosomi. Si ritrova nelle cellule della linea germinale, nelle cellule staminali e nell'80-85% dei tumori conosciuti.

L'*alternative lengthening of telomere* (ALT) invece si ritrova nelle cellule del restante 15-20% dei tumori ed è poco conosciuto dal punto di vista molecolare.
Basato sulla ricombinazione tra telomeri, è un meccanismo in grado di aggiungere ripetizioni telomeriche all'estremità cromosomiche mediante interazione tra telomeri appartenenti a cromatidi fratelli dello stesso cromosoma o a cromosomi differenti.

Il bersaglio più importante delle radiazioni ionizzanti (IR) nelle cellule eucariotiche è il DNA.

Esse, infatti, sono in grado di interagire con il materiale nucleare danneggiandolo mediante un danno di tipo diretto o indiretto, secondo la tipologia di radiazione. I raggi-X, in particolar modo, tendono a interagire con il DNA in modo indiretto, innescando la lisi delle molecole di acqua presenti nel mezzo biologico, con successiva genesi di specie reattive dell'ossigeno (ROS), in grado di causare danno al DNA tramite induzione di stress ossidativo. Quest'ultimo è stato dimostrato essere capace di indurre accorciamento telomerico. Infatti, il più frequente danno ossidativo al DNA nelle cellule umane è rappresentato dall'8-oxoguanina (8-oxoG). Il telomero evidenzia un'elevata presenza di residui guaninici e una bassa efficienza nel riparare il danno al DNA. Questo lo rende il target di favore per l'accumulo di basi ossidate in grado di indurre rotture del DNA a livello telomerico.
Studi precedenti hanno dimostrato che radiazioni ionizzanti particellari (tipo protoni) sono in grado di indurre allungamento telomerico mediante l'attivazione di un meccansimo simil-ALT, mentre dati riguardanti i soli raggi-X indicano che questi sono in grado di indurre modulazione delle lunghezze telomeriche a tempi lunghi senza però approfondire ulteriormente i meccanismi dietro tale modulazione.
Per chiarire bene la causa e la cinetica di tale modulazione, sono stati trattati fibroblasti umani primari fetali di prepuzio (HFFF2) con 4Gy di raggi-X ed è stata eseguita un'analisi delle lunghezze telomeriche mediante Q-FISH dopo 3,4,5,6,7,8,10 e 13 giorni dal trattamento.
I risultati hanno mostrato una modulazione del telomero durante tale periodo, con un iniziale accorciamento a 3 giorni, un allungamento a 4, un nuovo accorciamento durante 5 e 7 giorni ed un ulteriore allungamento, che si è mantenuto stabile dagli 8 fino ai 13 giorni.
Per valutare il danno al telomero, sono stati analizzati i TIFs (telomere induced dysfunctional foci), mediante colocalizzazione tra telomero e proteine yH2AX e 53BP1, dimostrando una stretta corrispondenza tra accorciamento e danno telomerico.
Ci si è quindi interrogati su quale fosse il meccanismo attivato nelle cellule dopo irraggiamento, in grado di allungare il telomero.
Inizialmente si è ipotizzata un'attivazione della telomerasi, ma l'analisi dell'attività di tale enzima mediante RTQ-TRAP assay non ha mostrato nelle cellule trattate un aumento dell'attività di tale proteina.
Tale risultato si trova in accordo con i tanti dati in letteratura, dove è stato ampiamente dimostrato che cellule di origine mesenchimale, come i fibroblasti appunto, difficilmente attivano la telomerasi come

meccanismo di mantenimento telomerico, poiché probabilmente durante il differenziamento delle cellule a livello embrionale, i geni deputati all'attivazione e regolazione di tali enzimi sono strettamente silenziati.
L'attenzione si è spostata quindi sul secondo meccanismo in grado di indurre allungamento telomerico: l'ALT.
Sono state analizzate quindi alcune proteine notoriamente implicate in tale meccanismo oltre a due importanti *markers* di ALT: i *telomere sister chormatid exchanges* (T-SCE) mediante CO-FISH e gli *ALT-associated PML bodies* (APBs) mediante immunoFISH.
I risultati hanno mostrato una correlazione con l'aumento delle lunghezze telomeriche; nello specifico ad un incremento delle lunghezze corrispondeva un aumento delle frequenze dei due *markers*.
A conferma di ciò, l'analisi di proteine quali ATRX, RAD51 e RPA2, mediante western blot, ha evidenziato un profilo simile a quello che si ritrova all'interno di cellule ALT-positive. Tali risultati sono prova dell'attivazione di un meccanismo ALT in cellule normali, basato sulla ricombinazione tra telomeri, probabilmente simile a quello che si ritrova in cellule tumorali.
Si è voluto infine indagare circa il ruolo dello stress ossidativo nella modulazione telomerica osservata dopo trattamento con raggi-X.
Un'analisi mediante diclorofluoresceina-diacetato (DCFH-DA) delle specie reattive dell'ossigeno, ha evidenziato un persistente stress ossidavo per i primi 4 giorni dopo trattamento, che risulta essere, effettivamente, il periodo di tempo in cui si ritrova accorciamento e maggior danno telomerico.
La somministrazione di N-acetilcitosina (NAC), un noto antiossidante, 30 minuti prima e ogni 24 ore dopo trattamento con raggi-X, invece, non ha mostrato nessuno aumento dello stress ossidativo e l'analisi delle lunghezze telomeriche mediante Q-FISH non ha mostrato nessun *trend* di accorciamento ed allungamento.
Questi risultati consentono di concludere che fibroblasti umani primari fetali di prepuzio HFFF2, dopo trattamento con raggi-X, sono in grado di generare ROS ed uno stress ossidativo persistente, in grado a suo volta di danneggiare indirettamente il DNA e in particolare modo il telomero.
Di fronte a tale danno telomerico, la cellula, che potrebbe andare incontro a senescenza e/o apoptosi, è invece indotta ad attivare un meccanismo ALT, atto al riallungamento delle lunghezze telomeriche.
L'attivazione di tale meccanismo in cellule primarie in seguito a danno non è scontata, ma è un risultato estremamente interessante.
Nonostante l'ALT sia conosciuto canonicamente come un

meccanismo di mantenimento telomerico attivo in cellule tumorali, questo progetto di dottorato, insieme ad altri recenti e pochi lavori in letteratura, ne definisce un nuovo ruolo.
Infatti, mentre in cellule tumorali l'ALT è deputato al mantenimento telomerico e quindi risulta avere un ruolo centrale nell'immortalizzazione e nello sviluppo neoplastico (probabilmente dovuto ad una deregolazione dell'intero meccanismo che sfugge al controllo cellulare); in cellule primarie, invece, l'ALT potrebbe rappresentare un sistema finemente regolato e controllato di "riparazione telomerica" in risposta al danno.

SUMMARY

Telomeres are nucleoprotein structures localized at the end of chromosomes, organized in heterochromatic domains and involved in genome and chromosome stability.
Human telomeres consist of 2-30 Kbp of repeated TTAGGG sequences and telomeric proteins.
At every round of replication, telomeres become shorter and when they reach a threshold length, the cell goes through senescence and apoptosis, preventing DNA repair mechanisms activation at telomeres (which could induce chromosome and genome instability) and thus avoiding cellular transformation.
Sometimes cells could escape from senescence and apoptosis activating some mechanisms that can elongate telomeres again, counteracting the natural and physiological shortening.
These mechanisms are called telomeres maintenance mechanisms (TMMs). Two TMMs are mainly known: telomerase and alternative lengthening of telomere (ALT).

Telomerase is the most common TMM used by cells to elongate and maintain telomeres and it is well known from a molecular point of view.
It consists of a ribonucleoproteic complex with an enzymatic activity that uses an RNA template to add telomeric TTAGGG sequences at the 3'-overhang ends of chromosomes.
Telomerase is activated in germinal cells, stem cells and in the 80-85% of tumors.

The alternative lengthening of telomere (ALT) pathway has been found in the 15-20% of the remaining tumors and molecular factors and causes of its activation are still not well known.
Based on homologous recombination, this mechanism is able to add telomeric sequences at chromosome ends by interaction of telomeres, which belong to sister chromatids or different chromosomes.

The most important target of ionizing radiations (IRs) in eukaryotic cells is the DNA.
In fact, IRs can interact and induce DNA damage by a direct or an indirect effect, depending on the ionizing radiation typology.
X-rays can interact with DNA by an indirect way, giving rise to water radiolysis and generating reactive oxygen species (ROSs) that are able

to induce DNA damage. It has been demonstrated that oxidative stress can cause telomere shortening. In fact, 8-oxoguanine (8-oxoG) is the most common oxidative DNA damage in human cells and telomere shows high presence of guanine residues and low DNA repair efficiency.
In this way, telomere is able to accumulate oxidized bases, which can induce telomeric breaks.
Previous studies demonstrated that particle ionizing radiations (like protons) are able to induce telomere lengthening by activation of an ALT-like mechanism, while literature data about X-rays indicate just the capability of this kind of IR to induce telomere length modulation without deepen the mechanisms behind such modulation.
To better understand the cause and the kinetics of this modulation human primary fetal foreskin fibroblasts (HFFF2) were treated with 4 Gy dose of X-rays and it was performed an analysis for telomere length by Q-FISH at 3,4,5,6,7,8,10 and 13 days after treatment.
Results showed a telomere length modulation with a trend of shortening and elongation during this time period.
A TIFs (telomere dysfunctional induced foci) analysis performed by colocalization between telomere, yH2AX and 53BP1 proteins (as a telomere damage index), showed a close correlation between telomere shortening and telomere damage.
We first supposed that the main cause for this telomere modulation could be telomerase activation after X-rays treatment. But an analysis performed by RTQ-TRAP assay did not show any activity of this enzyme.
This was expected, according to many literature data. In fact, it has been fully demonstrated that cells with mesenchymal embryonic origin (like fibroblasts) do not activate telomerase as TMM, probably because genes involved in telomerase activation and regulation are tightly silenced during cellular differentiation.
The possibility of ALT activation was then investigated, analyzing some proteins involved and two different hallmarks of ALT: telomere sister chromatid exchanges (T-SCEs) by CO-FISH and the ALT associated PML bodies (APBs) by immunoFISH.
APBs and T-SCEs showed a correlation with telomere elongation.
Also the analysis of ALT related proteins such as ATRX, RAD51 and RPA2 by western blot showed a pattern similar to the ALT-positive cell lines.
These results proved that X-rays treatment is able to activate an ALT pathway similar to the one found in ALT-positive tumors.
Finally, the way by X-rays are able to induce telomere length

modulation and ALT activation was investigated.
Thus, a dichlorofluorescein-diacetate (DCFH-DA) analysis for ROS put in evidence a persistent oxidative stress during the first 4 days after X-rays treatment, that was the same time period in which telomere shortening and the highest telomere damage was detected.
On the contrary, administration to HFFF2 of N-acetyl-cysteine (NAC), an antioxidant molecule, 30 minutes prior and every 24 hours after X-rays treatment, did not show any oxidative stress and any modulation of telomere length observed by Q-FISH.
All these results allow to conclude that X-rays are able to induce a persistent oxidative stress, which can induce DNA damage, in a particular way at telomeres.
Thus, cells characterized by telomeric damage can activate the ALT pathway in order to elongate telomeres again.
Finally, ALT activation inside primary cells has to be considered an interesting result.
Since ALT has been found in tumor cells, this PhD project describes (together with other few and recent works in literature) a new role for this pathway.
In fact, if in tumors ALT is involved in telomere maintenance as well as in cellular immortalization and cancer development (probably because it is completely deregulated); inside primary cell lines, ALT could act as a strictly regulated pathway involved in damage-induced telomere repair.

INTRODUCTION

1. TELOMERE

1.1 Telomere structure

Genome integrity is essential for life. It has long been known that chromosome ends require a protection against degradation by nuclease and end-to-end fusions. In 1938, Muller realized that chromosome ends are specialized structures with a particular function that are able to seal chromosome *termini*. He coined the term "telomere" derived from greek words: *telos* and *meros*, that mean "end" and "part" [1]. In 1941, Barbara McClintock observed that broken ends in Zea mays are able to fuse each other giving rise to dicentric chromosomes. This extremity was called "sticky"; whereby normal chromosome ends were called "no sticky" [2]. Only 37 years later Blackburn and Gall discovered the sequences that form telomeres [3]. In human, telomeres consist of 2-30 Kbp conserved, non coding, regions composed by tandem repeats of the G-Rich exanucleotide (TTAGGG)n [4] **(Fig. 1)**

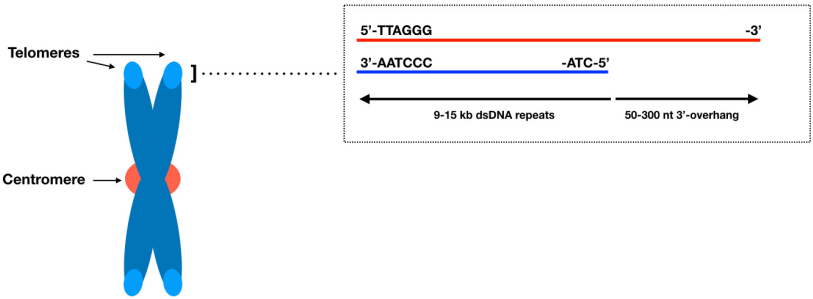


Figure 1: Schematic representation of mammalian sequences of telomere.

Repeats are oriented 5' to 3' towards the end of the chromosome, ending in a fundamental 3' single G-stranded overhang, which length is species-specific. In human telomeres has a variable G-stretch of 50 to 100 nucleotides [5].

Studies by electron microscopy suggested that the end of chromosomes forms a large loop (t-loop, telomeric loop) generated by the invasion of the 3'-overhang strand into the telomeric duplex itself,

resulting in the displacement of the TTAGGG repeats strand, forming a second loop (d-loop, displacement loop).
The circular segment of the loop is composed by duplex DNA **(Fig. 2)**.

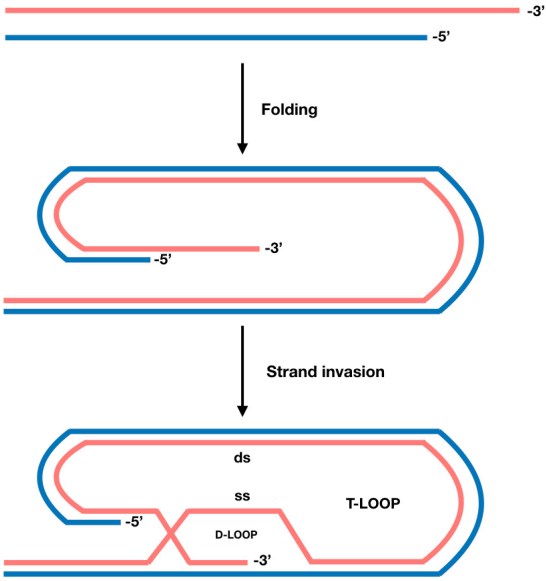


Figure 2: Telomeric loop formation. The 3'-overhang single strand of telomeric DNA invades the telomeric duplex, forming first the telomeric loop (t-loop) and then the displacement loop (d-loop).

1.2 Telomere proteins

Telomeres DNA interacts with different proteins that together form the "shelterin complex", involved in chromosome end integrity and dynamics [6], t-loop formation and telomere protection [7].
The shelterin complex is made up of six different proteins: TRF1 and TRF2 (telomeric repeats binding factor 1 and 2, respectively) RAP1 (repressor activator protein 1), TIN2 (TRF1-interactin protein 2), TPP1 (tripeptidyl peptidase 1) and POT1 (protection of telomeres 1) [6] **(Fig. 3)**.

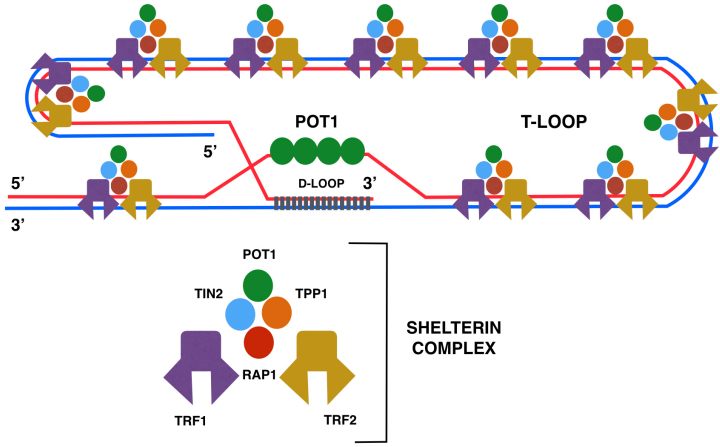


Figure 3: Telomeric loop. Telomere structure is composed by the DNA and the telomeric "shelterin complex".

The shelterin complex is a very important component of telomere since it interacts with other DNA associated factors in order to remodel and change the telomere structure, maintaining its main role in telomere protection [8]. Proteins of the shelterin complex bind telomeres because of their higher specificity for the telomere TTAGGG sequence. In fact, TRF1 and TRF2 directly bind the telomere duplex as a homodimer [8]; also POT1 has a strong specificity, but it binds to single stranded telomeric sequences. TRF1 and TRF2 play together an important role in binding telomeres [9]. Indeed, despite they have similar functional domains, with small but significant differences (for example TRF1 binds more strongly the DNA than TRF2 [10]), they have different functions [11].

TRF2 is essential for the formation of the t-loop; it was observed at the tail-loop junction stabilizing the d-loop and allowed the invasion of the single-strand overhang into the telomeric duplex; it also protects the 3'-overhang [12]. TRF1 binds the circular segment of the duplex that is also composed by duplex array of TTAGGG. This protein is required for the efficient replication of telomeres [13,14].

TIN2 is a linker protein, it interacts with TRF1, TRF2 and TPP1 holding and stabilizing the complex [15]. Also TPP1 is a linker protein, it interacts with POT1 and TIN2 forming a link between the 3'-overhang and the duplex telomeric repeats [15]. Finally, RAP1 interacts with TRF2 [15].

At first, it seems that the six proteins act as a single functional unit, because the disruption of just one protein can affect the role of the others leading to an unprotected telomere, even if each single component has developed a specific function in telomere replication and end protection [16].
Other proteins, most of them associated with the DNA damage repair, are located at the end of chromosomes: tankyrase 1 and 2 (TANK1-2), DNA- dependent protein kinase (DNA-PK), Ku 70/86 that form the complex DNA-PK, ATM (ataxia telangiectasia mutated), PARP-1 (poly-ADP ribose polymerase -1), EXCC1/XPF, RAD51, WRN, BLM and the MRN complex (MRE11/RAD50/NBS) [7].

1.3 Telomere functions and replication

Telomere is involved in different functions within the cell including chromosome stability, correct chromosome segregation, apoptosis, cellular division, cellular senescence and tumor development.
As mentioned before, its main role is to protect the end of linear chromosomes. In fact, the t-loop ensures chromosome stability and avoids that chromosome ends could be recognized as free, sticky and damaged ends (like double strand breaks, DSBs), which could give rise to end-to-end fusions [17]. Telomere is affected by a natural shortening because of the "end replication problem". In fact, semi-conservative replication of eukaryotic DNA generates two different strands: the leading strand that is completely replicated, and the lagging strand that is not fully replicated. This difference is due to the inability of the conventional DNA polymerase to fully replicate the extreme 3'-end of the DNA in absence of a template strand [18]. In fact, the RNA primer of the most distal Okazaki fragment is removed during the end of replication; leading to a loss of some DNA bases every round of replication **(Fig. 4)**.
Indeed, it is known that at the end of each cycle of replication, about 100-200 bases of TTAGGG repeats are lost [19-21], bringing to a continuous telomere shortening [22]. Thus, when in somatic cells telomeres become critically short (after many rounds of cell division), they are sensed as DNA damage by the DNA damage repair machinery and the cell enters senescence or dies (apoptosis) [23], preventing immortalization of cells and then tumorigenesis.

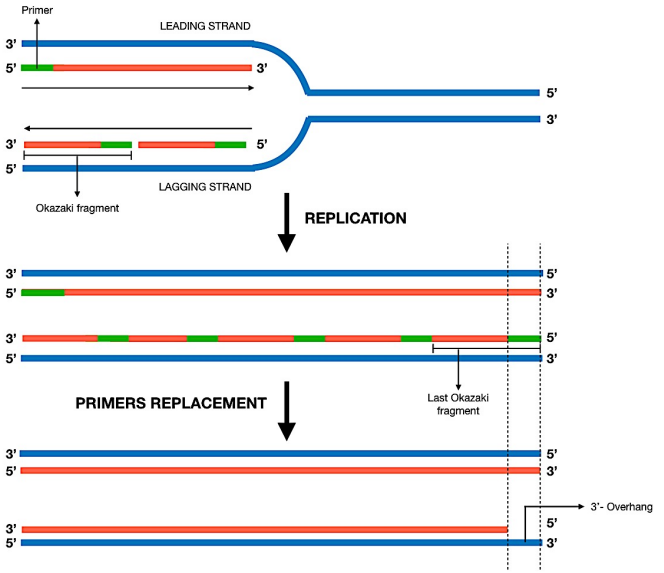


Figure 4: The "end replication problem". The removal of the last Okazaki fragment in the lagging strand leaves a gap of unreplicated DNA.

In addition, Hayflick and Moorehead discovered in 1961 that human cells derived from embryonic tissues could only divide a finite number of times in culture [24]. This phenomenon is known as "Hayflick limit" and acts as a "mitotic clock" within the cells. Different studies, including the mathematical approach of Olovnikov, demonstrated that this limit is determined by the initial length of telomeres and by the rate of telomere shortening [18,25].

So, as it was said before, when telomeres become too short and they reach the Hayflick limit, they can face chromosome fusions and rearrangements that could lead to chromosome instability and alteration of gene-expression patterns.

If this happens, cells with dysfunctional telomeres can escape senescence and further divide beyond their "replicative limit" entering in a phase called "crisis". Crisis is characterized by massive genome instability [20,26] and, during this step, cells could activate mechanisms able to elongate telomeres again, making themselves immortal [20]. These mechanisms are known as telomere maintenance mechanisms (TMMs). In this way, immortalized cells continue to divide accumulating DNA damage and mutations, giving rise to

further chromosome instability: all these modifications could lead to tumor development [27] **(Fig. 5)**.

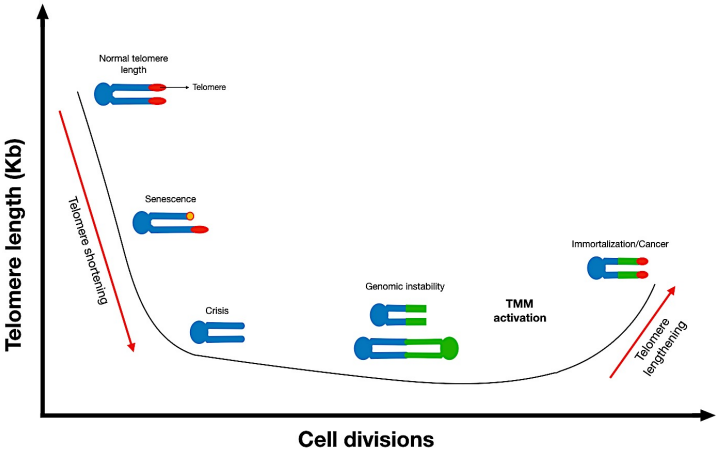


Figure 5: At each cell division telomere becomes shorter. When telomeres are too short, cells activate a mechanism by which they enter in senescence or apoptosis to avoid chromosome fusions and rearrangements. Cells with dysfunctional telomeres continue to divide and enter a stage termed "crisis". This step is characterized by genome instability that could bring to TMMs activation.

1.4 Telomere maintenance mechanisms

Unlimitedly proliferating cells need to acquire a telomere DNA maintenance mechanism to counteract the natural shortening through multiple rounds of replication and segregation of linear chromosomes. For this reason, human cancer cells are characterized by one of the two known TMM mechanisms. Most human cancer cells express telomerase whereas other cells utilize the alternative lengthening of telomeres (ALT) pathway to elongate telomeric DNA.

Telomerase is a ribonucleoprotein complex that regulates telomere length maintenance adding telomeric repeats to the 3'-end of chromosome using a RNA template [28]. Telomerase activity was primary discovered by Greider and Blackburn in 1985 in *Tetrahymena thermopile*. They found that telomeric ends were elongated without a DNA template. In fact, telomerase is a reverse transcriptase that consists of a non-coding RNA (TERC) that provides the template for *de novo* synthesis of telomeric DNA sequences and an enzymatic part (TERT). Other proteins, for example dyskerin, take part to the formation, stabilization and function of telomerase **(Fig. 6)**.

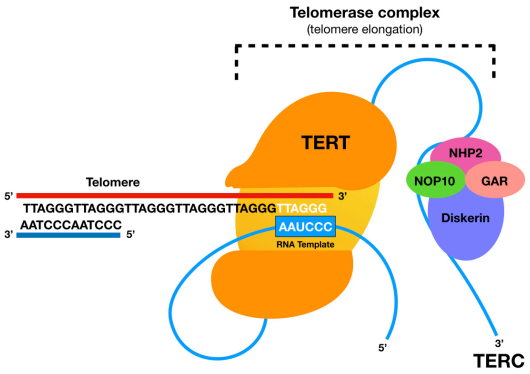


Figure 6: Telomerase complex. Telomerase is a ribonucleoprotein complex composed by an RNA template (TERC) and a catalytic subunit (TERT). Dyskerin and other proteins take part to telomerase function.

Telomerase is inactive in somatic cells, due to the lack of the catalytic protein subunit gene transcription; this contributes to the telomere shortening at each cellular division. On the other hand, stem cells and germ line cells show telomerase activity. In fact, in this type of cells telomerase is essential to maintain the integrity of telomeres, to prevent the loss of genetic information and to ensure chromosome stability [29]. Finally, the 80-85% of cancers show telomerase activity.

2. ALTERNATIVE LENGTHENING OF TELOMERE (ALT)

In the remaining 15-20% of tumors telomere length is maintained by the alternative lengthening of telomere (ALT) mechanism.
This mechanism is based on homologous recombination (HR) dependent exchange and/or HR dependent synthesis of telomeric DNA [30].
Basically, in this process, in a damaged or partially replicated telomeric strand the 3'-overhang end is able to invade the duplex telomeric region of another telomere (that belongs to a sister chromatid or to another chromosome) to initiate DNA synthesis, yielding a net gain in telomere length [rewied in Nabetani, 31] **(Fig.7).**

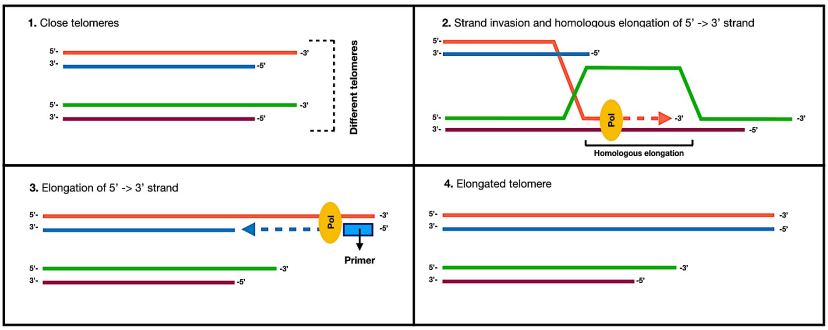


Figure 7: Alternative lengthening of telomere. This mechanism is based on recombination that leads to telomere elongation.

Alternatively, linear or circular extra-chromosomal telomeric DNA fragments (that have been found in ALT cells) could serve as templates for telomere synthesis by rolling circle amplification [32].
As previously mentioned, ALT-positive tumors occur less frequently than telomerase-positive tumors, but surveys of human cancers have identified evidence of ALT in glioblastoma multiforme (GBM) [33], diffuse intrinsic pontine gliomas (DIPG) [34], pancreatic neuroendocrine (PanNET) tumors [35], human epidermal growth factor receptor-2 (HER-2) positive breast carcinoma [36], cancers of mesenchymal origin including osteosarcoma, astrocytoma, and liposarcoma, and tumors from Li–Fraumeni syndrome patients [37].
Although it is unknown exactly how ALT cells elongate their telomeres, cells that utilize ALT display an array of unique features and these hallmarks have provided valuable insights into telomere maintenance by the ALT pathway [38]. In particular, ALT telomeres

are characterized by a high telomere length heterogeneity, telomere sister chromatid exchanges (T-SCEs), presence of PML bodies at telomeres (APBs) and presence of extra chromosomal telomere repeats (ECTRs).
In addition, specific proteins (such the ones involved in homologous recombination) have been found to be involved in the ALT mechanism.

2.1 Telomere heterogeneity

Telomere length in ALT-positive cells is different from those that are telomerase-positive. The mean length of telomere in human telomerase-positive cancer cell lines is usually <10 Kbp. In contrast, ALT cells have longer and more heterogeneous telomeres and the mean length is 20 Kbp [39]. This suggests that ALT is a lengthening mechanism different from telomerase.
Fluorescent *in situ* hybridization (FISH) experiments on metaphase chromosomes demonstrated the remarkable heterogeneity of telomere lengths in ALT cells. The signal strength of the telomere foci in ALT cells varies markedly, whereas those in telomerase-positive cells are comparable between chromosome ends [40,41].

2.2 Telomere-Sister Chromatid Exchanges (T-SCEs)

Since ALT is based on homologous recombination between telomeres, ALT positive cells show to be positive for sister chromatid exchanges at telomere (T-SCEs).
To detect T-SCEs, chromosome orientation (CO)-FISH analysis is used and applied at telomeres. It permits to visualize the *de novo* synthesized telomeric G- or C- strands specifically using two different probes marked with two different fluorochrome for either 5'->3' and 3'->5' telomere DNA sequences **(Fig. 8)**.
During metaphase, chromosomes of ALT-positive cells show a high level of T-SCEs that is not found in telomerase-positive cells [42-45]. Therefore, hyper-recombination between sister telomeres was recognized as one of the features of ALT cells.

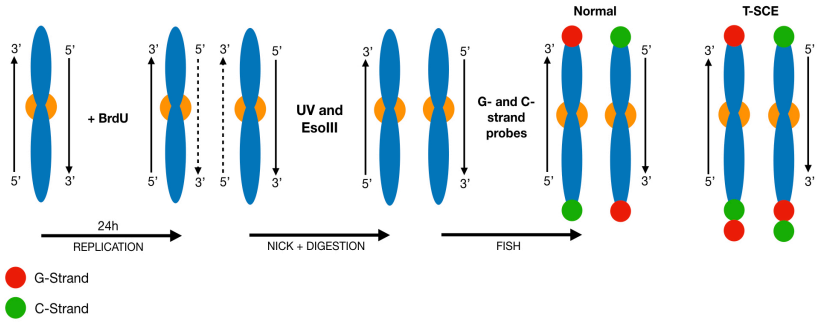


Figure 8: Schematic representation of the CO-FISH technique used to label telomeres produced by lagging-strand and leading-strand DNA synthesis. A sister chromatid exchange within telomeric DNA (T-SCE) will lead to combined green and red fluorescence.

2.3 ALT-Associated PML Bodies (APBs)

PML body is a nuclear aggregate of PML and other proteins.

This protein was first discovered as the product of the PML gene, which fuses with the RARα gene in the t(15;17) chromosomal translocation.

This chromosomal abnormality causes the human acute promyelocytic leukemia (APL), where a fusion PML/RARα protein is expressed [46-49].

In normal cells, the PML protein aggregates with nuclear structures called PML nuclear bodies (PML-NBs), where it interacts with multiple protein partners to accomplish a wide variety of functions, including regulation of transcription and p53 activation [50,51].

PML also participates in DNA damage response and is overall regarded as a tumor suppressor [52-54]. In ALT cells the interaction between the PML protein and telomeres is clearly recognized and PML is present within telomeric bodies named as ALT-associated PML nuclear bodies (APBs) [55].

However, the specific role played by the PML protein in this context is still under investigation; but it is supposed to be able to build a kind of "platform" able to host two different telomeres and to bind different proteins involved in ALT, such as the ones involved in HR, leading to telomere sequence exchanges within the PML body **(Fig. 9)**.

A recent study was able to visualize a dynamic movement of telomeres inside ALT-positive cells nuclei demonstrating that telomeres are able to move inside a PML body in order to recombine,

while a similar telomere movement has not been shown in telomerase-positive cell lines [58].
This data confirm the role of PML and APBs as a scaffold for telomere recombination. The same study used transfected cell lines (ALT-positive and normal cell lines), which express FOK1 restriction enzyme able to cut DNA and to induce DSBs specifically at telomeres, showing that DSBs lead to telomere movement and APBs formation, suggesting that telomere damage is able to increase the frequency of this hallmark [58]. In addition, the incorporation of the thymidine analogue bromodeoxyuridine (BrdU) was observed in the APBs [58] showing nascent DNA synthesis inside APBs themselves. New DNA synthesis was suppressed by inhibitors of ATM and ATR [59], which are members of phosphoinositide 3-kinase related kinase (PIKK) crucial for the activation of DNA-damage response (DDR). Furthermore, telomeres of ALT cells colocalized with proteins of DDR, for example γH2AX and 53BP1 [60,61]. These results suggest that damage signaling could regulate ALT and the new telomeric DNA synthesis inside APBs.

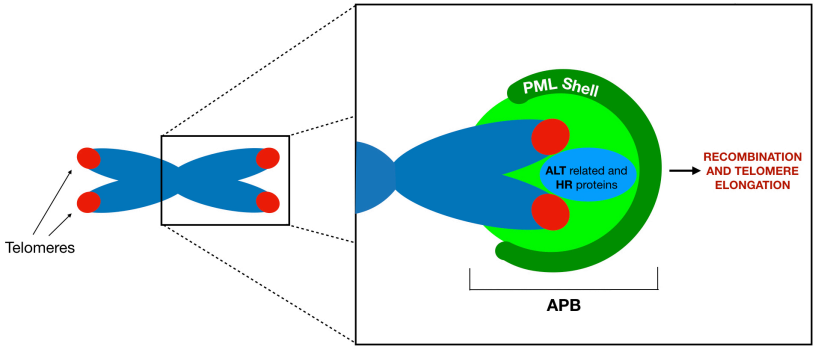


Figure 9: Schematic representation of an APB. PML builds a shell able to host different telomeres and to bind ALT and HR proteins leading to telomere recombination and elongation.

Finally, it was investigated whether PML-NBs exert a telomeric function in normal cells. Evidence for the presence of the PML proteins at telomeres of non-neoplastic cells have been reported in human endothelial cells and mouse embryonic stem cells, where the PML protein appears to be relevant for telomere stability [62-64].

2.4 Extra Chromosomal Telomere Repeats (ECTRs)

Evidence of unusual telomere DNA in ALT cells are the extrachromosomal telomere repeats (ECTRs). FISH analyses of metaphase chromosomes of ALT cells suggested the presence of significant telomere DNA repeats other than chromosome ends [65]. In fact, ALT cells are characterized by small double strand linear telomere DNA in the soluble fractions of the cell extract, which can be separated from bulk chromatin [66]; but circular DNA molecules were occasionally pointed out in ALT cells by electron microscopy and these are thought to correspond to circular forms of telomere DNA (T-circle) determined by two-dimensional (2D) gel electrophoresis [67,68].

These T-circles are regarded as a marker for ALT cells; although they can be observed in telomerase-positive cells that have a defect in TRF2 or that contain extensively elongated telomere DNA [68,69].

Moreover, ALT cells are characterized by particular single strand structures, resulting from circularization of C-strands telomeric DNA, called C-circle [70].

A sensitive method to detect circular C-strands was developed and applied to examine various cell lines [71]. Interestingly, ALT cell lines were positive for the circular C-strands whereas telomerase-positive cells were negative.

This suggests that C-circles can be considered a more specific ALT marker than T-circles.

2.5 Proteins related to ALT

Different proteins have been found to be important for ALT mechanism.

Most of them were examined when function and/or expression of their coding genes were suppressed or induced.

ALT related proteins are involved in HR, DNA structural maintenance, chromatin remodeling and histone chaperoning.

ATRX/DAXX chromatin remodeling complex has been proposed to have numerous diverse functions, including chromatin remodeling, viral resistance, a role in chromatin separation during cell division, as well as binding to tandem DNA repeats [72-75]. ATRX/DAXX genes are mutated and thus inactivated in ALT-positive tumors and cell lines [76-78]. In addition ATRX loss was also highly correlated with ALT in a panel of 19 ALT /telomerase cell line hybrids [79].

It was demonstrated that knockdown of ATRX in SV-40 transformed fibroblasts led either to an increased frequency of ALT activation or

to a decrease of crisis period prior to immortalization via ALT. Just as importantly, the transient expression of exogenous ATRX in three independent ALT-positive, ATRX-negative cell lines, led to a reduction in C-circles and ALT-associated PML bodies (APBs). These data provide the first functional evidence that ATRX acts as a repressor of the ALT mechanism in cells of mesenchymal origin [80]. Must be said anyway that knockdown of ATRX in telomerase positive epithelial cells and epithelial cells transformed by SV-40 did not show any ALT phenotype [80] confirming that epithelial cells most likely activated telomerase and not ALT as a TMM [81].
The hypothesis that ALT involves HR is supported by evidence that genes encoding HR proteins (including Rad50, Rad51, Rad52 and Sgs1) are essential for telomerase-negative yeast survivors, and HR proteins (including MRE11, RAD50 and NBS1 which form the MRN complex), SMC5 and SMC6 (which form a heterodimer) and others are also essential for telomere length maintenance in human ALT cells [reviewed in 82].
Circumstantial evidences have been provided by the observation that in ALT cells many HR proteins (RAD51, RAD52, RPA, NBS1, SLX4, BLM, MRN, BRCA1 and BRIT1) are present, along with telomeric DNA and telomere-binding proteins, in ALT-associated PML bodies (APBs) [83-86].
The ASF1 (anti-silencing factor 1) paralogs ASF1a and ASF1b are histone chaperones that assist in the transfer of H3.1-H4 or H3.3-H4 histone dimers for nucleosome assembly [87,88]. It was demonstrated that ASF1a/b depletion can lead to manifestation of all phenotypes consistent with activation of telomere maintenance by ALT pathway, including APBs, ECTRs, T-SCEs and telomere heterogeneity [89]. The defective chromatin assembly, triggered by ASF1 depletion, could impact the transmission of epigenetic histone modification [90] activating other genes related to ALT [89].

2.6 ALT hallmarks in not cancer cells

ALT pathway displays its characteristic features: namely heterogeneous telomere length, ECTRs, APBs and T-SCEs and specific proteins involved.
Recently, in different cases, it was demonstrated that elongation of telomeres can occur in telomerase-negative non-cancerous cell lines by a mechanisms similar to ALT but that lack one or more ALT features mentioned above [91-93]. For example, one cell line was reported to lack APBs but displayed other ALT characteristics

[92,93]. Remarkably, though lacking APBs, this telomerase-negative SV40-immortalized fibroblast cell line still showed nuclear aggregates of APB components at telomeres.
Moreover, low levels of ECTRs were detected in telomerase-positive and mortal cells [91,94,95].
Other studies proved that protons ionizing radiations can modulate telomere length in human primary fibroblasts [96,97] and induce an ALT phenotype, confirmed by induction of ALT hallmarks such as T-SCEs and APBs [98].
Notably, recent studies provided evidence that ALT biomarkers can be activated inside primary cells after replication stress and DNA damage [89,99].
In order to distinguish this phenotype from the “canonical” ALT known inside cancer cells, the “ALT-like” or “non-canonical ALT” name was coined [57].

3. IONIZING RADIATIONS

Ionizing radiations are energy propagations, through electromagnetic waves or particle flux that carry enough energy to kick out an electron from an atom. The borderline between ionizing and non-ionizing radiations is wide. One reliable landmark is around 10 eV energy, close to the ionization energy of hydrogen or oxygen (14 eV), or 12.6 eV, the ionization energy of the water. Beyond this threshold, radiations are supposed to be effective to damage biological biomolecules such as DNA.

There are two kinds of ionizing radiations: "electromagnetic radiations" and "charged particles" radiations. Mainly, both of them injury the living matter through the inelastic collisions of the secondary electrons (δ-electrons) produced at the primary interaction with the medium. The photon transfers all its own energy in one event of interaction with matter, while one charged particle do it all along its trajectory, a bit at time, through several physical collisions. The outcome is a difference in the spatial distribution of the energy deposition, and consequently the spatial arrangement of the ionizations: spread and uniform for the electromagnetic radiations, clustered in tracks for the particulate type.

The International Commission of Radiation Units & Measurements [100], defines the adsorbed dose in units of Gray (1Gy = 1 J/Kg) according to formula [100]:

$$D[Gy]=\bar{\varepsilon}/m$$

Where "$\bar{\varepsilon}$" is the mean energy transferred by radiation to the "m" mass.

About consequences of ionizing radiations on the living matter they can be summarized in one physical-chemical and one biological stage. In the first step, the energy is deposited on an atom and an electron is kicked out (the secondary electron or δ-electron); δ-electron is scattered and rapidly loses energy producing further ionizations until it reaches the thermal equilibrium. In simple terms, secondary electrons are the principal damage effectors. Afterwards, the newborn species diffuse and react both each other and with the solvent.

3.1 Electromagnetic ionizing radiations

y-rays, X-rays, UV, visible light, infrared, microwaves and radio waves are electromagnetic radiations characterized by different wave length, frequency and energy **(Fig. 10)**.

The X- and γ- rays flux into the medium undergoes an exponential decay depending on the energy of the incident photons, the arresting power of the medium and the depth of penetration. They randomly interact with the matter in different ways but in water-based environments, such as the cells, the main photons injury mechanism is by mean of the reactivity of the water radiolysis compounds. Water radicals and water ions (primary radiolysis products) are generated by the interaction of the IR with the water molecules.

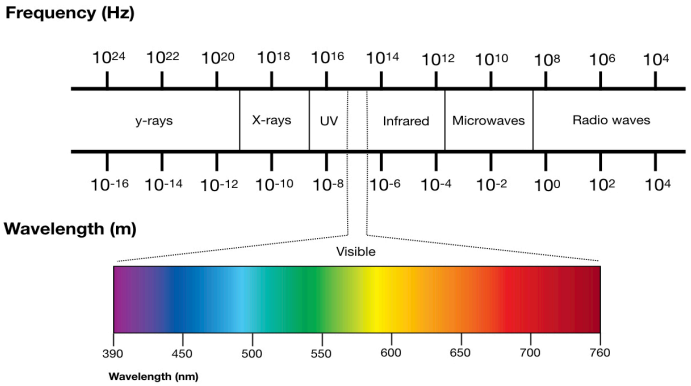


Figure 10: Electromagnetic spectrum

These compounds are then further decomposed into their primary products, composed both of radical and non-radical molecules **(Fig. 11)**.

In oxygenated solutions (such as in physiological conditions) electrons react with molecular oxygen to form superoxide anion, one further highly reactive oxygen specie (ROS).

In the biological matter, such as the cell, is not the direct photons interaction with matter itself able to damage components of cells like protein or DNA, but the indirect interactions of water radiolysis compounds generated from electromagnetic ionizing radiation within a water-like system, like the cytosol.

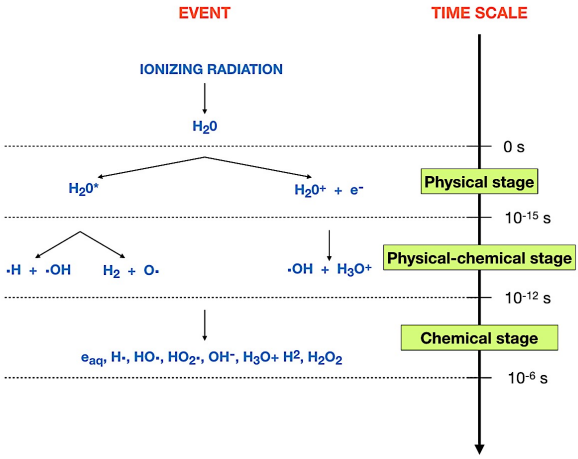


Figure 11: Representation and time scale of radiolysis of water

3.2 Charged particles interaction with living matters

Particulate radiations (proton or heavier accelerated ions), are classified as densely ionizing or high-LET radiations because they transfer their own kinetic energy to the hit matter, through several direct collisions along their straight trajectory (direct damage), in contrast both with the scattered path of the electrons, and the random interaction of the photons.

Briefly, the interaction of the particle with the matter depends on the mass of the particle, particle energy, the charge and the depth of the particle into the matter [101].

3.3 Ionizing radiations and DNA damage

DNA is the main cellular target that realizes the most important consequences on cell viability when harmed. DNA damage comes in several different varieties: base lesions, intra- and inter strand cross-links, DNA-protein cross-links, and both SSBs and DSBs [102] **(Fig. 12)**.

As mentioned before, ionizing radiations can injury DNA both by indirect and direct damage prevalent for electromagnetic and particle radiations, respectively. In the latter case the radiation ionizes and breaks directly the DNA molecule by direct interaction, while in the first case the ionization occurs by mean of reaction of the DNA with water radiolysis compounds released in solution, that are able to diffuse for nanometers from the origin point [103]. The level of

combination and the proximity of the lesions determinate the severity of the injury, in fact, since the information on both strands of the DNA molecule is complementary, all injuries only on one side of the double strand can potentially be repaired by using the information on the intact complementary strand. Single-strand breaks (SSBs) are considered less severe than double-strand breaks (DSBs), for the integrity of chromosome preserved. In fact, DSBs are considered critical events for the induction of lethal lesions since they are responsible for the structural chromosomal aberrations; just only one of those may be enough to inactivate the cell and to upset the whole chromosomal asset [104].

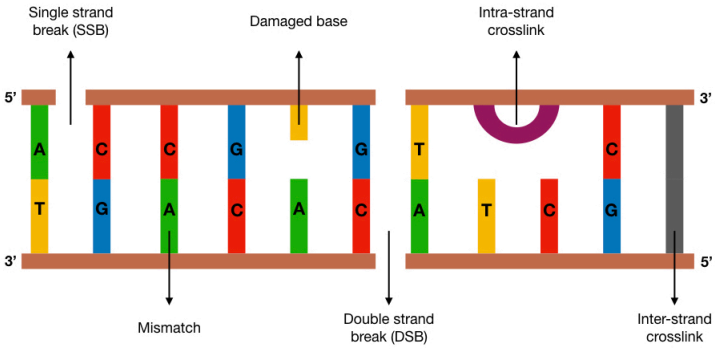


Figure 12: Different types of damage that can occur in the DNA

There are different mechanisms that are involved in DSBs and SSBs repair.

The homologous recombination (HR) or the non-homologous end joining (NHEJ) can repair DSBs [105,106]; while the base excise repair (BER) and the nucleotide excision repair (NER) are involved in the SSBs repair [107,108]. It is known that a not repaired SSB could become a DSB. It is clear that an unrepaired breakage on the DNA strand could be very dangerous for the cell and can give rise to genomic instability [109].

4. OXIDATIVE STRESS

Oxidative stress represents an imbalance between the production of ROS and the ability of the biological system to readily detoxify these reactive molecules. This imbalance is then able to damage cell components.

Both the exogenous oxidative agents and the normal biological process can increase the levels of ROS within the cells. This happens because O_2 is involved in many chemical reactions important for cell viability and if molecular oxygen is not much reactive, cellular metabolism or external agents, such as temperature, radiation and chemical agents, can transform it in ROS [110]. These molecules are highly reactive and unstable [111] and can damage the different cellular components, such as proteins, lipids and the DNA.

4.1 Oxidative stress, ionizing radiations and DNA damage

Oxidative DNA damage constitutes the majority of DNA damage in human cells [112]. ROS represent a source of oxidative damage due to the production of SSBs anywhere in the genome [113] either directly or as an intermediate step in the repair of oxidative base modifications [114]. Not repaired SSBs can become DSBs leading to genomic instability [109].

One form of DNA damage induced by oxidative stress is the modification of DNA bases to species such as 8-oxoguanine (8-oxoG), thymine glycol, and 5-hydroxy-methylluracil. The 8-oxoG is recognized as typical biomarker of oxidative DNA damage [115]. It can be repaired correctly by 8-oxoganine glicosylase (OGG1) that removes the modified base; a polymerase synthesizes a new nucleotide and a ligase links all together [116-119]. If it not occurs, it induces single or double stand breaks, and GC-AT mutation that results in genomic instability [120].

Both electromagnetic and particle ionizing radiations can induce a persistent oxidative stress [121].

For X-rays and in general for electromagnetic ionizing radiation it has been shown that radiation exposure alters intracellular levels of ROS in human tumor and immortalized cells [122-124]. The reason for this increase is likely to be mitochondrial damage associated with a change in the membrane potential [125,126]. The involvement of ROS has been shown in rodent models after X-rays exposure *in vitro* [127-129] and *in vivo* [130,131]. In addition, a connection between elevated levels of ROS, an impeded oxidative defense and the

accumulation of chromosomal damage has been reported in X-rays irradiated human tumor cells [124].
Moreover, even for particle ionizing radiation, such as protons and carbon ions, it has been shown that radiation exposure is able to unbalance ROS within the cell and to induce oxidative stress [132]. In this case a possible explanation in increasing cellular ROS levels could be a stronger impairment of the anti-oxidative capacities of the exposed cells [132].

4.2 Oxidative stress and telomeres

As it was said above, in somatic cells telomeres get shorter at each cell division due to the end-replication problem [19]. This telomere shortening could be enhanced, even in a significant way, by environmental agents such as radiation or chemical agents [133,134].
Literature data demonstrated that oxidative stress could accelerate telomere shortening in replicating fibroblasts *in vitro* through the induction of SSBs at telomeres [135]. Seen that the principal DNA damage that occurs after oxidative stress is the formation of 8-oxoG, the probability of the accumulation of this lesion within telomeres during oxidative stress is enhanced by the high incidence of guanine residues in telomeric DNA sequences [136]. Moreover, the guanine expressed in sequences GG or GGG are more susceptible to oxidation than single guanine [137,138], supporting the hypothesis that telomeric sequence is the first target of oxidative damage [139]. Furthermore, due to the telomeric heterochromatin state, the oxidative damage is less efficiently repaired compared to the rest of the genome. All of these features make telomere the preferential sequences damaged by oxidative stress [140,141]. The presence of 8-oxoG leads to telomere shortening and consequently to the alteration in telomere maintenance and function [19]. In fact, this lesion within telomeric DNA interferes with the replication fork at telomeres and aborted replication may lead to strand breaks and loss of telomere repeats (telomere shortening) [133]. Moreover, this telomeric oxidative lesion may interfere directly with the recognition by TRF1 and TRF2 proteins of telomere repeats [141] leading to telomere dysfunction **(Fig.13)**.

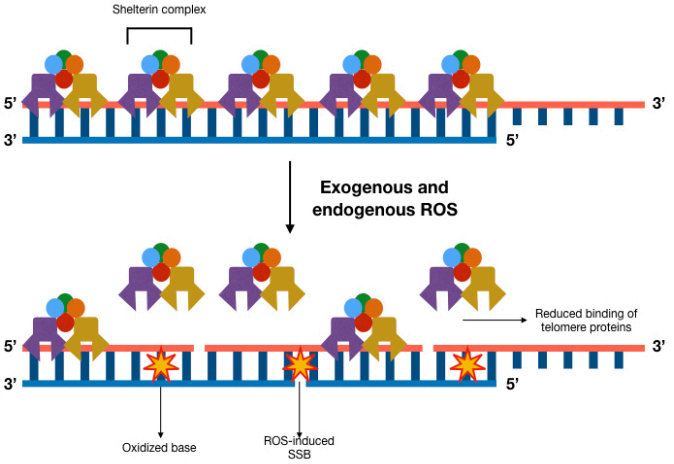


Figure 13: Endogenous and exogenous oxidative stress induces the formation of oxidised bases in telomeric region. The presence of these base modifications lead to SSBs formation, telomere shortening and reduce the binding of telomeric proteins.

5. DNA DAMAGE RESPONSE AT TELOMERES

After DNA damage detection, the DNA damage response (DDR) pathway directs the DNA repair activities and arrests the cell cycle progression in proliferating cells until the DNA damage has been completely repaired [142]. The activation of the DDR can be detected thanks to the association of DNA damage factors to the damaged chromatin such as 53BP1 (p53 binding protein 1) that is a protein involved in detection and processing of DSBs [143] and to the phosphorylation of the histone variant H2AX in γH2AX. Initially it was thought that H2AX was phosphorylated only in response to a DSB, but Ward and Chen [144] found that H2AX phosphorylation occur also in response to replication arrest [144,145] **(Fig. 14)**.

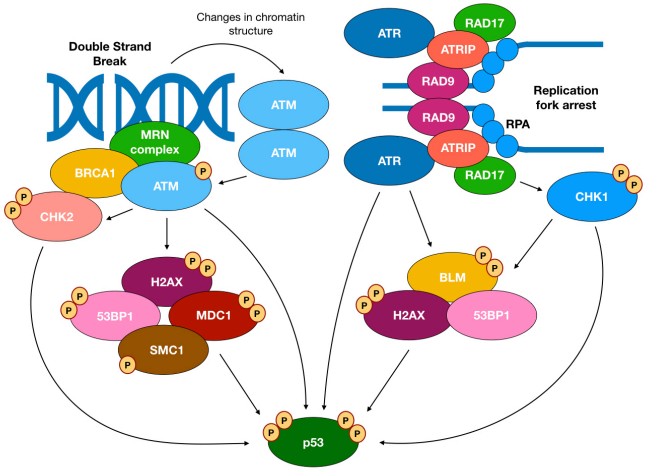


Figure 14: The DNA damage signaling pathway of DNA DSBs (left) or replication stress (right). ATM is first activated by DSBs, instead ATR is activated by replication fork arrest. Changes in chromatin structure lead to the autophosphorilation of ATM, that localizes to the DNA damage site and phosphorylates H2AX and 53BP1. The replication stress recruit the ATR-ATRIP complex that localizes at the damaged sites. In this case, H2AX is phosphorylated in a ATR/ChK1-dependent manner and recruit transducer proteins.

In somatic cells telomere becomes shorter at each cell division until it reaches a critical length; at this point it is recognized as a DSB and activates the DDR pathway, leading to replication senescence [19,23]. The presence of the t-loop at the end of the chromosomes represses

the activation of the DDR pathway, protecting chromosomes **(Fig. 15)**.

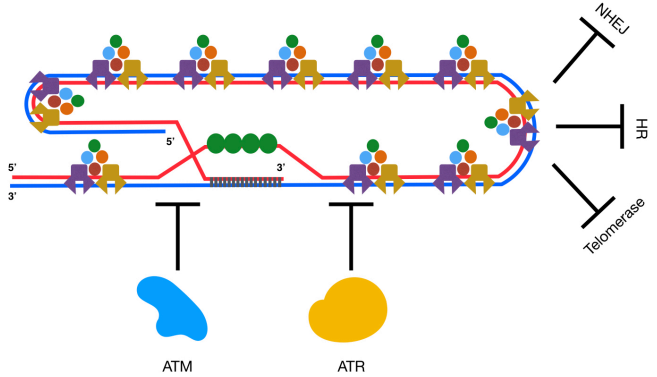


Figure 15: The shelterin complex, that form the t-loop, suppresses the activation of the DNA damage signalling (activation of ATM/ATR) and protects the end of chromosomes from end-to-end fusions and elongation (activation of NHEJ, HR and telomerase).

Dysfunctional telomeres are recognized by the association of the two DNA damage factors (53BP1 and yH2AX) at telomeric regions, increasing the telomere-dysfunctional induced foci (TIFs) [146].
The presence of TRF2 is essential to suppress the activation of the ATM kinase pathway [147] and the NHEJ pathway [148], in fact evidences in mouse embryonic fibroblasts (MEFs) showed that the absence of TRF2 increased the amount of end-to-end fusions [149]. POT1 suppresses the ATR pathway [146] and TRF1 with TPP1/POT1 heterodimers repress the activation of ATR kinase pathway in case of replication; as previously reported, TRF1 is required for the efficient telomeric replication. Previous studies indicated that also stalled replication fork, due to the presence of an oxidized base or to an alteration of TRF1 during telomere replication, lead to the activation of the DDR [150]. This block of the replication fork can collapse into a DSB and again activate a DDR [151]. Evidences in MEFs demonstrated that deletion of TRF1 activates the ATR kinase pathway, increasing the amount of yH2AX and 53BP1 foci at telomeres [13,14]. In this last case, foci appear when cells pass the S phase without TRF1, indicating that the activation of the DDR is replication-dependent [14] and derives from telomeric site of replication stress [152].

AIM OF THE PROJECT

The ends of linear chromosomes are protected by “telomeric cups”.
In somatic cells telomeres become shorter at each cell division; moreover there are chemical and psychical agents that can accelerate telomere shortening.
A cell with very short telomeres can enter senescence or apoptosis or can elongate telomere again activating some mechanisms (counteracting the natural shortening at each cell division), becoming immortal.
These mechanisms are known as telomere maintenance mechanisms (TMMs) and are: telomerase and the alternative lengthening of telomere (ALT)
Ionizing radiations (IRs) can damage DNA especially by an indirect effect, due to the production of reactive oxygen species (ROS) after the radiolysis of H_2O molecules within cells.
Telomeres are particular sensitive to oxidative DNA lesions, such as 8-oxoguanine (8-OxoG), that can block replication fork, leading to unreplicated telomere, or can induce telomere breaks.
Moreover, previous studies demonstrated that particle ionizing radiations (like protons) are able to induce telomere lengthening by activation of an ALT-like mechanism, while literature data about X-rays indicate just the capability of this kind of IRs to induce telomere length modulation without deepen the mechanisms behind such modulation.

The aim of this project is to understand if the main role of this X-rays induced telomere modulation can be entrusted to telomerase or ALT (according to the previous data on protons ionizing radiations) and to shed light about how this eventual mechanism can be activated by primary cells.
To reach this purpose we treated HFFF2 human primary fibroblasts with 4Gy of X-rays and we evaluated several endpoints:

1. Telomere length from 3 to 13 days after exposure (according to previous papers published by our laboratory), in order to confirm telomere length modulation.
2. Telomere induced dysfunctional foci (TIF) in order to study telomere damage.
3. Telomerase activity.
4. Two different hallmarks of ALT: APBs and T-SCEs.

5. Some proteins related to ALT: ATRX, RAD51 and RPA2.

Moreover, in order to understand the mechanism by which X-rays are able to induce telomere damage, we analyzed from 3 to 8 days after exposure:

6. Oxidative stress after X-rays treatment.
7. Oxidative stress after X-rays and antioxidant molecule (NAC) administration.
8. Telomere length after X-rays and antioxidant molecule (NAC) administration.

RESULTS

X-rays irradiation affects HFFF2 proliferation.

First it was studied whether X-rays treatment is able to affect HFFF2 cells proliferation. Proliferation was analyzed by cell growth curves, calculating the cumulative population doubling level (CPDL) and by cell cycle analysis.

Fig. 16a shows short-term growth curves for X-rays and sham-irradiated samples as evaluated in the first 168 hours after irradiation. As expected, data showed that X-rays were able to significantly affect cells proliferation, reducing cell growth by 70% at 96 hours. Long-term (up to 16 days) growth curves allowed us to calculate cumulative population doubling level (CPDL) of irradiated and unirradiated cells. Data indicated that irradiated cells continue to growth after irradiation but, as expected, with a slower proliferation rate than sham irradiated samples **Fig. 16b**.

Cell cycle analysis was performed in order to further characterize short- and long-term (1-8 days) X-rays effect on cell proliferation. Data showed that control cells linearly increased G_1 cell population over-time whereas IR exposed cells displayed a G_2-M phase cell accumulation coupled with an emptying of G_1-phase **Fig. 16c.**

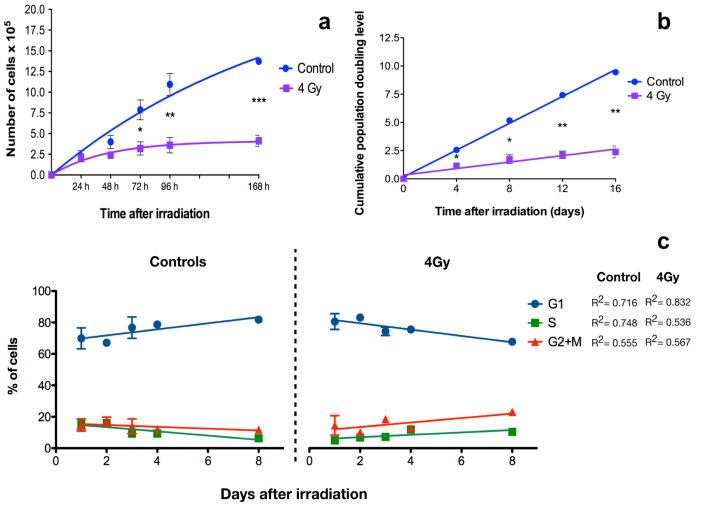


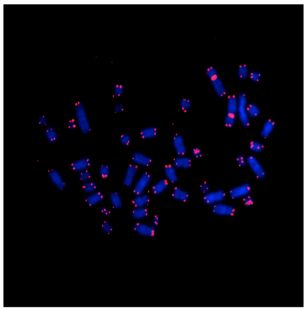
Figure 16: Growth curves and *Cumulative Population Doubling Level* of X-rays treated HFFF2.
a) The graph shows the growth curve for X-ray treated HFFF2.
Untreated cells were used as controls. Curves were obtained seeding cells after the X-rays irradiation and counting them at 24, 48, 72 and 168 hours after treatment.
Results show a significant decrease at 72h ($p<0.05$), 96h ($P<0.01$) and 168h ($P<0.001$) of the cell growth. Four experiments were performed.
b) The graph shows the cumulative population doubling level (CPDL) of X-rays treated HFFF2.
Untreated cells were used as controls. Population doublings were calculated counting and seeding again the same number of cells at 4, 8, 12 and 16 days after exposure and applying for each day the formula:

$$PDL = (logN - logN0)/log2$$

The final CPDL was obtained adding at each day results from the previous ones.
Results show a significant reduction of cell proliferation at 4 ($p<0.05$), 8 ($p<0.05$), 12 ($p<0.01$) and 16 ($p<0.01$) days after exposure. Two experiments were performed.
c) The graph shows data about cell cycle analysis.
DNA staining was performed by incubating cells with propidium iodide (PI), samples were then acquired with a Cytoflex (Beckman Coulter) equipped with a 488nm laser source. Cell cycle analysis was performed using Cytexpert v2.0 software. Three experiments were performed.
Results show an emptying trend of the G1 phase ($R^2=0.832$) and a consequently accumulation at G2+M phase ($R^2=0.567$).
Statistical analysis was performed between treated and control samples.
*$p<0.05$; **$p<0.01$; ***$p<0.001$ by t Student test.

X-rays irradiation induces telomere length modulation.

We performed a telomere length analysis by centromere-calibrated Q-FISH (**Fig. 17a**) to test whether X-rays treatment is able to affect telomere length in a time period between 3 to 13 days after exposure.
The kinetics of telomere length is shown in **Fig. 17b**.
A statistical significant telomere shortening, compared to the normalized average of the control samples, was observed at 3 and 6 days after exposure, while telomere elongation was detected at 4, 7, 8, 10 and 13 days after treatment.
Data at 5 days were similar to the normalized average of the controls, but values showed a significant telomere length reduction compared to the previous 4 days time-point.
All together these data demonstrate that X-rays are able to induce a telomere length modulation in HFFF2 during the time period of analysis.



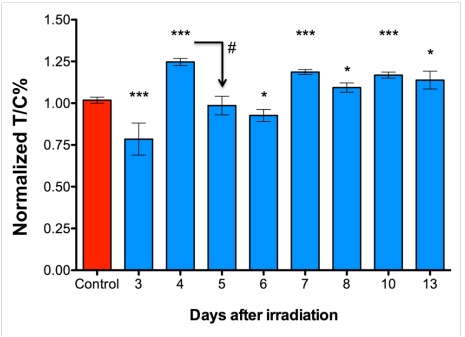
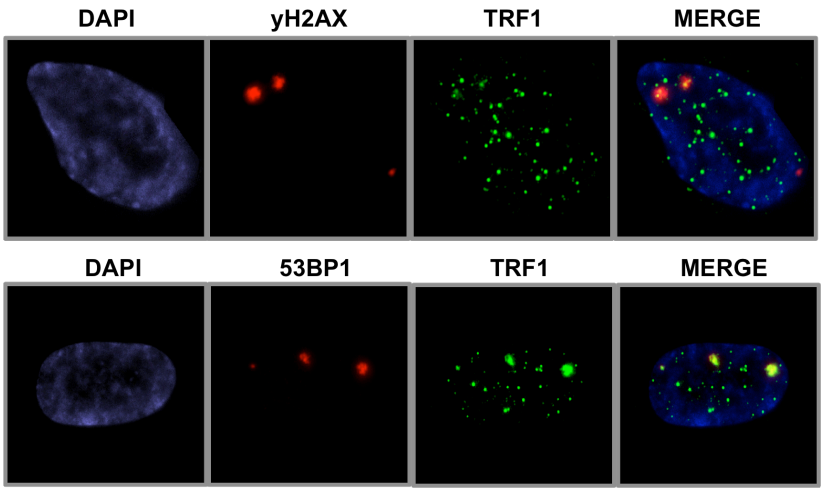


Figure 17: Quantitative-Fluorescence *in situ* Hybridization analysis for telomere lengths.
(a) Example of a metaphase stained by Q-FISH.
(b) The histogram represents results obtained by the analysis of telomere length after X-rays treatment. Telomere lengths (T/C%) were calculated as the ratio between the total telomere fluorescence (T) and the fluorescence of the centromeres of the two chromosomes 2 (C), used as internal reference in each metaphase analyzed; data obtained from treated samples were normalized with the value of each matched control samples. For each time-point 10 metaphases were analyzed.
Data at 3 and 6 days after exposure showed a significant (3 days, $p<0.001$; 6 days, $p<0.05$) reduction of the telomere length, while data at 4, 7, 8, 10 and 13 days after exposure showed a significant telomere elongation (4 days, $p<0.001$; 7 days, $p<0.001$; 8 days, $p<0.05$; 10 days, $p<0.001$; 13 days, $p<0.05$). Finally data at 5 days after treatment were no different from controls but values show a significant reduction ($\#p<0.05$) compared to the 4 days data.
Errors bar were calculated using standard error propagation rules.
Three experiments were performed.
Statistical analysis was performed between normalized treated samples and the average of all the normalized matched control samples (left bar).
$*p<0.05$; $**p<0.01$; $***p<0.001$ by t Student test.

X-rays irradiation induces damage at telomeres, related to telomere shortening.

To test if telomere erosion observed resulted in telomere dysfunction we performed TIFs (telomere induced dysfunctional foci) co-immunostaining with antibodies against telomeric repeat binding factor 1 (TRF1) and two different DNA damage markers (53BP1 and yH2AX) (**Fig. 18a**).
Results are shown in **Fig. 18b,c**.



a

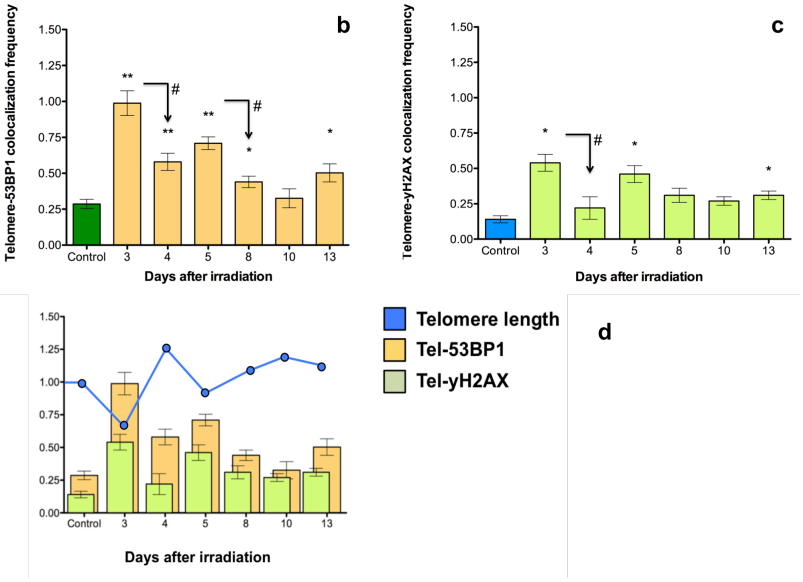


Figure 18: Co-immuno staining for telomere induced dysfunctional foci (TIFs).
(a) Example of nuclei stained by Co-immuno staining for TIFs analysis.
(b) The histogram represents results obtained by the analysis of TIFs after X-rays treatment, counting the colocalization between the fluorescent spots of the telomeric protein TRF1 and of the DDR signaling protein 53BP1.

Data obtained from treated samples show a significant increase of TIFs frequency at 3, 4, 5, 8 and 13 days after exposure (3 days, $p<0.01$; 4 days $p<0.01$; 5 days $p< 0.01$; 8 days, $p<0.05$; 13 days, $p<0.05$) compared to the average of matched untreated controls. Data at 10 days were similar to the average of matched control samples. The decrease at 4 and 8 days are statistical significant compared to 3 (#$p<0.05$) and 5 (#$p<0.05$) days data.
(c) The histogram represents results obtained by the analysis of TIFs after X-rays treatment, counting the colocalization between the fluorescent spots of the telomeric protein TRF1 and the ones of the DDR signaling protein yH2AX.
Data obtained from treated samples show a significant increase of TIFs frequency at 3, 5 and 13 days after exposure (3 days, $p<0.05$; 5 days $p< 0.05$; 13 days, $p<0.05$) compared to the average of matched untreated controls. Data at 4, 8 and10 days were similar to the average of matched control samples. Decrease at 4 days is statistical significant compared to 3 days (#$p<0.05$) data.
For each time-point 50 nuclei were analyzed and at least 3 experiments were performed. Three experiments were performed.
Statistical analysis was performed between treated samples and the average of the untreated matched control samples (left bar).
*$p<0.05$; **$p<0.01$; ***$p<0.001$ by t Student test.
(d) The histogram is a comparing graph between telomere lengths (Fig. 17) and TIFs data.
It highlights an inversely proportional relation between the two sets of data.

Analysis for TRF1/53BP1 showed a statistical increase of telomere damage at 3, 4, 5, 8 and 13 days after X-rays irradiation, compared to the average of matched controls (left bar). Moreover, it was pointed out a significant reduction of telomere damage at 4 days compared to 3 days, and at 8 days compared to 5 days. While analysis for TRF1/yH2AX showed a statistical increase of telomere damage at 3, 5 and 13 days after X-rays exposure, compared to the average of matched controls (left bar). Furthermore, the decrease of telomere damage at 4 days was statistical significant compared to 3 days.
In both the two co-immuno stainings the highest TIFs frequency was detected at 3 and 5 days after exposure.
Still, all TIFs data (both TRF1/53BP1 and TRF1/yH2AX) are inversely proportional compared to telomere length data (**Fig. 18d**).
In fact, at longer telomere lengths correspond lower telomere damage, while shorter telomeres are related to higher damage values.

X-rays do not induce telomerase activity.

To evaluate if the observed telomere elongation after X-rays treatment could be due to telomerase activation, telomerase activity was measured by RTQ-PCR TRAP assay (**Fig. 19**).

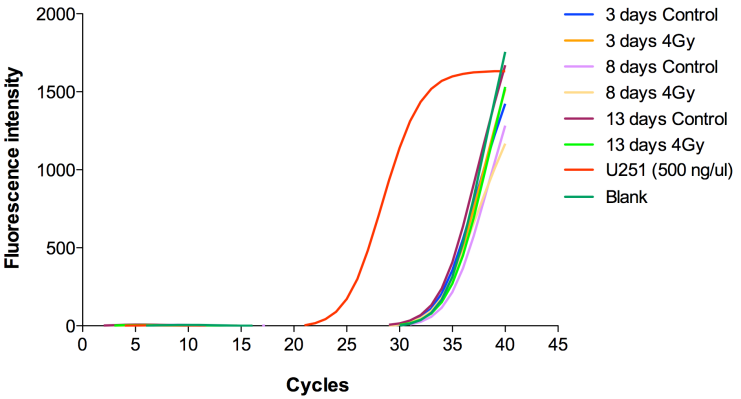


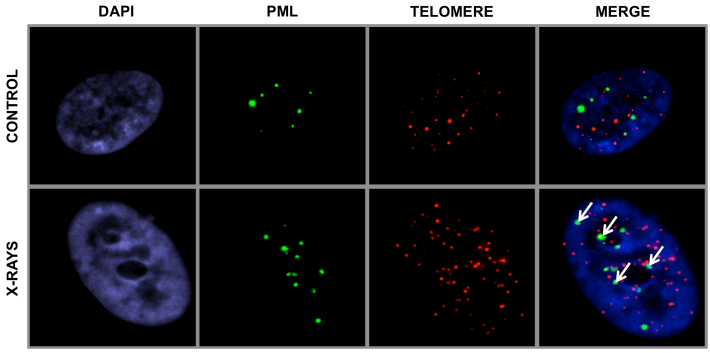
Figure 19: RTQ-PCR Trap assay analysis for telomerase activity.
The graph displays data on telomerase activity in X-rays treated HFFF2.
The Trap assay amplifies the forward primer complementary to the telomerase RNA.
Samples are compared with the telomerase-positive U251 cell line.
Telomerase activity of U251 starts to be detected around the 23th round of amplification, while the telomerase activity of all the control and treated samples (related to 3, 8 and 13 days after exposure) starts to be detected around the 33th round of amplification, which is known to be an artifact.
These data indicate that HFFF2 samples do not show any telomerase activity.
Two experiments were performed.

Both untreated control and X-rays treated samples (at 3, 8 and 13 days after exposure) were compared with U251, a telomerase-positive tumor cell line, which displays a high telomerase activity. Telomerase activity (reported by SyBR green fluorescence intensity) of U251 started to be detected around the 23th round of amplification, while the telomerase activity of all the control and treated samples (related to 3, 8 and 13 days after exposure) starts to be detected around the 33th round of amplification, which is known to be an artifact. Thus, in every sample, results did not showed telomerase activity.

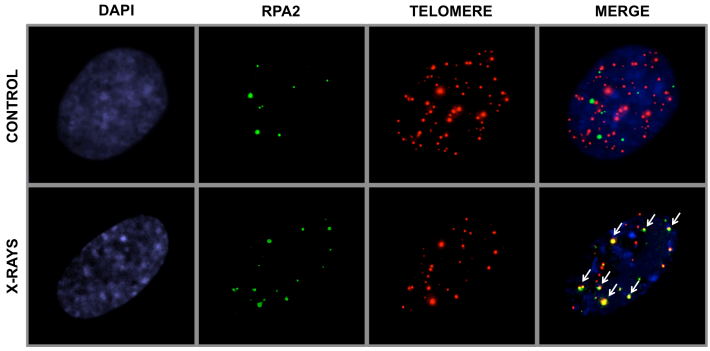
X-rays irradiation is able to induce ALT-associated PML bodies (APBs).

We investigated by two immuno-FISH (marking telomere with a telomeric probe and two proteins involved in APBs formation using antibodies against PML and RPA2) the possible induction of APBs in irradiated samples (**Fig. 20a,b**).

APBs frequency was analyzed counting the colocalization between the fluorescent spots of the PML or RPA2 proteins and the ones of the telomeric sequences.
Results were similar for both telomere-PML and telomere-RPA2.
In fact, **Fig. 20c** shows that X-rays are able to induce a significant increase of APBs at 4, 8, 10 and 13 days after exposure compared to the average of untreated matched control samples (left bars).



a



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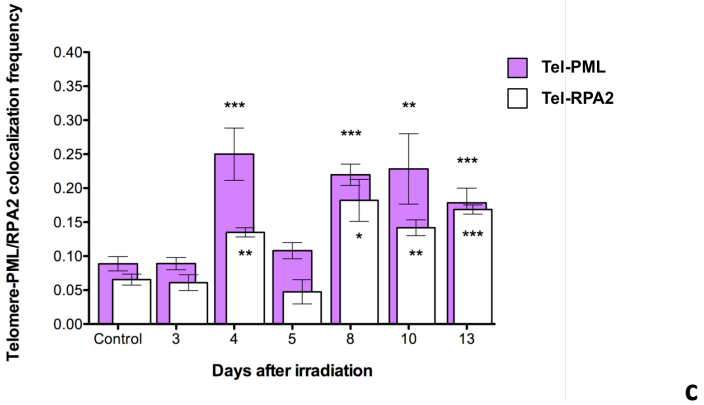

Figure 20: Immuno-FISH analysis for ALT Associated PML Bodies (APBs).
(a) Nucleus stained by immuno-FISH for APBs by PML and Telomere.
(b) Nucleus stained by Immuno-FISH for APBs by RPA2 and Telomere.
(c) The histogram represents results obtained by the analysis of ALT Associated PML bodies (APBs) after X-rays treatment
APBs frequency was analyzed counting the colocalization between the fluorescent spots of the PML or RPA2 proteins and the ones of the telomeric sequences marked with a specific telomeric probe.
Data obtained from treated samples show a significant increase of APBs frequency at 4, 8, 10 and 13 days after exposure, both for telomere-PML (4 days, $p<0.001$; 8 days $p<0.001$; 10 days $p< 0.01$; 13 days, $p<0.001$) and telomere-RPA2 (4 days, $p<0.01$; 8 days $p<0.05$; 10 days $p< 0.01$; 13 days, $p<0.001$) compared to the average of matched untreated controls. Data at 3 and 5 days were similar to the control samples.
For every endpoint and time-point 50 nuclei were analyzed.
Three experiments were performed.
Statistical analysis was performed between treated samples and the average of all the untreated matched control samples (left bars).
*$p<0.05$; **$p<0.01$; ***$p<0.001$ by t Student test.

X-rays irradiation induces telomere-sister chromatid exchanges (T-SCEs).

In order to test whether X-rays are able to induce T-SCEs we performed a chromosome orientation FISH (CO-FISH) that permits to visualize the *de novo* synthesized G- and C-telomeric strands (**Fig. 21a,b,c**).
T-SCEs frequency was analyzed counting the presence of both G- and C- fluorescence telomeric spots on each chromatid couple of every chromosome in the analyzed metaphases.

Results showed in **Fig. 21d** demonstrated a significant induction of T-SCEs in treated samples at 4, 10 and 13 days after X-rays exposure compared to the average of untreated control samples.

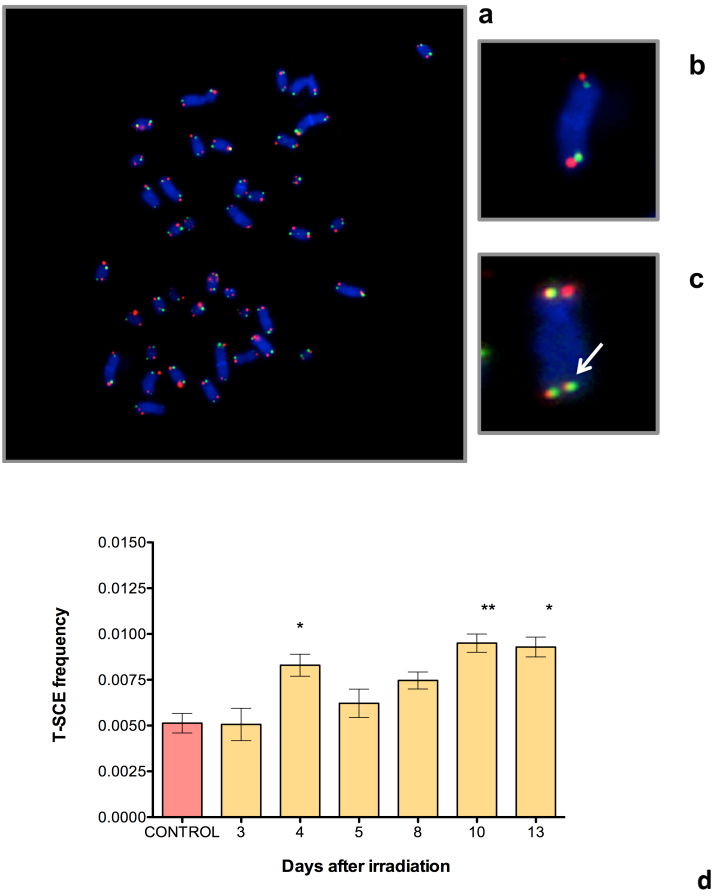

Figure 21: CO-FISH (chromosome orientation FISH) analysis for telomere-sister chromatid exchanges (T-SCEs).
(a) Metaphase stained by CO-FISH to measure the T-SCEs.
(b) Chromosome negative for T-SCE with different fluorescent spots at G- and C-telomeric strands on the two chromatids.
(c) Chromosome positive for T-SCE with a simultaneous green and red spots on the same G- and C- telomeric strands on the arrowed chromatids.
(d) The histogram represents results obtained by T-SCEs analysis in X-ray treated HFFF2.

T-SCEs frequency was analyzed counting the presence of both G- and C- fluorescence telomeric spots on each chromatid couple of every chromosome in the analyzed metaphases.
Data obtained from treated samples show a significant increase of T-SCEs frequency at 4, 10 and 13 days after exposure (4 days, $p<0.05$; 10 days $p< 0.01$; 13 days, $p<0.05$) compared to the average of all matched untreated controls. Data at 3, 5 and 8 days were similar to the control samples.
For each time-point at least 1000 chromosomes were analyzed.
Three experiments were performed.
Statistical analysis was performed between treated samples and the average of all the untreated matched control samples (left bar).
*p<0.05; **p<0.01; ***p<0.001 by t Student test.

APBs and T-SCEs data are coherent with telomere length results.

Looking at APBs and T-SCEs results together with telomere length data, it was clearly put in evidence a correlation between these different endpoints (**Fig. 23**). In fact, at those days that showed telomere elongation (4, 8, 10 and 13 days after exposure) corresponds a match with the increasing frequency of APBs and T-SCEs.
On the other side, time points that showed a telomere shortening did not show induction of the two makers.

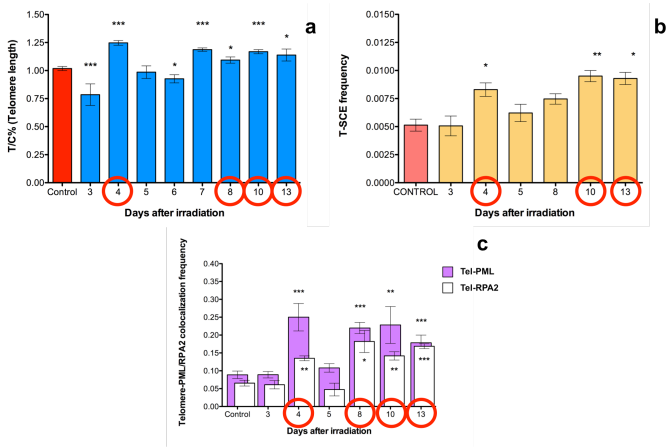

Figure 23: Comparison between telomere length, APBs and T-SCEs data.
This figure compares data on telomere length **(a)**, T-SCE **(b)** and APBs **(c)** of X-rays treated cells. It highlights that a significant telomere length is associated with a statistical significant increase of APBs and T-SCEs frequency, while those days that show a telomere shortening and any telomere modulation do not show any APBs or T-SCEs induction.

X-rays treated cells show a profile similar to ALT-positive cell lines for some ALT related proteins.

Several proteins have been discovered to be involved in ALT pathway. Thus, in order to deeper investigate the ALT activation in our irradiated samples, we analyzed, by western blot, some specific proteins related to ALT:

-ATRX, a chromatin remodeling protein known to be an inhibitor of the ALT mechanism;

-RAD51 and RPA2, two proteins of the HR repair system, which participate in telomere recombination between ALT telomeres.

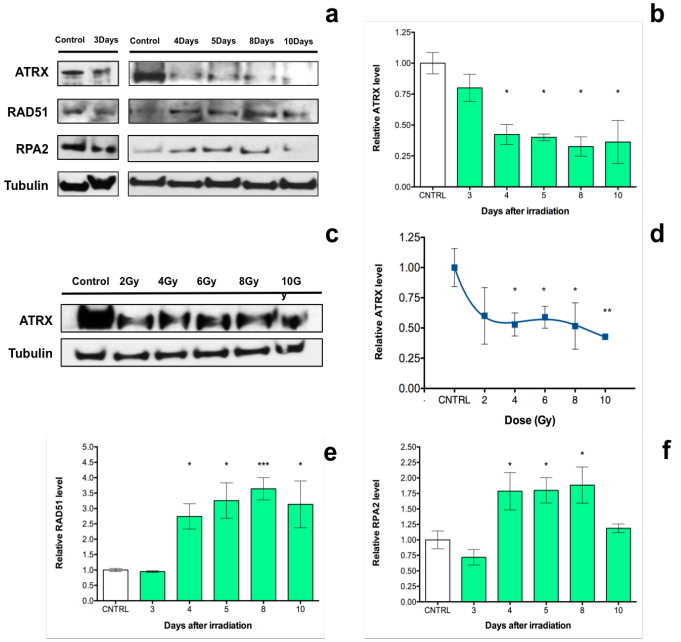

Figure 24: Western Blot analysis for different ALT related proteins (ATRX, RAD51 and RPA2) inside HFFF2 treated with X-rays.

(a) Western blot results on film for ATRX, RAD51 and RPA2. β-tubulin is used as the control protein.

(b) Graph shows results for ATRX protein in X-rays treated samples.

Data demonstrate that from 4 to 10 days ($p<0.05$) after exposure, X-rays are able to reduce ATRX level in the cells, while results at 3 days were similar to the average of normalized untreated controls.

(c) Western blot results on film for dose-response ATRX analysis. β-tubulin is used as the control protein.
(d) Graph shows results for ATRX dose-response analysis.
Data demonstrate that in an X-rays dose range from 4 to 10 Gy (4, 6 and 8 Gy, $p<0.05$; 10 Gy, $p<0.01$) the protein level decrease up to 50%, while data at 2 Gy are not statistical significant.
(e) Graph shows results for RAD51 protein in X-rays treated samples.
Data demonstrate that from 4 to 10 days (3, 4 and 10 days, $p<0.05$; 8 days, $p<0.01$) after exposure, X-rays are able to increase RAD51 level in the cells while results at 3 days are similar to the average of normalized untreated controls.
(f) Graph shows results for RPA2 protein in X-rays treated samples.
Data demonstrate that from 4 to 8 days ($p<0.05$) after exposure, X-rays are able to increase RPA2 level in the cells while results at 3 and 10 days are similar to the average of normalized untreated controls.
Results are normalized to the control values.
Three experiments were performed.
Errors bar were calculated using standard error propagation rules. Statistical analysis was performed between normalized treated and untreated control samples.
*$p<0.05$; **$p<0.01$; ***$p<0.001$ by t Student test.

β-tubulin was used as the control protein and results were normalized on control values.
Fig. 24a,b shows that ATRX level was lower in every sample after X-rays irradiation but not at 3 days after exposure, compared to average of the untreated controls.
Moreover, a dose-response analysis for ATRX showed a stable decrease of this protein level up to 50% **(Fig. 24c,d).**
On the other hand, RAD51 and RPA2 levels were higher in treated samples compared to the average of untreated controls. But, for both proteins, 3 days data were similar to the control ones **(Fig. 24e,f)**.
Finally, RPA2 level did not show a significant difference with controls at 10 after X-rays exposure.

X-rays irradiation induces a persistent oxidative stress (OS).

In order to understand the main cause able to trigger telomere length modulation and ALT biomarkers, we performed an analysis for oxidative stress administrating DCFH-DA (dichlorofluorescin-diacetate), a fluorescent molecule able to bind ROS produced by cells and to generate a fluorescence intensity related to the ROS concentration. Results are showed in **Fig. 25** and demonstrate that X-rays irradiation is able to induce a persistent oxidative stress in treated samples compared to the untreated controls.

In fact, OS was significant higher at 3 and 4 days after exposure, compared to untreated controls, while from 5 to 13 days after exposure OS level was similar to OS controls values.

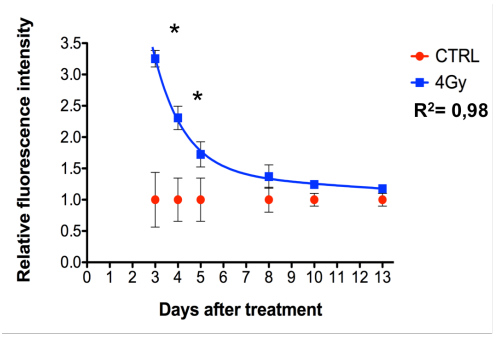

Figure 25: Oxidative stress analysis by dichlorofluorescein diacetate (DCFH-DA).
Graph shows data for oxidative stress in HFFF2 treated with X-rays.
Analysis is performed administrating DCFH-DA able to bind ROS and to release fluorescence relative to ROS concentration in the cellular environment. The fluorescence intensity is detected by a Victor apparatus.
Results show an higher oxidative stress level at 3 days ($p<0.05$) after irradiation that starts to decrease at 4 days ($p<0.05$).
From 5 to 13 days after X-rays treatment, OS level is considered similar to the level of the untreated control samples.
Still, the curve describes a logarithmic function with $R^2=0.98$.
Results were normalized to the control values.
Three experiments were performed.
Errors bars were calculated using standard error propagation rules. Statistical analysis was performed between treated and the average of normalized control samples.
*$p<0.05$; **$p<0.01$; ***$p<0.001$ by t Student test.

NAC (N-acetyl-cysteine) administration prevents cells from OS.

In order to inhibit OS, we administrated NAC, an antioxidant molecule, 30 minutes prior and every 24 hours after X-rays treatment. Results (**Fig. 26**) were normalized on control values and showed that NAC is able to prevent cells from X-rays induced OS, since any differences in OS level could be detected between treated and controls samples.

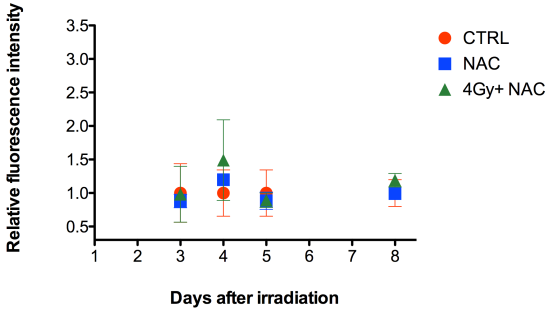

Figure 26: Oxidative stress analysis by dichlorofluorescein diacetate (DCFH-DA) in X- rays treated HFFF2 after administration of N-acetyl-cysteine (NAC).
Graph shows data for oxidative stress in HFFF2 treated with X-rays after administration of the NAC antioxidant molecule.
Analysis is performed administrating DCFH-DA able to bind ROS and release fluorescence relative to ROS concentration in the cellular environment. Fluorescence is detected by a Victor apparatus.
Results do not show oxidative stress from 3 to 8 days after X-rays irradiation.
Results were normalized to the control values.
Three experiments were performed.
Errors bar were calculated using standard error propagation rules. Statistical analysis was performed between normalized treated and control samples by t Student test.

X-rays treated cells, prevented from OS by NAC administration, do not show telomere length modulation.

Finally, in order to understand if X-rays-induced OS is the main cause of telomere length modulation, we performed a new telomere length analysis by Q-FISH on treated samples administrated with NAC 30 minutes prior and every 24 hours after irradiations, preventing them from oxidative stress. Results, normalized on control values (**Fig. 27**) did not highlighted a trend of telomere shortening and elongation after X-rays irradiation, strictly linking OS to telomere damage, to the consequent ALT mechanism activation and to telomere elongation.

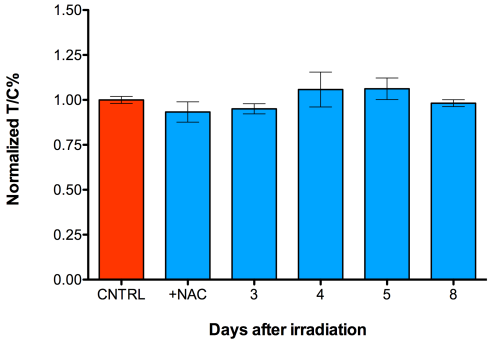

Figure 27: Quantitative-Fluorescence *in situ* Hybridization analysis for telomere lengths after N-acetyl-cysteine (NAC) administration.
The histogram represents results obtained by the analysis of telomere length after X-rays treatment and administration of NAC antioxidant molecule. Telomere lengths (T/C%) were calculated as the ratio between the total telomere fluorescence (T) and the fluorescence of the centromeres of the two chromosomes 2 (C), used as internal reference in each metaphase analyzed; data obtained from treated samples were normalized with the value of each matched control samples.
Data do not show any statistical significant trend of telomere shortening and elongation in the time period from 3 to 8 days after X-rays exposure after the NAC administration.
For each time-point 10 metaphases were analyzed.
Three experiments were performed.
Errors bar were calculated using standard error propagation rules. Statistical analysis was performed between treated samples and the average of all the normalized untreated matched control samples (left bar) by t Student test.

DISCUSSION

Telomeres are nucleoprotein structures localized at the end of eukaryotic chromosomes [4].
Every time that a mortal cell splits and during DNA replication, telomeres become shorter until cell activates senescence and apoptosis pathways [23], preventing telomeric DNA repair mechanisms activation able to induce chromosome and genome instability that could give rise to a cellular transformation [27].
Sometimes cells can avoid senescence and apoptosis activating telomere maintenance mechanisms (TMMs) in order to counteract the natural and physiological shortening [20]. Two TMMs are mainly known: Telomerase and Alternative Lengthening of Telomere (ALT).
It is well known that, while there are genetic contributions to the maintenance and inheritance of telomere length and functions, different lifestyle factors and environmental stress have been shown to negatively impact on them [153,154]. In particular, ionizing radiations (IRs) belong to these environmental factors able to affect telomeres. In fact, telomeres are considered radiosensitive structures responding to radiation exposure that acts directly or indirectly on telomeric DNA, impairing or modulating telomere functions [155,156]. Different studies showed that sparsely IRs cause telomere shortening *in vitro* [157,158]. Furthermore, a study performed on workers chronically exposed to different types of ionizing radiations (both sparsely and densely IRs) highlighted telomere shortening after exposure [159]. In the same way, studies performed on leukocytes from Hiroshima atomic bomb survivors [160] and from Chernobyl accident survivor workers [161] found similar results demonstrating telomere shortening in the exposed group.
On the other hand several studies showed telomere lengthening after IRs exposure both *in vivo* and *in vitro* [96,97,161,162]. Some of them speculated about the possible selection of radio-resistant cells with longer telomeres; while few studies demonstrated telomere lengthening because of the activation of telomerase in mouse splenocytes after X-rays [156] and of alternative lengthening of telomere (ALT) pathway in human primary fibroblasts after protons irradiation [98].
Regarding X-rays, it has been found that they are able to cause telomere shortening at short-term [158] and telomere elongation at long-term from exposure in human primary fibroblasts [96,158].

Considering the role of telomere in chromosome stability, our aim was to understand the mechanism(s) of telomere length modulation after irradiation. Starting from the above findings, in our work we focalized our attention on the possible mechanism involved in telomere length modulation induce by X-rays in human primary foreskin fetal fibroblasts (HFFF2) at different times from irradiation.
Therefore, we first analyzed telomere lengths by telomeric quantitative-FISH (Q-FISH) after X-rays exposure.
Data showed telomere shortening at 3 days and telomere elongation at 13 days confirming literature data discussed above [96,158]. Moreover, these data highlighted a trend of telomere shortening and elongation during the whole period of analysis.
We later analyzed whether X-rays are able to induce telomere dysfunctional induced foci (TIF) [146] in HFFF2 by the analysis of colocalization between the telomeric protein TRF1 and 53BP1/yH2AX, two proteins involved in the DNA damage response (DDR) [143-145]. Results showed for both 53BP1 and yH2AX a correlation between TIF increase and telomere shortening, linking the latter to an increase of telomeric DNA damage. On the other hand, at longer telomeres corresponded a significant decrease of TIF frequencies, suggesting a reduced telomeric damage after telomere elongation.
Broadly (especially for 53BP1 results), TIF frequencies were higher than control values during the whole period of analysis. This is expected, since telomeric DNA damage persists longer then the other sites [157,163,164] and are repaired less efficiently [165,166], probably because of the heterochromatic nature of telomere [167] and/or inhibition of non-homologous end-joining (NHEJ) by TRF2 [168-170].
In order to understand the mechanism responsible of telomere lengthening, we tested our cells both for telomerase activity and ALT biomarkers. A previous study (on HFFF2 fibroblasts treated with densely ionizing radiations like protons) demonstrated ALT mechanism activation and any telomerase activity [98]. Also, for X-rays, any telomerase activity was detected by TRAP-assay analysis in our treated samples. Consequently, we studied the involvement of ALT mechanism testing our samples for two different ALT biomarkers: telomere-sister chromatid exchanges (T-SCEs) [42-45] by CO-FISH**;** and associated PML bodies (APBs) [55] by Immuno-FISH between telomere and PML/RPA2 proteins.
Results showed a matched increase of T-SCEs and APBs frequency with those days that showed telomere elongation.

In order to better characterize the TMM mechanism, we analyzed some proteins usually related to ALT.
ATRX/DAXX chromatin remodeling complex has been proposed to have numerous diverse functions, including chromatin remodeling, viral resistance, and fidelity of chromatin separation during cell division, as well as binding to tandem DNA repeats [72-75]. ATRX gene has been found to be mutated and inactivated in ALT-positive tumors and cell lines [76-78].
Moreover, since ALT is based on recombination between homologous repeated telomeric sequences, is well known that many proteins of the H
homologous recombination (HR) DNA repair pathway (such as RAD51, RAD52, MRN complex, BLM, BRCA1) are involved in ALT [Reviewed in 82].
In addition, the mammalian replication protein A (RPA) involved in single stranded DNA sequence stabilization during DNA replication and in the interaction with repair and recombination components during replication stress [82,171], has been found to be involved in the first steps of ALT activation [172].
Due to the important role of these proteins in the ALT mechanism, to improve our results on ALT activation, we analyzed ATRX, RAD51 and the second RPA subunit (RPA2).
Data showed a similar profile to ALT-positive cell lines with a significant reduction of ATRX level in treated samples (confirmed by a dose-response analysis on cells treated with 2-10 Gy of X-rays), a significant increase of RAD51 and a similar increase of RPA2 level.
ALT mechanism is know to be activated as a TMM by the 15-20% of the known tumors, but our results enrich the scientific literature that found evidence of an ALT-like mechanism in not cancer cells. For example, telomerase-negative fibroblasts immortalized by SV-40 were reported to lack APBs but still showed nuclear aggregates of APBs components at telomeres and featured all the other ALT characteristics [91-93].
Moreover, low levels of extra chromosomal telomere repeats (ECTRs) were detected in telomerase-positive and mortal cells [91,94,95].
Another study proved that protons ionizing radiations are able to modulate telomere length in human primary fibroblasts and to induce a transient ALT phenotype, confirmed by an induction of ALT hallmarks such as T-SCEs and APBs [98]. Notably, recent studies provided evidence that ALT biomarkers can be activated inside primary cells after replication stress and telomeric DNA damage [89,99]. In this work, all results (telomere lengthening, ALT

biomarkers induction and a protein profile similar to ALT-positive cells) allowed us to point out an ALT-like mechanism, transient in its activation and milder in its phenotype than ALT-positive cancer cells, in HFFF2 exposed to X-rays. Furthermore, considering data on cell cycle and on cell proliferation, as expected, we highlighted an affected growth of X-rays treated cells compared to the untreated controls, displaying an accumulation at G2 phase. This stacking in G2 probably could allow and ensure HR pathway (and so ALT) even at telomeres.

We believe that the induction of an ALT-like mechanism following X-rays irradiation could be a response to telomeric DNA damage and could act as a "telomere-repair system".

Very recent studies suggested this role for ALT that seems to be activated (in primary and telomerase-positive cells) or enhanced (in ALT-positive cells) following telomeric damage. In fact, was demonstrated that induced telomere damage [176] or replication stress [89] lead to ALT hallmarks detection both in primary proliferating and telomerase-positive cells and, consequently, to telomeric DNA damage reduction resulting in TIF frequency decrease [176].

The expression of the TRF1-FOK1 system, able to cut and to induce DSBs specifically at telomeric sequences, is a powerful and current tool that permits to study effects of telomeric DNA damage. With this system mouse embryonic fibroblasts (MEFs) unleashed an ALT-like phenotype showing T-SCEs after telomeric DSBs induction [99] while ALT-positive cells were characterized by an "enhanced" ALT phenotype displaying homology-directed directional telomere movement inside APBs [58] and break-induced new telomere synthesis [177].

The TMM mechanism involves both ALT or telomerase activation. The ALT mechanisms evidenced in fibroblast could be explained considering that fibroblasts are characterized by a mesenchymal embryonic origin and it is well known that cancers arising from mesenchymal tissues including bone, soft tissues, neuroendocrine systems, peripheral nervous system and central nervous system, generally do not activate telomerase but ALT as TMM [173], hypothesizing that a tighter regulation of telomerase expression in mesenchymal tissues may force these cells to choose for ALT when telomere lengthening is needed [41].

Another question of this project was to understand the trigger of telomere shortening after X-rays irradiation. It is known that both sparsely and densely ionizing radiation are able to induce a persistent oxidative stress (OS) [121-124,132].

Furthermore it is also well known the capability OS to affect telomere. The principal DNA damage that occurs after OS is the formation of 8-oxoG and the probability of an accumulation of this lesion within telomeres during oxidative stress is enhanced by the high incidence of guanine residues in telomeric DNA sequences [136]. Moreover, the guanine expressed in sequences GG or GGG are more susceptible to oxidation than single guanine [137,138] supporting the hypothesis that the telomeric sequence is an important target of oxidative damage [139].

To study whether specifically OS is involved in telomere shortening observed in our cells, we first analyzed by dichlorofluorescein-diacetate (DCFH) assay the level of reactive oxygen species (ROS). Results showed a persistent oxidative stress up to 4 days after X-rays exposure, approximately corresponding to the period of telomere shortening and highest telomere damage. Then, the further test was to administrate to cells (before and every 24 hours after X-rays treatment) a well-known antioxidant molecule, the N-acetyl-cysteine (NAC), in order to prevent ROS production after irradiation and to protect cells from OS. As expected, the DCFH assay analysis on NAC-administrated cells did not show any OS after X-rays irradiation compared to untreated controls. Interestingly, the NAC-administrated HFFF2 did not show any trend of telomere shortening and elongation after X-rays exposure. These data on the OS persistence and the results obtained after antioxidant treatment strongly support the idea that the telomere shortening observed after irradiation is due to the ionizing radiation-induced oxidative stress.

Our data are in agreement with previous results that indicated oxidative stress (and the consequent generation of 8-oxoG lesions) as the responsible of persistent telomere damage [174] and of the ALT-like phenotype induction [175]. In addition, other authors demonstrated that this lesion within telomeric DNA interferes with the replication fork at telomeres and aborted replication may lead to strand breaks, loss of telomere repeats and to telomere shortening [133]. Moreover, 8-oxoG may interfere directly with the recognition by TRF1 and TRF2 proteins of telomere repeats [141] leading to telomere dysfunction and activation of DDR at telomeres.

Thus, we concluded that ionizing radiation-induced oxidative stress can provoke oxidative damage at telomeres leading to telomere dysfunction and consequently to telomere shortening. This damage could be responsible of TMM activation in order to stabilize telomere structure that in fibroblasts, probably due to their embryonic origins, is the ALT mechanism.

CONCLUSION

Considering the role of chromosome ends in genomic stability, this PhD project was focused on understanding the mechanism involved in telomere maintenance after DNA damage in primary cells. In this work we used X-rays as DNA damage-induced agent, because it was previously demonstrated the modulation of telomere length after X-rays irradiation. In fact, X-rays are responsible of DNA damage both *via* direct and indirect effects due to their capability to induce oxidative stress (OS). It is well known that telomere is one of the main targets of OS because its G-rich structure.
Data obtained demonstrated an initial telomere shortening and a subsequent telomere elongation after 4Gy of X-rays in the frame of two weeks. Because of the high incidence of guanine residues, we demonstrated that radiation-induced OS is responsible of telomere shortening.
In fact, this effect was not observed with a pre-treatment with an antioxidant molecule. Probably, radiation-induced OS causes oxidative lesions, affecting telomere integrity and functions, and leading to telomeric damage.
The second question was to understand the mechanism of telomere elongation observed immediately afterwards telomere shortening. We demonstrated no telomerase activation but the activation of the ALT pathway, probably able to counteract the OS-induced shortening effect on telomere. Moreover, the ALT activation in fibroblasts could be due to the origin of these cells that, as mesenchymal, have usually genes involved in telomerase activation strongly silenced.
We hypothesized that cells activate a transient ALT mechanism able to re-elongate and at the same time heal telomere, reducing telomeric damage **(Fig. 28)**, genomic instability and, probably cell death.
From this perspective, the modulation in telomere length is the result of the interaction between the "shortening and elongation forces" of OS and ALT, respectively, on telomeres.

Since we found evidence of a mechanism (canonically known in cancers) in primary cells, these findings are pretty interesting and this work contributed, with other few studies, to understand not only telomere dynamics but also to describe a new role of ALT in normal cells. In fact, our idea is that, while ALT works as a TMM within the completely functionally deregulated ALT-positive cancer cells, contributing to immortalization and cancer development; in primary

cells ALT could act as a “telomeric-repair system”, based on proteins already committed to DNA repair, that can promptly “switched on/off” when needed.

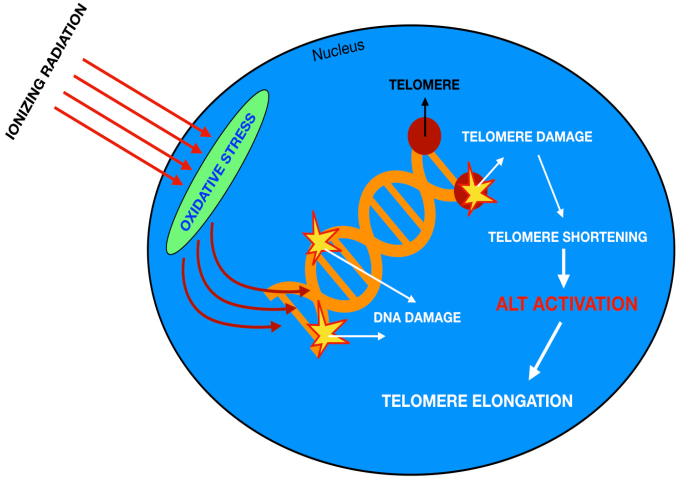

Figure 28: Schematic representation of our final conclusions.

APPENDIX - MATERIALS AND METHODS

Cell Culture
Human fetal foreskin fibroblasts (HFFF2) (ECACC, UK) were cultured in D-MEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine and grown in a 95% air/5% CO_2 atmosphere at 37° C. All the media and the supplements for cell culture were purchased from Euroclone. In these conditions, the cell doubling time, T_d, as determined from the growth curves, was 24 ± 1 h. Cells used in this work were at passage 29.

Irradiation Procedure
For X-rays irradiation, cells were seeded in plates at least 48h before treatment and irradiated at room temperature (RT) using a Gilardoni apparatus (250 kV, 6 mA, dose-rate 0.53 Gy/min). Cells were irradiated with a dose of 4 Gy and unirradiated cells were used as controls.

Long-term proliferation assessment
Irradiated and control cells were grown for 16 days with 3 intermediate passages after 4, 8 and 12 days of culture. After harvesting, cells were counted using a scepter handheld automated cell counter (Millipore). The cumulative population doubling level (cPDL) after 4, 8, 12 and 16 days after X-rays treatment was calculated as fallows:

$$cPDL = \log2(N_f/N_0)$$

where N_f is the final cell number and N_0 is the initial number of seeded cells. The experiment was repeated two times.

Growth curves
Cells at 80% of confluence were treated with X-rays, detached by standard trypsinization and seeded again in different 60 mm plates (10^5 cells). Cells were then grown for 168h and detached every 24h and counted with a scepter handheld automated cell counter (Millipore). Doubling time was determined as slope of the straight region of the growth fitted function in the semilog chart. Results were obtained from four independent experiments.

Flow Cytometry

For cell cycle analysis 1 x 10^6 cells for each samples were washed twice with PBS, fixed dropwise with cold ethanol (70%) and rehydrated with PBS. DNA staining was performed by incubating cells for 30 min at 37°C in PBS containing 0.18 mg/ml propidium iodide (PI) and 0.4 mg/ml DNase-free RNase (type 1-A). Samples were acquired with a Cytoflex (Beckman Coulter) equipped with a 488nm laser source. Cell cycle analysis was performed using a Cytexpert v2.0 software. Doublet discrimination and exclusion was performed by an electronic gate on FL3-Area vs. FL3-Height. Three independent experiments were performed.

Collection of chromosome spreads

Chromosome spreads were obtained following 30 min incubation in Calyculin-A (30 μM; Wako), a protein phosphatase inhibitor, which induces chromosome condensation irrespectively of cell-cycle phase. In the present paper only G_2 condensed chromosomes have been scored in cytogenetic analysis. Prematurely condensed chromosomes (PCC) were collected by a standard procedure consisting of treatment with hypotonic solution (75 mM KCl) for 30 min at 37 °C, followed by fixation in freshly prepared Carnoy solution (3:1 v/v methanol/acetic acid). Cells were then seeded onto slides and utilized for cytogenetic analysis.

Telomeric quantitative FISH (Q-FISH)

After seeding (48h), the slides were rinsed with PBS, pH 7.5, and fixed in 4% formaldehyde for 2 min. After two rinses in PBS, the slides were incubated in acidificated pepsin solution for 10 min, rinsed, and dehydrated through graded alcohols. Slides and probes (Cy3-linked telomeric and chromosome 2 centromeric PNA probe, Panagene and DAKO Cytomatation, respectively) were codenatured at 80°C for 3 min and hybridized for 2h at RT in a humidified chamber. After hybridization, slides were washed twice for 15 min with 70% formamide, 10mM Tris pH 7,2 and 0,1% BSA, followed by three washes (in 0,1 M Tris pH 7,5, 0,15 M NaCl and 0,08 % Tween 20) for 5 min each. Slides were then dehydrated with an ethanol series and air-dried. Finally, slides were counterstained with 4,6-diamidino-2 phenylindole (DAPI) in Vectashield (Vector Laboratories). Images were acquired at 63x magnification using an Axio Imager Z2

microscope (Carl Zeiss) equipped with a Cool Cube 1 CCD camera (MetaSystems). Telomere size analysis was performed with the ISIS software (MetaSystems). The software calculates telomere lengths as the ratio between the total telomere fluorescence (T) and the fluorescence of the centromeres of the two chromosomes 2 (C), which was used as the internal reference in each metaphase spread analyzed, and expressed as percentage (T/C%). At least 10 metaphases were analyzed for each sample in three independent experiments.

TIF co-immuno staining

Cells were fixed with 4% paraformaldehyde (Sigma Aldrich), permeabilized with 0,5% Triton-X and blocked in PBS/BSA 1%. Samples were then co-immunostained over night at 4°C, using a rabbit telomeric protein TRF1 antibody (Santa Cruz Biotechnology) in combination with mouse yH2AX (Millipore) or a mouse 53BP1 antibody (Millipore). After washes in PBS/BSA1% samples were incubated with the secondary antibodies (anti-mouse Alexa 546 and anti-rabbit ALexa 488, Invitrogen) for 1h at 37°C. Finally, slides were washed in PBS/1% BSA, counterstained with DAPI and analyzed with fluorescence microscopy using an Axio-Imager Z2 microscope (Cal Zeiss) equipped with Cool Cube 1 CCD camera (MetaSystems).
The frequency of foci and colocalization dots per cell were scored in 50 nuclei in three independent experiments.

Real Time Quantitative–Telomerase Repeat Amplification Protocol assay (RTQ-TRAP)

Telomerase activity was measured by the SyBR green RTQ-TRAP assay, which was conducted as described elsewhere (Wege et al., 2003) with minor modifications. Briefly, the reaction was performed with protein extracts (1,000 cells), 0.1 µg of telomerase primer TS, and 0.05 µg of anchored return primer ACX, in 25 µl of SyBR Green PCR Master Mix (Biorad). The primer sequences were those reported by Kim and Wu (Kim and Wu, 1997). The reaction was performed using the Agilent AriaMx real-time PCR system (Agilent Technologies), samples were incubated for 20 min at 25°C and amplified in 35 PCR cycles with 30 sec at 95°C and 90 sec at 60°C (two step PCR). The threshold cycle values (Ct) were determined from semi-log amplification plots (log increase in fluorescence as a function of cycle number) and compared with standard curves generated from serial dilutions of telomerase-positive U251MG cell extracts. Each sample was analyzed in triplicate in two independent experiments. Telomerase activity was expressed relative to the

telomerase-positive samples.

Chromosome orientation-FISH (CO-FISH) Analysis
Sub-confluent HFFF2 cells were exposed to X-rays and sub-cultured in the presence of 5'-bromo-2'-deoxyuridine (BrdU, Sigma Aldrich) at a final concentration of 2.5 x 10^{-5} M and were then allowed to replicate their DNA once at 37°C overnight. Calyculin A was added at a final concentration of 30 μM during the final 30 min. Cells were then collected and chromosome spreads were prepared as described above. CO-FISH was performed as described previously (Bailey et al., 2004) first using a (TTAGGG)3 probe labeled with FITC and then using a (CCCTAA)3 probe labeled with Cy3 (Panagene). Images were captured at 63x magnification using an Axio-Imager Z2 microscope (Cal Zeiss) equipped with a Cool Cube 1 CCD camera (MetaSystems) and analyzed by ISIS software (MetaSystems)
T- SCEs were scored only when the double signals were visible with both the Cy3 and FITC probes. Experiments were repeated three times and 1000 chromosomes were analyzed for each sample.

PML-Telomere Immunofluorescence-FISH staining
At different times after irradiation, cells were fixed for 20 min with 4% paraformaldehyde (Sigma Aldrich) in PBS at 4°C and permeabilized with 0.1% Triton X-100 in PBS at RT. After blocking with 10% BSA at 37°C for 20 min, cells were incubated with a rabbit polyclonal antibody against PML (H-238:sc5621, Santa Cruz Biotechnology) diluted 1:100 in PBS, for 3h at RT. After washing with 0.05% Triton X-100 in PBS for 5 min, cells were incubated with Alexa 488 anti-rabbit antibody diluted 1:300 in blocking solution for 1h at 37°C (for PML detection). After the immunostaining, telomeric FISH was performed as described above. Images were captured with fluorescence microscopy using an Axio-Imager Z2 microscope (Carl Zeiss) equipped with a Cool Cube 1 CCD camera (MetaSystems).
At least 50 nuclei in three independent experiments were analyzed to identify events of possible colocalization.

RPA2-Telomere Immunofluorescence-FISH staining
At different times after irradiation cells were fixed with 4% paraformaldehyde (Sigma Aldrich), then permeabilized with 0.2% Triton X-100, and blocked in 1% BSA in PBS for 30 min at RT. Samples were incubated overnight at 4 °C with a mouse mono-clonal anti-RPA2 antibody (Abcam; Cambridge) diluted 1:100. Coverslips

were then washed in 0.05% Triton X-100/PBS and incubated for 1h at 37 °C with an Alexa 488 anti-mouse antibody (Invitrogen, Life Technologies) diluted 1:200 for 1h at 37°C. After the immunostaining, telomeric FISH was performed as described above. Images were acquired using an Axio Imager Z2 microscope (Carl Zeiss) equipped with a Cool Cube 1 CCD camera (MetaSystems). Images were then analyzed to identify colocalizations between RPA2 foci and telomere signals. The frequency of foci and colocalization dots per cell were scored in at least 50 nuclei and in three independent experiments.

Whole cell extracts and western blotting

Cells were harvested with trypsin, quickly washed in PBS, counted with a scepter handheld automated cell counter (Millipore) and directly lysed in LDS sample buffer (Life Technologies) at 10^4 cells per µl. Proteins were gently homogenized using a 25-gauge syringe), denatured for 10 min at 70°C and resolved by SDS-Page electrophoresis, transferred to nitrocellulose, blocked in 5% skim milk for 20 min and probed. For secondary antibodies, HRP-linked anti-rabbit, or mouse (Amersham) were used, and the HPR signal was visualized with Supersignal ECL substrate (Pierce) as the manufacturer's instructions. Three experiments were performed.

Western primary antibodies:

Target	Species	Source, Cat#	Diluition
RAD51	Rabbit	Santa Cruz,#sc-8349	1:500
ATRX	Rabbit	Santa Cruz,#sc-7152	1:500
RPA2	Mouse	Abcam,#ab2175	1:1000

Intracellular ROS determination

Cells were seeded at the density of 4 x 10^3 inside 96-multiwell plates. Culture medium was then discarded and a new medium containing 10 µM dichlorofluorescein 2'-7'-diacetate (DCFH- DA) (Sigma-Aldrich) was added. Samples were incubated for 30 min in the dark, to allow probe uptake. Cells were washed twice with PBS buffer and recovered for 30 min in the dark before analysis. DCFH-DA diffusion into cells was allowed by acetyl groups, while deacetylation by intracellular esterase activity prevented the DCFH exit from cells (Gnocchi et al., 2012). Emission analyses were performed by the automatic plate

reader Victor 3V (Perkin Elmer) and Wallac 1420 software. Excitation and emission wavelengths were set at 498 nm and 530 nm. To assess ROS content variations after X-ray exposure, cells were irradiated and analyzed at different times.
For each sample, the analysis was repeated three times in three independent experiments.

N-acetylcysteine (NAC) administration
ROS content variations were valuated even after N-acetyl-cysteine (NAC, Sigma-Aldrich) antioxidant molecule administration.
NAC was administrated 30 min prior and every 24 h after irradiations at the final concentration of 2mM.

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ACKNOWLEDGEMENTS

I would like to express my special appreciation and thanks to my "Boss" Prof. Antonella Sgura (Department of Science, University of Rome "Roma Tre"), who gave me the opportunity to study and follow this project, encouraging my research.
Thanks to Prof. Antonio Antoccia (Department of Science, University of Rome "Roma Tre"), for his scientific support.
A special, big, huge thanks goes to the guys of my laboratory: Francesco, Elisa, Silvia, Jessica, Valentina, Antonella, Daniela, Sabrina and Ion for their assistance and suggestions; and to Elisa and Arianna for the help in this project.
I would like to thank who collaborated with us, Dr. Stefano Leone (Department of Science, University of Rome "Roma Tre"); Dr. Haroldo Silva and Dr. David Halvorsen (SENS Foundation, Mountain View, California).
I would like to thank Dr. Roderick J. O'Sullivan (Hillman Cancer Center, Pittsburgh), who gave me the opportunity to spend six months in his lab, working on ALT related proteins.
A special acknowledgement goes to my family, which always supported me.