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TELOMERE METABOLISM IN RESPONSE TO DNA DAMAGE INDUCED BY LOW- AND HIGH-LET RADIATIONS

METABOLISMO TELOMERICO IN RISPOSTA AL DANNO AL DNA INDOTTO DA RADIAZIONI IONIZZANTI DI DIVERSA QUALITÀ

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Abbreviations

ALT	Alternative Lengthening of Telomeres
APBs	ALT associated PML Bodies
AT	Ataxia Telangiectasia
BER	Base Excision Repair
BFB	Break Fusion Bridge
BrdU	Bromo Deoxyuridine
CO-FISH	Chromosome Orientated FISH
DAPI	4',6-diamidino-2-phenylindole
D-loop	Displacement loop
DSB	Double Strand Break
ECTR	Extra Chromosomal Telomere Repeat
FISH	Fluorescence *in situ* Hybridization
FITC	Fluorescein isothiocyanate
Gy	Gray
HR	Homologous Recombination
IR	Ionizing Radiation
LET	Linear Energy Transfer
LMDS	Local Multiple Damaged Site
MN	Micronucleus
NBS	Nijmegen Breakage Syndrome
NHEJ	Non Homologous End Joining
PML	Promyelocytic Leukaemia
Q-FISH	Quantitative FISH
RBE	Relative Biological Effectiveness
ROS	Reactive Oxygen Species
RQ-TRAP	Real Time Quantitative TRAP
SCE	Sister Chromatid Exchange
SSB	Single Strand Break
TIF	Telomere dysfunction Induced Focus
T-loop	Telomeric loop
TRAP	Telomerase Repeat Amplification Protocol
TRF	Telomere Restriction Fragment
T-SCE	Telomeric SCE

Summary

1 Italian

Le radiazioni ionizzanti (IR) sono un noto agente genotossico, ampiamente studiato dato l'ampio spettro di possibili applicazioni in campo medico (radioterapia, diagnostica), e soprattutto per i possibili effetti sulla salute dell'uomo (esposizione occupazionale, esplorazione dello spazio). In base alla loro capacità di cedere energia alla materia le IR possono essere suddivise in radiazioni a basso- o alto- Trasferimento Lineare di Energia (LET). Nella prima categoria ricadono radiazioni in grado di cedere alla materia fino a 10 keV/μm, ad esempio raggi-X e raggi-γ, mentre nelle seconda categoria sono incluse radiazioni che rilasciano alla materia fino 200 keV/μm (protoni, ioni pesanti).
A livello cellulare, la differente capacità di cedere energia alla materia determina effetti diversi. In particolare il danno al DNA indotto da radiazioni ad alto-LET risulta più complesso e più difficile da fronteggiare rispetto a quello indotto da radiazioni a basso-LET, e tale maggiore complessità risulta in effetti cellulari più severi. Numerosi *endpoint* cellulari, quali mutazioni geniche, malsegregazione cromosomica, aberrazioni cromosomiche, sopravvivenza cellulare, sono stati presi in esame nello studio comparativo di radiazioni a basso- ed alto-LET. Tuttavia altri importanti aspetti come l'influenza di tali radiazioni sul metabolismo telomerico e sulla modulazione delle lunghezze telomeriche fino ad oggi, sono stati scarsamente investigati.
I telomeri sono complessi nucleoproteici localizzati alle estremità cromosomiche ed altamente conservati tra gli eucarioti. Consistono di sequenze ripetute in tandem dell'esanucleotide TAAGGG e sono strutture di fondamentale importanza per il mantenimento dell'integrità cromosomica; infatti, perdita di sequenze telomeriche o mutazioni in proteine che legano il telomero attivano una cascata di eventi che includono fusioni cromosomiche, instabilità genomica ed in ultima istanza, possono compromettere la proliferazione e/o la vitalità cellulare o condurre alla trasformazione tumorale.
Nel presente lavoro sono state utilizzate "radiazioni sparsamente ionizzanti" o a basso-LET, quali raggi-X e protoni da 62 MeV (pochi keV/μm), e radiazioni "densamente ionizzanti" o ad alto-LET, quali protoni da 3 MeV e ioni carbonio (LET da 28 a 39 keV/μm). Per gli esperimenti con sorgenti di

radiazione diverse dai raggi-X, ci si è avvalsi della collaborazione con ricercatori dell'INFN, e gli irraggiamenti sono stati effettuati presso i Laboratori Nazionali di Legnaro e Catania.
Nella prima parte del lavoro sono stati analizzati gli effetti genotossici indotti da radiazioni a basso ed alto LET in fibroblasti primari umani HFFF2. In particolare, le cellule sono state esposte a dosi crescenti (0.25-2 Gy) di raggi-X e protoni (3MeV, 28.5 keV/µm) e successivamente è stata valutata la cinetica di riparazione del danno al DNA e l'induzione di micronuclei. L'induzione di rotture alla doppia elica del DNA (DSBs) e la cinetica di riparazione di tali rotture sono state valutate seguendo la defosforilazione dell'istone H2AX nel tempo. I risultati mostrano che seppure la radiazione ad alto-LET induca inizialmente un minore numero di DSBs rispetto ai raggi-X, tali DSBs sono riparate con una cinetica più lenta nelle prime 24 ore dal trattamento. Dati ottenuti dal test del micrronucleo hanno confermato il più elevato potere clastogenico delle radiazioni ad alto-LET. L'esposizione ai protoni, infatti, è stata in grado di indurre un numero approssimativamente doppio di micronuclei rispetto a quello indotto dai raggi-X, almeno fino alla dose di 1.5 Gy. I dati ottenuti mostrano come il danno al DNA indotto da radiazioni ad alto-LET, ed in particolare da protoni a bassa energia, sia riparato in modo meno efficiente e generi danno citogenetico nel corso del primo ciclo cellulare dopo il trattamento.
Nella seconda parte del lavoro è stato valutato l'effetto del danno indotto dai protoni a 3 MeV sulla modulazione delle lunghezze telomeriche. A tale scopo fibroblasti umani HFFF2 sono stati trattati con 4 Gy di radiazioni a basso- (raggi-X, protoni da 62MeV) ed alto- (protoni da 3 MeV) LET e sono stati effettuati esperimenti di Q-FISH (*Quantitative-Fluorescence In Situ Hybridization*) telomerica quantitativa a 24 ore dall'esposizione. I risultati ottenuti hanno mostrato che solo la radiazione ad alto-LET è in grado di indurre un aumento significativo delle lunghezze telomeriche (22%), mentre nessuna variazione è stata riscontrata dopo trattamento con radiazioni a basso-LET. Per comprendere quale meccanismo fosse alla base del fenomeno osservato, è stata valutata l'induzione dell'enzima telomerasi e l'attivazione di meccanismi di ricombinazione telomerica. I dati ottenuti hanno escluso che il trattamento con radiazioni ionizzanti fosse in grado di indurre sia la trascrizione del gene *TERT*, codificante per la subunità catalitica dell'enzima, sia l'attività dell'enzima stesso, come mostrato tramite saggio TRAP (*Telomerase Repeat Amplification Protocol*). Risultati più incoraggianti sono invece emersi analizzando il coinvolgimento della ricombinazione telomerica nel fenomeno di allungamento osservato. Esperimenti di CO-FISH (*Chromosome Orientated*-FISH) infatti hanno

mostrato che i protoni da 3 MeV sono in grado di indurre un aumento della frequenza di scambi tra cromatidi fratelli nella regione telomerica. Tale evidenza ha mostrato come le radizioni ad alto-LET siano in grado di indurre, in modo transiente, un meccanismo di ricombinazione telomerica con caratteristiche simili al pathway di allungamento telomerico ALT (*Alternative Lengthening of Telomeres*) presente in alcune cellule tumorali. Tali dati sono stati confermati attraverso esperimenti di immuno-FISH che hanno mostrato un'induzione da parte dei protoni da 3 MeV di un altro marcatore delle cellule ALT-positive: la colocalizzazione della proteina PML (*ProMyelocytic Leukaemia*) e del DNA telomerico.

Recenti evidenze sperimentali hanno mostrato come la proteina NBS1 è essenziale per il fuzionamento del *pathway* ALT. NBS1 è una proteina coinvolta nel processo di riparazione del danno al DNA radio-indotto e in particolare nella riparazione mediata da ricombinazione omologa. Per valutare se mutazioni nel gene *NBS1* influenzassero l'attivazione radio-indotta del pathway ALT, linee cellulari linfoblastoidi (LCLs) con differente genotipo per il gene *NBS1* ($NBS1^{+/+}$, $NBS1^{+/-}$, $NBS1^{-/-}$) sono state esposte a 0.5-4Gy di ioni carbonio (39 keV/µm). I risultati ottenuti hanno mostrato che solo nelle linee $NBS1^{+/+}$ e $NBS1^{+/-}$ viene indotto un'allungamento telomerico significativo, supportando l'ipotesi del coinvolgimento del pathway ALT.

L'analisi della modulazione delle lunghezze telomeriche mediante Q-FISH è stata successivamente effettuata anche a tempi più lunghi (3/4 e 15 giorni) dall'esposizione alle diverse sorgenti di radiazioni. I dati ottenuti hanno mostrato che l'allungamento telomerico osservato a 24 ore dal trattamento con radiazioni ad alto-LET viene mantenuto nel tempo almeno fino al 15° giorno, sia in cellule primarie che immortalizzate.

Un più complesso *pattern* di modulazione delle lunghezze telomeriche è stato invece osservato dopo esposizione a radiazioni a basso-LET. Infatti a 3 o 4 giorni dal trattamento (3 giorni nel caso di cellule primarie HFFF2 e 4 nel caso dei LCLs) è stato riscontrato un accorciamento telomerico, seguito poi da un allungamento, come rivelato dall'analisi a 15 giorni. Risultati analoghi sono stati ottenuti su popolazioni clonali di linfoblasti umani TK6 derivati da singole cellule sopravvissute al trattamento con 4Gy di raggi-X. I dati ottenuti suggeriscono che uno dei processi implicati nella modulazione delle lunghezze telomeriche indotta da radiazioni a basso-LET sia una accresciuta radioresistenza delle cellule che nella popolazione iniziale mostravano lunghezze telomeriche medie maggiori.

2 English

Ionizing radiations are a well known genotoxic agents, widely studied for the great impact of their applications (*i.e.*, radiotherapy and hadrontherapy) and effects (*i.e.*, exposure risk for astronauts in space missions). Exposure to ionising radiations (IR) can result in the deposition of energy to DNA molecules, thus leading to DNA damage. IR-induced DNA damage is localized, and the level of localization is believed to increase with increasing linear energy transfer (LET) values of the radiation. Because LET is a measure of the energy released to an object along the path of the radiation, high-LET radiation can deposit more energy than low-LET one. Condensed or concentrated energy deposition results in cluster of ionization events. When the target is the DNA, the site of such lesions is termed "clustered DNA damage" or "locally multiply damaged site", which consists in two or more lesions localized in close proximity on the DNA duplex.

In order to study the biological effects of high-LET radiations, several endpoints have been evaluated both in rodent- and in human-irradiated cells, including chromosomal aberrations, micronuclei (MN), chromosomal non-disjunction, mutations, DNA fragmentation, clonogenic survival, and cell cycle effects. However, aspects related to telomere length modulation and telomere metabolism have been so far poorly investigated both in primary and in immortalized cells exposed to low- and high-LET radiations.

The aim of the first part of the study was to analyze the DNA-damage and the genotoxic effects induced by graded doses (0,25-2 Gy) of low-energy protons (high-LET radiation), and X-rays (low-LET radiation) in human primary fibroblasts. DSB induction and repair as mesured by scoring for γ-H2AX foci indicated that 3MeV protons, with respect to X-rays, yielded a lower number of DSBs per Gy, which showed a slower kinetics of disappearance in the first hours from irradiations. Furthermore, irrespective of dose delivered, a higher fraction of unrejoined DSBs persisted in sample harvested 24 hours from exposure to protons. The higher clastogenic effect of protons was in agreement with the extent of micronuclei (MN) induction in binucleated cells up to 1,5 Gy. Our results support the notion that DNA DNA damage produced by 28.5 keV/μm protons appears less amenable to be repaired and could be transformed in cytogenetic damage in the form of MN in the first cell cycle from irradiation .

After confirming the greater biological effectiveness of high-LET radiations compared to low-LET ones, we focused our attention on studying telomere metabolism within 24 hours from the exposure to both types of radiations.

Interestingly, data obtained showed a different kinetics of telomere length modulation in cells exposed to low- or high-LET radiations. Moreover, the phenomenon observed appeared to be conserved both in primary and in immortalized cell lines. Interestingly, exposure of human primary fibroblasts to 4Gy high-LET radiation determined a telomere elongation respect to untreated cells, whereas no telomere length modulation was observed in low-LET treated fibroblasts. In order to investigate the molecular mechanism underlying the observed elongation, the expression levels of the telomerase (*i.e.,* hTERT) and its enzymatic activity were evaluated. Results obtained excluded the involvement of the telomerase in the observed telomere lengthening induced by high-LET radiation, thus supporting the activation of a telomerase-independent mechanism. Some mammalian cells lacking in any telomerase activity are able to maintain the length of their telomeres for many population doublings (PDs). This indicated the existence of one or more non-telomerase mechanism(s) for telomere maintenance, further termed Alternative Lengthening of Telomeres (ALT). To date, clear evidences of the existence of an ALT activity has been demonstrated only in human tumours and immortalized cell lines, and in telomerase-null mouse cell lines. To analyze whether a recombinational mechanism could be responsible for the high-LET-induced telomere lengthening observed in human primary fibroblasts, two types of experiments were performed. On one side, the incidence of recombinational events at telomeres (T-SCE) was measured, and on the other side the colocalization of telomeres and PML bodies (that are considered as an hallmark of cells with activated ALT pathway), was analyzed. Strikingly, our results indicated that the DNA damage induced by high-LET radiation is somehow able to induce telomere lengthening through the transient activation of an ALT recombinational pathway.

Recent reports demonstrated that NBS1 is essential for the correct functioning of the ALT pathway. *NBS1* gene, mutated in the NBS human chromosome instability disorder, encodes for the NBS1 protein, a central player in the response to the ionizing radiation-induced DNA damage, as well as in the homologous recombination repair. In order to confirm the high-LET-induced recombinational ALT pathway, telomere length was evaluated in Lymphoblastoid Cell Lines (LCLs) heterozygous ($NBS1^{+/-}$) and homozygous ($NBS1^{-/-}$) for a mutation of the *NBS1* gene, as well as in normal cells ($NBS1^{+/+}$) exposed to 4 Gy of carbon ions (39keV/µm). Remarkably, a telomere elongation was observed in $NBS1^{+/+}$ and $NBS1^{+/-}$ cells, but not in $NBS1^{-/-}$ones. These data evidenced that the process of telomere lengthening induced by high-LET radiation is NBS1-dependent,

thus supporting the hypothesis that telomere elongation is mediated by recombinational mechanisms.
Beside the analysis performed at 24 hours, telomere length modulation was followed up to 15 days from the irradiation of both human primary fibroblasts and LCLs. Dynamics of telomere lengths modulation appeared to be different after low- and high-LET irradiation. Our data showed that the telomere lengthening observed in high-LET-treated cells seems to be maintained at 3-4 days, as well as 15 days after exposure. Interestingly, the time-course of the low-LET radiation-induced telomere length modulation appeared to be more complex than the high-LET one. In fact, after 3-4 days telomere erosion was reported, whereas after 15 days from the exposure a telomere lengthening was observed in primary as well as in immortalized cell lines.
To explain the time course of low-LET-induced telomere length modulation we have hypothesized that a direct correlation between telomere length and radioresistance/radiosensitivity could account for this phenomenon. To test our hypothesis, we decided to perform experiments in TK6 lymphoblast cells, since they represent a good and widely used radiobiological cellular model. Data obtained brought us to suggest a model: the radioresistance of cells with longer telomeres drives a selection process that led to an increased telomere length in clones survived to low-LET radiation exposure. A direct correlation between telomere length and radisensitivity/radiresistance has already been proposed in some published reports and imply that telomeres length measurement could be potentially used as a tool to predict clinical radiation response in radiotherapy.

Introduction

1 Telomeres

Telomeres or the ends of linear eukaryotic chromosomes, were first described almost 70 years ago since the pioneering studies of the geneticists Hermann Joseph Muller and Barbara McClintock in the fruit fly *Drosophilia melanogaster* and *Zea maize*, respectively [1, 2]. Muller observed that the ends of chromosomes rarely interacted with breaks that resulted from ionizing radiation (*i.e.*, X-ray-induced chromosomal aberrations never included deletions or inversions involving the terminal regions of the chromosomes). Thus, he proposed that chromosome ends are specialized structures that he coined "telomeres", from the Greek, telo = end, and mere = part [1]. The concept of "telomere" meant not only the physical ends of the chromosome itself but also in Muller's words "a terminal gene with a special function, that of sealing the end of the chromosome" [1]. Also by the end of 1930s, Barbara McClintock, studying chromosomal aberrations induced by X-rays in maize, found that broken chromosomes frequently fused to their sister chromatids, creating breakage-fusion-bridge (BFB) cycles, which were always accompanied by the loss of the terminal regions at the fusion site, demonstrating that broken chromosomes (*i.e.*, without "end caps") were subject to fusion events [2-6]. These observations lead to the idea that the ends of chromosomes or telomeres were "capped" and therefore protected from fusion reactions characteristic of ends created by chromosome breakage events. In this way, telomeres were defined as the terminal regions or physical ends of eukaryotic chromosomes, which protected them from fusion with either broken chromosomal fragments or other telomeres. Nowadays, in the light of molecular biology studies, telomeres are defined as specialized nucleoproteic complexes localized at the physical ends of linear eukaryotic chromosomes maintaining their stability and integrity [7].
Telomere biology has evolved from peripheral, albeit interesting branch of cell biology studied by a few groups to a major field involving hundreds of laboratories worldwide. Novel techniques have been developed and understanding of the structures, functions and roles of telomeres have evolved rapidly. In addition, the attention of the wider public has been stimulated through proposed links between telomeres and cancer.

1.1 Structure and functions

Telomeres are composed of both repeated DNA elements and specific DNA-binding proteins, which together form the ends of eukaryotic chromosomes [8-12]. Molecular dissection of telomeres started with the discovery of the telomeric DNA sequence of the ciliated protozoan Tetrahymena thermophila, (TTGGGG)n, by Blackburn and Gall in 1978 [13]. Telomeric DNA is characterized by being a G-rich double strand DNA composed by short fragments tandemly repeated with different sequences depending on the species considered [14, 15].
In all vertebrates, telomeres consist of tandem repeats of the hexanucleotide sequence (TTAGGG/CCCTAA)n and associated proteins [10, 16-22]. The arrays of TTAGGG repeats, first identified at human chromosome ends by Moyzis et al. in 1988 [22], are oriented 5′ → 3′ towards the end of chromosomes [19, 23] and form a 3′ single-strand G-rich overhang found at both chromosomal ends [24, 25]. The C-rich telomere strand is at the 5′ end and the G-rich telomere is at the 3′ end of each chromosomal DNA strand. These single-strand, G-rich 3′ overhangs result from both the “end replication problem”, that is the inability of DNA polymerase to replicate the very end of the telomeres, and postreplication processing [26, 27]. Thus, removal of the most distal RNA that primes lagging-strand synthesis leaves an 8- to 12-base gap at the 5′ end that, if not filled in, leads to a small loss of DNA in each round of DNA replication [28] (see paragraph *1.1.2 Regulation of cellular life spans*).
The physical structure of the telomere was revealed by electron microscopy to be a large duplex loop [29-31], called t-loop, which is created when a telomere's end loops back on itself and the single-strand overhang invade an interior segment of the duplex telomeric DNA. The t-loop structure of mammalian telomeres is thought to repress the non homologous end-joining (NHEJ) DNA double-strand breaks (DSBs) repair process at chromosomal ends, thus rendering telomeres nonrecombinogenic (Fig. 1).
The length of the double strand telomeric repeat varies greatly among species [7]. For example, in the ciliate Oxytricha, it is only 20 base pairs (bp) long [64] in *Saccaromyces cerevisiae*, it is a few hundred bp long [32] while, in vertebrates, individual telomeres may extend to more than 100 kb, such as in some mouse cells. [33]. In normal primary human cells, the DNA at each chromosome terminus spans 5–20 kb in length [22], terminating in a 3′ single-strand overhang 100–400 nt in length [34]. In human tumor cells which use telomerase for telomere maintenance (see paragraph *1.2.1 Telomere maintenance*), telomere length varies from 1 to 20 kb [35-37]. It

has been shown that in humans and mice, the length of telomere repeats at individual chromosome ends in individual cells is highly variable [38-42] and that mouse and human cell lines exhibit subpopulations of cells with different telomere lengths [43]. It was shown that, at cellular level, a stable hierarchy exists in the form of a telomere length profile of the human karyotype [44]. This rank order is conserved between different human cell types and individuals, maintained throughout a lifetime, and seems to be genetically determined [44] and [45]. The longest human telomere is found at the long arm of chromosome 4, whereas the shortest one is at the short arm of chromosome 17 [46].

The homeostasis of mammalian telomeres is regulated by a number of telomere associated proteins (Fig. 1). Among these proteins, Telomere Repeats binding Factors, TRF1 and TRF2, directly bind double-strand telomere DNA and interact with a number of proteins to maintain telomere length and structure [47, 48]. It has been shown that the amount of telomere-bound TRF1 correlates with telomere length. Overexpression of TRF1 shortened telomeres in human cells, whereas dominant negative TRF1 led to elongated telomeres [49-51]. TRF1 may control the length of telomere repeats through multiple mechanisms. For example, TRF1 can control telomerase access through its interaction with TIN2, PTOP/PIP1, and the single-strand telomere DNA-binding protein POT1 [52-54]. TRF1 may also regulate telomerase activity through its interaction with PINX1 [55]. In comparison, TRF2 has an essential role in telomere end protection and t-loop formation [29, 47, 56]. Interference of endogenous TRF2 activity by expressing dominant negative forms of TRF2 markedly increase the rate of telomere end-to-end fusions [57]. Consistent with this role of TRF2, TRF2 forms a complex with RAP1 and associates with several proteins involved in DNA damage and repair responses, notably MRE11/RAD50/NBS1 (MRN complex), Ku86, and ERCC1/XPF [58-60]. These findings have pointed to distinct biological functions of TRF1 and TRF2. Some recent findings, however, suggest a more complex picture. In mouse embryonic stem cells, the conditional knockout of TRF1 led to significantly reduced levels of TRF2 at the telomeres, suggesting that TRF2 telomere localization may be partially regulated by TRF1 [61]. In addition, chromosome end-to-end fusion was detected in TRF1 knock-out cells, indicating that telomere end protection was compromised. Despite the wealth of information, the functional relationship between TRF1 and TRF2 in telomere maintenance remains unclear. Notably, a recent report demonstrated a direct interaction between TRF2 and the TRF1-interacting protein, TIN2 [62]. Such findings further suggest that cross-talk probably

occurs between the TRF1 and TRF2 complexes. However, whether TIN2 can simultaneously associate with both TRF1 and TRF2 in the same complex remains to be demonstrated. In addition to TRF1, several other telomeric proteins have been shown to be regulators of telomere length [47, 48, 53, 54, 63-66].

Fig. 1 The mammalian telomeric complex. The fluorescence image shows the location of a telomere within a chromosome. Mammalian telomeres consist of TTAGGG repeats with a single stranded 3' overhang of the G-rich strand. Specific protein complexes bind to the double-and single- stranded telomeric DNA. The components of the shelterin complex are shown in bold text. The single-stranded overhang can invade the double-stranded portion of the telomere, forming protective loops — such as t-loops with displacement loops (D-loops) — at the invasion site. The telomerase complex (which contains the telomerase RNA template and the reverse transcriptase TERT) interacts with the overhang and is regulated by shelterin and other telomeric proteins. Other factors that can interact with telomeres are listed. Bidirectional arrows indicate interactions. From [I].

Both inhibition of endogenous RAP1, TIN2, POT1, or PTOP expression through RNA interference and expression of dominant negative forms of these four proteins resulted in elongated telomeres in cultured cells [49, 52-

54, 60, 67]. These observations suggest that RAP1, TIN2, POT1, and PTOP may function in the same pathway. All four proteins, RAP1, TIN2, POT1, and PTOP, directly or indirectly associate with TRF1 or TRF2 [53, 54, 64], pointing to a possible functional connection among these six telomeric proteins.

1.1.1 Main role of telomeres

Recently, the term 'telomere capping' emerged to describe the protective role of telomeres [8, 9, 68, 69], since telomeres provide a protective "cap" for the ends of chromosomic DNA. The main role of telomeres is to preserve the integrity of the chromosomes, protecting them from degradation, recombination or fusion [47] by preventing the ends of linear chromosomes from being recognized as DSB by the DNA repair machinery, i.e., they distinguish natural DNA ends from DNA ends resulting from breakage events [1, 2]. Thus, telomeres. Thus, "capping" refers to the ability of telomeres to protect chromosome ends from DNA damage responses, prevent inappropriate repair and recombination between internal DNA breaks and native chromosomal or the ligation of chromosomal ends. Accordingly, when telomeres become dysfunctional, fusions between two telomeres and between a telomere and a DSB occur, as shown by recent studies using the chromosome orientation, CO-FISH, technique [70, 71]. In mice, one of the consequences of impaired telomere function is the formation of Robertsonian-like chromosome fusions [72-74]. Therefore, the maintenance of telomere function is crucial for genomic stability and cell viability. Cells respond to dysfunctional telomeres by undergoing senescence, cell death, or genomic instability [74-86]. Cellular response to dysfunctional telomeres is governed by proteins that also control the DNA damage response [10, 15, 17, 87, 88]. The domain of telomere-associated DNA damage factors has been termed telomere dysfunction-induced focus or "TIF" by Takai et al. [87] who showed that DNA damage foci form at telomeres uncapped by TRF2 inhibition, and that uncapping of telomeres occurs in late S/G_2.

1.1.2 Regulation of cellular life span

In 1972, James Watson wrote, "While 5' to 3' oriented growth should proceed smoothly to the end of its template, I see no simple way for 3' to 5' growth to reach the 3' end of its template" [26]. Thus, he correctly predicted that the lagging strand of linear chromosomes copied by the semi-conservative replication machinery would not be fully replicated 26]. In 1973, A. M. Olovnikov proposed the 'marginotomy theory of ageing',

suggesting that 'telogenes' located at opposite ends of DNA molecules carry no genetic information and fullfil a buffer function. He stated that these telogenes are stochastically shortened during each mitotic cycle, providing a mechanism for ageing [27] (Fig. 2). Observations made by L. Hayflick in 1961 suggested that human cells derived from embryonic tissues can only divide about 50 times, and this became known as the Hayflick limit [89]. Since then, the assumption that the Hayflick limit is determined by the initial length of the telomeres and the rate of telomere shortening, as laid out in the mathematical approach of A. M. Olovnikov [27], has been proved experimentally [90, 91]. It is well established that critically short telomeres cease to function as protective units and cause the cell to die or to arrest permanently. Telomeres are now known to have many more roles than simply buffering against DNA loss; however, the initial concept of replication associated telomere shortening was correct. At present, we refer to the inability of conventional DNA polymerases to replicate linear molecules fully as the 'end-replication problem'. This is caused by the deletion of the RNA primer of the most distal Okazaki fragment and results in the loss of about five bases of terminal genetic material per population doubling [90-94]. However, the sequence loss that is predicted to occur as a result of the end-replication problem is considerably less than that which has been observed in primary human cells, which lose about 100–200 bases of TTAGGG repeats per cell division [95-97]. Consequently, replication-associated terminal sequence loss is caused by a combination of the end-replication problem and the processing that must occur to create the G overhang on the telomeres generated by leading- and lagging-strand synthesis. When telomeres become critically short, they are detected by the cellular DNA-damage repair machinery [47]. As demonstrated in *S. cerevisiae*, chromosomes that lose a telomere are often eliminated, despite checkpoint and DNA damage repair machineries [98]. In human cells, p53- and RB1- (retinoblastoma 1) dependent pathways are responsible for monitoring telomere function (Fig. 2), whereas p53 seems to be the main sensor in mouse cells [80]. The minimal functional telomere length, and whether this length varies among cell types, has not been clearly defined. But even in senescent human cells, telomeric double-stranded repeats are readily detectable, suggesting that several kilobases of TTAGGG repeats are required at all times. Replicative senescence can be viewed as a mechanism to limit the potential number of population doublings a cell can undergo, hypothetically rendering it a powerful tumour-suppressor mechanism [99]. Each time a cell divides, telomeres shorten as a result of the end-replication problem and end processing. After

telomeres have become critically short, they are detected by the DNA-damage repair machinery, and the cell dies or enters senescence. At present, senescence in human cells is regarded as an irreversibly arrested state, effectively inhibiting the generation of immortal cells and therefore cancer formation. As a result, major tumour suppressive mechanisms need to be deactivated before a cell can overcome this block to immortality.

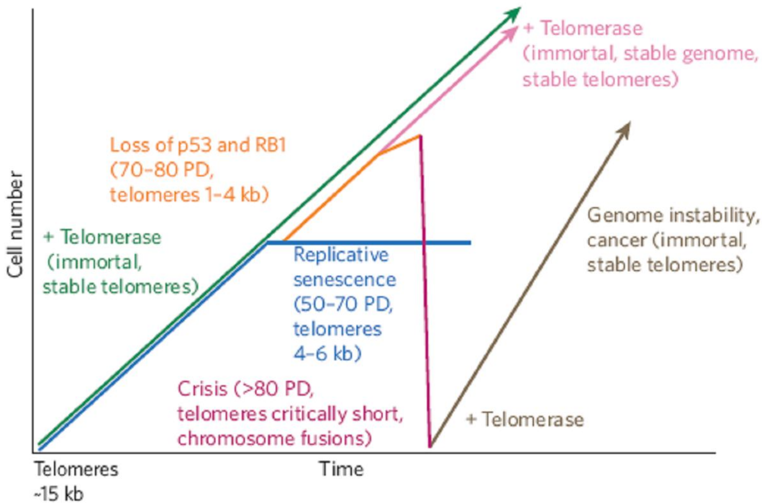

Fig. 2 Telomere shortening, senescence and cancer. Primary cells divide exponentially, and telomeres shorten from ~15 kilobases (kb) until they reach a critical length, 4–6 kb. Irreversible cell-cycle arrest then occurs (blue). Activation of telomerase before senescence allows cells to divide indefinitely and maintain a stable genome (green). If, instead, the p53 and RB1 pathways are suppressed, cells continue dividing (orange) until end protection is completely lost, resulting in telomeric crisis, cell death and massive genomic instability (dark pink). If telomerase is activated before erosion is complete, this rescues the genome from instability by re-establishing telomere maintenance (light pink). Activation of telomerase after the accumulation of mutations results in an unstable genome, allowing clones that carry multiple mutations to escape cell death (that is, to become immortal). Such cells are predisposed to oncogenic transformation (brown). PD, population doublings. From [I].

1.1.3 Additional functions of telomeres

Besides the above-mentioned functions, telomeres contribute to maintenance of chromosome topology in the cell nucleus and play a fundamental role in the proper alignment of chromosomes for recombination during the first meiotic prophase [7, 24, 75, 100-103]. An outstanding feature of telomeres is that they silence genes flanking the telomere repeat sequence [104-106]. This phenomenon, called the “telomere position effect”, is thought to be modulated by telomere length and local heterochromatin structure [107-109]. It has been shown that mutations in the encoding genes for Ku proteins lead to disruption of nuclear organization of telomeres and loss of telomeric silencing [110, 111]. Moreover, in mammalian cells, loss of Ku leads to aberrant telomere–telomere fusions [112, 113].

1.2 Telomere maintenance

1.2.1 Telomerase

The loss of telomeric repeats is usually prevented by telomerase, a specialized reverse transcriptase-like enzyme, containing a RNA subunit (TR) and a catalytic protein subunit called telomerase reverse transcriptase (TERT) which is the rate-limiting factor for the enzyme activity (Fig. 3). This enzyme, first discovered by Greider and Blackburn in 1985 in *Tetrahymena* [114], works via an RNA template – using exclusively single-strand 3′ telomeric overhangs as primers [9, 11, 18, 24, 115, 116] – by adding TTAGGG repeats to the telomere. Although repressed in the majority of normal somatic cells (with the exception of a transient S phase activity thought to maintain the single-stranded overhang [117], telomerase is present in immortal cell lines, germline cells, stem cells, activated lymphocytes, and most of the tumor cells analyzed so far [35- 37, 118-120]. Telomerase activity favours 3′ overhangs over blunt DNA ends for addition of telomere sequence, at least *in vitro* [121, 122]. Recent observations indicate that telomerase exists as a complex tetramer composed of two RNA subunits and two catalytic subunits [123-125]. These subunits act in concert to elongate telomeres by reading from the RNA template sequence carried by the RNA subunit and synthesizing a complementary DNA strand. Mutations in conserved reverse-transcriptase catalytic residues found in telomerase eliminate the enzymatic activity of telomerase [126-129].

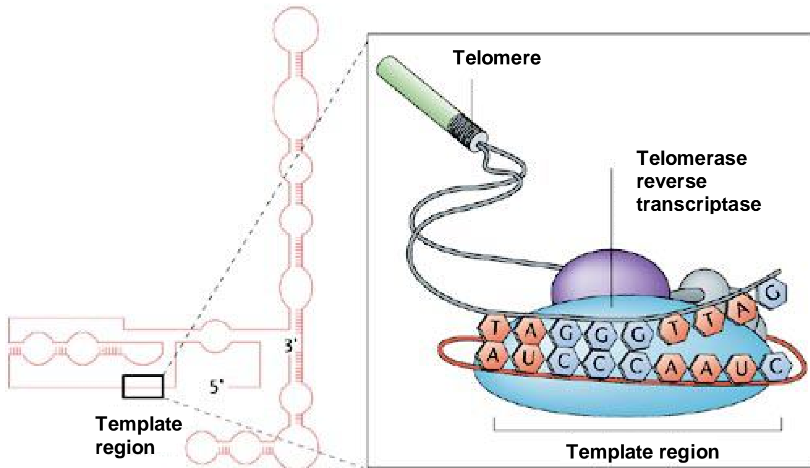

Fig. 3 Human telomerase is a cellular reverse transcriptase. It is composed of two essential components: telomerase reverse transcriptase catalytic subunit (hTERT) and functional telomerase RNA (hTR), which serves as a template for the addition of telomeric repeats (left side). From [II].

Other telomerase-specific motifs are also required for catalytic activity [123, 130-132]. In addition to these core components, several other proteins associate with the telomerase holoenzyme, including TEP1, p23, and Hsp90 [133, 134]; however, the physiologic function of these other proteins remains undefined because both biochemical and genetic experiments indicate that these other proteins are dispensable for telomerase activity [128, 135]. Telomere length is controlled by a mechanism involving telomerase and the telomere-binding proteins [18, 51]. Loss of telomerase enzymatic function leads to progressive telomere shortening over time, eventually resulting in the disappearance of detectable telomeric DNA and the formation of end-to-end chromosome fusions, followed by growth arrest or cell death [11, 136].

A telomere is a repeating sequence of double-stranded DNA located at the ends of chromosomes. Greater telomere length is associated with immortalized cell lines such as embryonic stem cells and cancer cells. As cells divide and differentiate throughout the lifespan of an organism or cell line, the telomeres become progressively shortened and lose the ability to

maintain their length. Telomerase is an enzyme that lengthens telomeres by adding on repeating sequences of DNA. Telomerase binds to the ends of the telomere via an RNA template that is used for the attachment of a new strand of DNA. Telomerase adds several repeated DNA sequences then releases and a second enzyme, DNA polymerase, attaches the opposite or complementary strand of DNA completing the double stranded extension of the chromosome ends. High levels of telomerase activity are detected in embryonic stem cells and cancer cells, whereas little or no telomerase activity is present in most mature, differentiated cell types. The functions of telomeres and telomerase appear to be important in cell division, normal development, and aging.

1.2.2 Chromosome healing

Besides maintaining pre-existing telomeres, telomerase can catalyze the addition of telomeric sequences directly on to non-telomeric DNA [137]. This process of direct addition of telomeric repeats to the ends of broken chromosomes by telomerase is called “chromosome healing” (a term coined by Barbara McClintock in 1941 to describe the phenomenon that halted the BFB cycles in the embryo of plants [2]), and has been observed in protozoans, yeast, plants, insects, and mammals [138-145]. Repair function of telomerase would lead to uncontrolled chromosomal fragmentation and karyotypic instability, because chromosome healing prevents repair of broken ends. Therefore, telomerase must be prevented from accessing internal DSBs. Slijepcevic and Al-Wahiby [146] proposed that Ku, a DSB protein which has a high affinity for DNA ends, acts to prevent telomerase from accessing internal DSBs. This model is supported by the fact that the efficiency of chromosome healing is extremely low, about 1% [147]. In fact, no evidence of “chromosome healing” was found in normal human lymphocytes [148] or in Ataxia-Telangiectasia (AT) cells [149], which display high radiosensitivity, exposed to ionizing radiation. The failure to recruit and/or activate telomerase at sites of DSB may contribute to the paucity of chromosome healing events.

1.2.3 “Telomere capture” and beyond

Lost telomeres in broken chromosomes can also be acquired by “telomere capture” and break-induced replication. Telomere capture is a process which involves the addition of telomeres at the site of DSB by subtelomeric cryptic translocations, undetectable by classical cytogenetic techniques [144-146, 150, 151]. In telomere capture, broken chromosomes are stabilized by the transfer of telomeres from normal chromosomes. This

phenomenon was first reported in human malignant melanoma cells [151], and has been recently observed in the leukocytes of chronic lymphocytic leukemia and chronic myeloid leukemia patients [152]. Telomere capture is essentially a non-reciprocal process, producing a chromosome with only one telomere (donor chromosome) and another one with a new telomere (recipient chromosome) plus an acentric fragment (a terminal fragment or terminal deletion, as seen by telomeric FISH, if the chromosome break occurs at G_0/G_1/early S phase, or a chromatid-type fragment, if the break takes place in late S/G_2). However, the donor chromosome may be involved in secondary recombination events [145, [151, 153] by the initiation of BFB cycles in this chromosome. Slijepcevic et al. [153] showed that only a small percentage of radiation-induced chromosome/chromatid breaks may be modified by "telomere capture".
In break-induced replication [154] and [155], the broken end of a chromosome invades a region of homology and initiates replication, thereby duplicating the end of that chromosome. Although not involving telomere restoration, the formation of ring or dicentric chromosomes can also compensate for telomere loss [156-158].

1.2.4 Alternatives to telomerase: the "ALT" mechanisms

An alternative method of telomere elongation in the absence of telomerase has been described in several tumor cells and immortalized cell lines and named "ALT" (for "alternative lengthening of telomeres") [159-166], although some tumors were found to posses neither telomerase activation nor ALT mechanisms for telomere length maintenance [167, 168]. Evidence hints that Homologous Recombination (HR) plays a primary role in most of the mammalian ALT pathways (Fig. 4). Several characteristics can be used to identify human cells using the recombination-based pathway of telomere length maintenance. First, they have no detectable telomerase activity and lack expression of the catalytic protein component, hTERT, or in some cases, they lack both hTERT and the integral RNA component, hTR [169]. Second, cells using the recombination-based pathway to maintain telomeres have very long and heterogeneous telomeres ranging in length from less than 2 kb to 50 kb [170]. Third, they contain extrachromosomal telomere repeats (ECTRs) that may be linear double-stranded fragments of telomeric DNA [171]. Fourth, cells using the recombination-based pathway to maintain telomeres have a novel type of promyelocytic leukaemia (PML) nuclear bodies called ALT-associated PML bodies (APBs) that contain telomeric DNA, telomere-associated proteins (*i.e.*, the telomere-binding proteins TRF1 and TRF2), and

recombination-associated proteins (*i.e.*, RAD50, RAD51, RAD52, MRE11, NBS1, BLM and WRN) [159]. The evidences that APBs appear at exactly the same time as the activation of the ALT mechanism during cell immortalization and contain HR associated proteins, suggest that PML may play a role in recombination mechanism in ALT [172-174]. Telomere exchange between sister chromatids has been suggested as a possible mechanism of ALT. It was recently found [175] that in ALT-negative cells the rates of sister chromatid exchanges in telomeres (T-SCE) are 10-fold higher compared to other DNA sequences. T-SCE rates are higher in ALT cells compared to normal cells [170, 171, 176] and has been suggested they play an important role in determining the proliferative potential of telomerase-negative cells [170, 171, 175, 176].

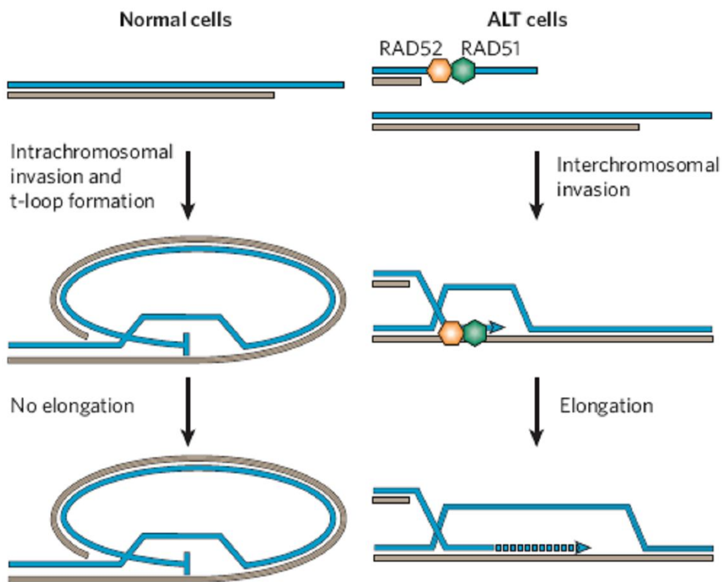

Fig. 4. ALT cells show an increased rate of sister chromatid exchange, suggesting that the homologous-recombination pathway is involved. From [I]

The increased telomere recombination, however, does not reflect a global increase in recombination frequencies because it was found that there was no increase in the rate of recombination at other genomic locations in ALT cells as compared to non-ALT controls [176-178]. However, while telomere recombination events are commonly called telomere sister chromatid exchanges, it is possible that the observed post replicative exchanges in ALT cells occurred with non-sister chromatids, or with the extrachromosomal telomeric repeat elements present in ALT cells. Many of these exchanges in ALT cells appear to be unequal, causing a reciprocal gain or loss of telomere sequence on the chromosome ends involved.

1.3 NBS1 and Homologous Recombination (HR) in telomere maintenance

MRN complex is required for the maintenance of telomere length in mammals, plants and yeast [179-181]. In yeast, the XRS2 complex (homologue in yeast of human MRN complex) plays a role in at least two pathways in telomere maintenance [181-184]. The first one is telomerase-dependent and consists in the generation of 3' ssDNA at the telomere for the recruitment and subsequent action of telomerase. In the second pathway, MRN takes part in the homologous recombination mediated telomeres elongation, which involves recombination between tracts of telomere repeats and is not dependent on telomerase function. In human, blood mononuclear cells from NBS patients analyzed for telomeres length, show shorter telomeres in comparison to cell from unaffected individuals. Primary fibroblasts isolated from NBS patients show an accelerated telomere shortening during *in vitro* colture [185].
In 2001 Ranganathan and co-workers demonstrated that only co-expression of NBS1 and TERT, the catalytic subunit of telomerase, leads to a significantly increase in telomere length whereas neither the introduction of NBS1 nor TERT, alone, has the same restoring effect [185]. These results suggest that the MRN complex may facilitate telomerase mediated telomere elongation by modifying telomere DNA ends or opening the T-loop [182, 186]. In 2003, Bai and Murnane elucidated another important aspect of NBS1 role in telomere maintenance. Cells expressing inducible NBS1 protein mutated at Ser278 and Ser343, showed an increased rate of telomere loss. Absence of detectable changes in the average telomere length suggest that this process is probably due to stochastic events, like complete telomere loss or loss of telomeric capping [187, 188]. These results have led to the

proposal that the MRN complex is involved in either establishment of the single stranded tail or in t-loop formation after telomeres replication.
NBS1 seems to play a key role also in ALT pathway, in yeast as well in mammalian cells. NBS1 was found to be co-localized with PML, as well as to be associated with a nuclear PML-binding protein, SP100, by the BRCT-domain at its C-terminus [189]. NBS1 also functions in recruiting other recombination proteins, including RAD50, MRE11, and BRCA1 to PML nuclear bodies. Moreover, recent reports demonstrated that NBS1 protein is essential for the correct functioning of the ALT pathway, which results impaired by depleting one of the three components of MRN complex [190].

2 Ionizing Radiations (IR)

Ionizing radiation consists of subatomic particles or electromagnetic waves that are energetic enough to eject one or more orbital electrons from the atom or molecule, this process is called ionization.
IR normally interacts with materials indirectly, via the formation of radical species. These species are mainly produced during radio-induced dissociation of the water surrounding the ultimate substrate. This process is termed “water radiolysis”. Although this indirect action can be modulated by the use of scavengers [191], in contrast to endogenous stress, these reactive species are not produced homogeneously, but are closely localised in spaces termed “clusters of ionisations”, which exist on a nanometre scale [192, 193]. In addition to water radiolysis, radiation interacts with materials in a direct way, which consists of the interaction between the radiation and the substrate itself (Fig. 5). The direct energy transfer from the radiation to the substrate can lead to either its excitation or ionisation. The relative importance of direct and indirect effects depends on the nature of the radiation, on DNA organisation and on DNA hydration, together with the scavenging properties of the environment. In order to explain the differing contribution of direct and indirect effects and the diverse biological effects observed between different ionizing radiation, the concept of linear energy transfer (LET) was introduced. LET is a macroscopic approach used to study the spatial distribution of ionisation and excitations produced along a linear path. It assumes that the energy is progressively and continuously deposited by the radiation through the matter and is expressed as the ratio between the energy loss and the corresponding path length in units of keV/µm. The LET delivered depends on several parameters such as the atomic number of the target and the velocity of the particle. It plays an important role in the understanding of the biological effects of radiation and thus allows a distinction between low-LET and high-LET radiations. For the International System of Units (SI) the unit of absorbed dose of IR is the Gray (Gy), 1 Gy is defined as 1 J kg^{-1}.

2.1 Low-LET radiation

Low-LET radiations include electrons and electromagnetic waves such as X- and γ-rays. X-rays are produced in electrical device that accelerate electrons to high energy and then stops them abruptly in a target, usually made of tungsten or gold. On the other hand γ-rays are emitted by

radioactive isotopes (^{32}P, ^{33}P, ^{14}C, etc.); they represent excess energy that is given off as the unstable nucleus breaks up and decays in its effort to reach a stable form. Their energy can range from 10 keV to 30 MeV. The interactions of low-LET radiation with matter depend on their nature (particles or photons) and their energy. Photons mainly interact with their biological target via the Compton process if their energy is between 20 keV and several MeV. For the lowest energetic photons, such as ultra-soft X-rays possessing energy ranging from 0.2 to 5 keV, photoelectric absorption is the preponderant process involved. Both modes of interaction result in excited and ionised matter. The trajectory of incident photons is deflected after each interaction, and dislodged electrons are emitted with a defined scattering angle and energy. Electrons mainly interact with biological material by electronic repulsion and, according to their energy, can either excite or ionise the matter. In the latter case, the dislodged electrons (termed δ electrons) can, in turn, further ionise or excite matter with deflection of the trajectory of the incident electron after each electronic interaction.

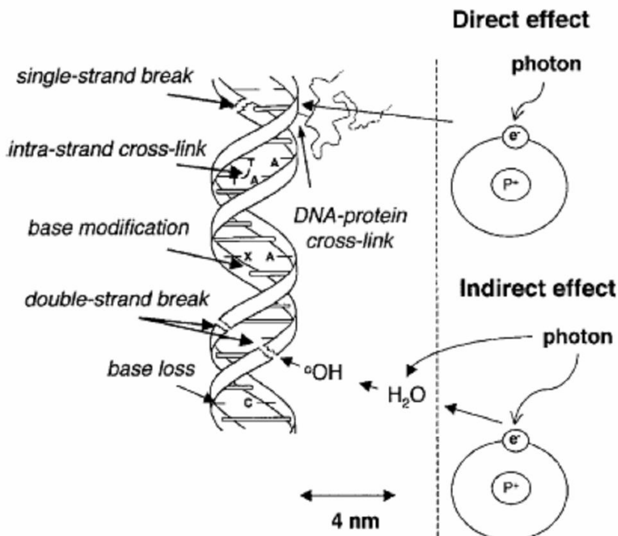

Fig. 5 - Schematic representation direct and indirect radio-induced DNA lesions. From [III].

Thus, the interaction of photons and electrons with biological material sets other electrons in motion, which in turn interact with matter, until their energy falls below 10 eV. As a consequence of their mode of interaction, these radiations are defined indirectly ionizing radiations and sparsely ionizing radiations. In fact that they do not produce chemical and biological damage themselves, but when they are absorbed in the material through which they pass they give up their energy to produce fast-moving charged particles. Moreover the ionisations and excitations produced by photons and electrons are sparsely produced in a large targeted volume (Fig. 6), and over a wide range. The LET of such radiation is low, with a range of values from <0.5 keV/μm (for ^{60}Co γ-rays) to a few keV/μm for X-rays.

2.2 High-LET radiation

High-LET radiations include helium nuclei (α-particles), neutrons, protons and heavy charged ions (^{198}Au, ^{56}Fe, ^{40}Ar, ^{12}C, ^{20}Ne, etc.) generated by accelerators. High-LET radiations interacting with materials slow down progressively until their complete arrest. They interact by coulombic repulsion with the electrons present in the atoms of the target, leading to either excitation or ionisation of matter. As the weight of such particles is very high compared to the weight of an electron, it is not deflected and the track of the particle is almost linear and the LET is high ranging from 20 keV/μm to several hundreds of keV/μm. This radiation are also defined *directly ionizing radiation* and *densely ionizing radiations* since they have sufficient kinetic energy to disrupt the atomic structure of the absorber in which they pass through and generate a denser ionization track compared with low-LET radiation induced one (Fig. 6).

2.3 LET and Relative Biological Effectiveness (RBE)

Already early in the 20th century it was demonstrated that densely ionizing (high-LET) radiations could have a greater biological effectiveness than sparsely ionizing (low-LET) X-rays or γ-rays. For a given biological effect, Relative Biological Effectiveness, (RBE) is the ratio between the dose of standard X-rays (250 kV) and the dose of the type of radiation of interest required to produce this biological effect. For human cells, the RBE of high-LET radiations has direct practical implications in therapy applications and in assessing risks from environmental and occupational exposures, and it also provides analytic information on the underlying mechanisms of

radiation biology. The numerical values of RBE for a given LET can vary by large amounts (even orders of magnitude) depending on other physical and biological conditions.

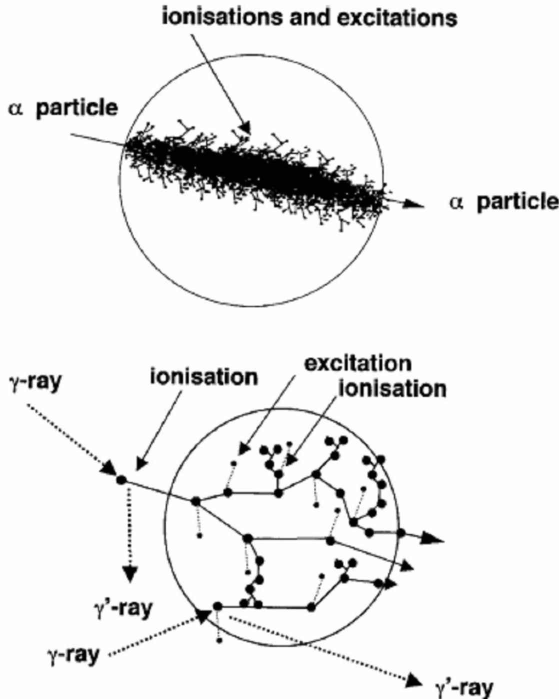

Fig. 6 - Schematic representation of track structure of low and high-LET radiations. From [III].

Values of less than unity up to a few hundred have been deduced from experimental data. Values of less than unity up to a few hundred have been deduced from experimental data. Common general tendencies in mammalian systems are [192]: for mutation RBEs to be greater than cell inactivation RBEs; for lighter ions (such as protons, or a-particles) to reach

their peak at lower LETs than faster ions (such as a-particles, or carbon-ions, respectively); for RBEs to be larger at lower doses and dose-rates; and for radiosensitive cells to show lower RBEs than radioresistant cells. There are, however, many exceptions to such generalities. Therefore, we should conclude that there are a number of competing mechanisms and diverse factors that determine the effectiveness of low- and high-LET radiations. Despite these variability in effectiveness, essentially all the biological differences between low- and high-LET radiations must arise from the track structures of the ionizing charged particles that are set in motion in the cells.

2.4 Radiation-induced DNA damage

2.4.1 Isolated and clustered DNA damage

Among all the biomolecules which make up a living organism, it is widely accepted that DNA, which carries the cells vital genetic information, is the primary target of ionizing radiation (IR). The acceptance of the fact that DNA is the target of IR has been guided by much direct and indirect evidence derived from the result of extensive research in radiation biology conducted over a long period of time [194]. Considering the spatial structure of energy transfer resulting from exposure to IR, discrete DNA lesions can be generated on DNA strands along the track of IR (i.e. isolated damage). In addition, multiple lesions can be produced on DNA strands when a single track of ionizing radiation hits the DNA directly or passes close by the DNA strand. This type of lesion is referred as clustered DNA damage, or as a locally multiply damaged site (LMDS) [195].

It has been suggested that clustered DNA damage is involved in the adverse biological effects of ionizing radiation. For instance, a double strand breaks (DSB) comprised of two closely opposed single strand breaks (SSB) is a typical clustered damage, and cells deficient in DNA repair are indeed hypersensitive to ionizing radiation [196]. Another class of clustered DNA damage consists of clustered base damage comprising closely spaced lesions of different types, such as oxidized base damage, abasic sites and SSB [197, 198]. Previous studies have shown that the abortive repair of clustered base lesions on opposing DNA strands results in DSBs and can lead to adverse biological consequences, indicating a crucial role for clustered base damage togheter with DSBs. [199-202].

However, high-LET radiation produces more severe biological consequences than low-LET one. In mammalian cells, the RBE as measured by cell killing rises with increasing LET values up to 100-200 keV/micron, and then decreases due to overkill effect. Considering that high-LET

radiations generates a denser ionization track than low-LET radiation, it is possible that high-LET radiation directly or indirectly produces detrimental clustered DNA damage more efficiently than low-LET radiation, thereby resulting in severe biological consequences. Furthermore, dense ionization events associated with high-LET radiation can increase the frequency of a DNA lesion within a damage cluster and increase the structural complexity of clustered DNA damage. Thus, the quantity and complexity of clustered DNA damage are two important but relatively poor understood parameters which determine the severity of the effects of ionizing radiation [203].

2.5 Biological relevance of high-LET radiation study

Hadrontherapy is a collective word and describes the many different techniques of oncological radiotherapy which make use of fast non elementary particles such as protons, neutrons and light nuclei (hadrons) used to locally control many types of tumours minimizing the irradiation of the surrounding tissues and avoiding intercepting vital organs. In particular depending on their physical properties beam of protons, or light ions such carbon ions, allows highly conformal treatment of deep-seated tumours with millimetre accuracy, giving minimal doses to the surrounding tissues.
Late effects of high-LET radiation are arguably an health risk not only for increasing number of cancer patients treated by hadrontherapy, including young adults and children, but also for the human space exploration.
Space travel encompasses exposure to a broad spectrum of radiation ranging from the infrared to galactic cosmic rays. The major component of galactic cosmic rays is the highly charged energetic particles ranging from energetic protons to iron nuclei with energies upwards to 1 GeV/nucleon.
Research in the field of biological effects of high-LET radiations is needed for both hadrontherapy and protection from the exposure to galactic cosmic radiation in long-term manned space missions. Although the exposure conditions (e.g. high- vs. low-dose rate) and relevant endpoints (*e.g.*, cell killing vs. neoplastic transformation) are different in the two fields, it is clear that a substantial overlap exists in several research topics.
However, an accurate risk calculation is required and in this respect a detailed investigation of both the physical aspects (patterns of energy deposition at the molecular/cellular level) and the biological response to high LET particles is necessary.

3 Biological effects of radiation on telomere metabolism

3.1 DNA repair and telomeres

The main function of telomeres is to protect the chromosome ends and to prevent activation of DNA damage response. Defined as the caps of linear chromosomes, they serve to distinguish normal ends from DSBs. As reported above, many proteins, involved in DNA repair and checkpoints, are also required for telomere maintenance. Therefore we could wonder how cells are able to discriminate normal from abnormal telomeres. Through the interaction between telomere maintenance and DNA repair, cells develop a sophisticated strategy to detect eroded or dysfunctional telomeres. Damaged telomere and proper repair failure might result in telomere dysfunction as shown in Fig. 7. It has been reported recently that telomere attrition or dysfunction results in the formation of the hallmark of DNA damage response [204]. Hence, uncapping or senescence elicits the formation of foci including several DNA repair proteins such as 53BP1, H2AX, ATM, MRN complex, Chk1/2. Correlation between accelerated shortening and hypersensitivity to IR in DSB repair deficiency syndromes argue in favour of a link between telomere maintenance and DNA repair [205].

3.1.1 Non Homologous End Joining (NHEJ)

NHEJ is one of most important pathway in the recognition and processing of DSBs in several organisms. In mammalian cells, NHEJ ensure alignment of DNA ends and ligation by end-joining involving several proteins and does not necessarily require sequence homology. After DSB formation, the complex Ku/DNA-PKcs (DNAdependent protein kinase catalytic subunit) is involved in initial recognition. Ku binds to DNA ends and recruits DNA-PKcs which can phosphorylate several targets [206]. This is followed by the removal of several base pairs and end-to-end ligation performed by DNA Ligase IV, XRCC4 and XLF [113, 207, 208]. Several kinds of IR-induced damage form complex DSBs which would be processed before ligation. Studies in mammalian cells revealed the role of NHEJ in the protection of chromosome ends. Several works demonstrated the implication of proteins of NHEJ in telomere maintenance.

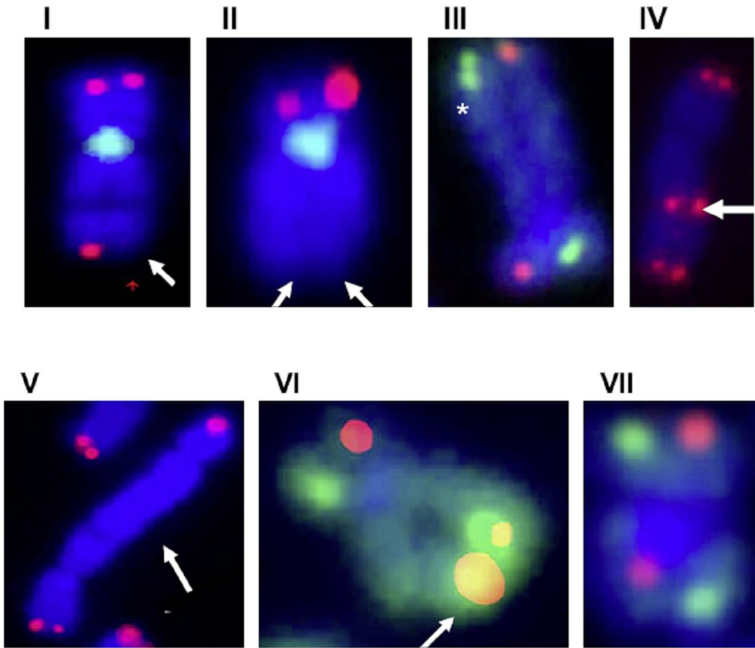

Fig. 7. Telomere dysfunctions in human cells. Metaphase spread with telomeric DNA detected by FISH (red); DNA stained with DAPI (blue). Examples of the chromosomal aberrations: (I) Loss of single telomere. (II) Loss of both telomeres. (III) Telomeres split/duplication. (IV, V) Dicentrics chromosomes with (IV) or without (V) TTAGGGq sequences at fusions junction. (VI, VII) CoFISH staining with C-rich probe (red) and G-rich (green) probe with (VI) or without telomere-SCE (T-SCE) (VII). From [IV].

For instance cells deficient in Ku86 and DNA-PKcs exhibited premature senescence, high proportions of chromosomal aberrations [209, 210], and an increase of telomere end-to-end fusions with telomeric sequences at the fusion point [112, 211-213]. In parallel deletion of Ku70 leads to and an increase of Telomere Sister chromatid Exchange (TSCE) (hallmark of HR events at telomeres) indicating that Ku70 prevents inappropriate recombination at telomeres [214]. Similarly, DNA-PKcs deficiency resulted in telomere uncapping in MEFs. Other components of NHEJ have been

shown to participate in telomere maintenance like Ligase IV [204] but not XRCC4 or XLF. According to all these data, NHEJ partners act as a genomic caretaker in the whole genome and particularly at telomeres. Deregulation of NHEJ components would alter telomere stability and lead to genomic instability.

3.1.2 Homologous Recombination (HR)

NHEJ and HR differ in their requirement for a homologous template DNA and in the fidelity of DSB repair. Whereas HR ensures accurate DSB repair, NHEJ does not. The relative contribution of these two DSB-repair pathways is likely to differ depending on the stage of the cell cycle [214 ,215]. However, the pathways are not mutually exclusive because repair events that involve both pathways can be detected. HR is most efficient in the S and G_2 phases of the cell cycle because of the availability of sister chromatids as repair templates [216]. As we mentioned previously concerning NHEJ, DSB repair proteins are tightly connected to telomere maintenance. T-loop is needed to protect telomere from inappropriate and deleterious recombinational events. Shelterin proteins prevent HR proteins from accessing the telomeres, and the modification/deregulation of the Shelterin complex generates aberrant events leading to uncontrolled shortening of telomeres. TRF2 mutation elicits the formation of T-loop extra chromosomal fragments that lead to a rapid deletion of telomere suggesting that uncontrolled HR alters telomere maintenance [217]. The participation of HR in telomere maintenance has been described in mammalian cells especially in mouse models. At this glance, HR has also been shown to regulate telomere length and capping but its contribution is less documented than NHEJ. The suppression of HR partners leads to telomere dysfunctions associated with telomere shortening. In mouse, $Rad54^{-/-}$ cells showed a significant increase of telomere end-fusions and have short telomeres compared to control cells [218]. Furthermore the disruption of Rad51D altered telomere length and caused telomere dysfunction. In addition, inhibition of Rad51D in alternative lengthening telomeres (ALT) cells induced cell death suggesting the importance of recombination in ALT cells [219]. Hence Rad54 and Rad51D would act in the regulation of telomere length and telomere capping in mammalian cells. Additionally it has been proposed that HR machinery plays a role in the achievement of telomere replication. The MRN complex plays an important role in HR, as it promotes the processing of DNA ends breaks to improve the recognition and exchange of sister chromatid.

3.1.3 DNA DSBs and telomeres

As we described above, the proteins well known to play a role either in the non-homologous end-joining pathway or in the homologous recombination pathway, are involved in the regulation of length and maintenance of telomeres. In general, the mutations of proteins involved in DNA repair induce telomere dysfunction phenotypes [146]. A mark of double-strand breaks, the phosphorylation of the protein histone H2AX (γ–H2AX), is found at dysfunctional telomeres [49]. Nevertheless the overexpression of the telomeric repeat factor 2 (TRF2), a specific protein of telomeric sequences, decreases the accumulation of γ-H2AX at photo induced breaks in human cells [220]. Furthermore, in human fibroblasts, high irradiation by UV with laser microbeam induces a relocalization of TRF2 at sites of photo-induced tracks. This localization of TRF2 is very early and transient after the induction of lesions [220]. However irradiation by gamma rays or alpha particles does not promote the relocalization of TRF2 at the lesion sites [221]. So the involvement of DNA DSB is controversial and the nature of the lesions inducing the recruitment of TRF2 remains to be defined. Moreover, if such a redistribution of proteins involved in telomeres occurred, the induction of telomere dysfunction could be expected. However, dicentrics containing telomere sequences located between the two centromeres are not induced by irradiation [222]. Such data keep active the hypothesis that the telomeres could have a role reserving DNA repair proteins [223]. Even if DNA repair proteins are present at telomeres, they are not processed as double-strand breaks. To complete the list of DNA repair members involved in telomere maintenance, we mention the impact of WRN in telomere maintenance. This helicase has been reported to participate in telomere maintenance and telomere replication [224]. The absence of WRN induced a single loss of G rich strand [225, 226] leading to premature senescence as reported in Werner Syndrome patients [79]. Table 1 gives a summary of the DNA repair proteins interacting with telomeres.

The question of the behaviour of telomeres compared to interstitials sequences after irradiation in mammalian cells is open. Indeed telomeres were less efficient than other sequences to repair SSB [227] due probably to their T-loop structure reducing access to DNA repair machinery at DNA lesions. So telomeres may fail to repaired DSB. In yeast, near the telomeres, Isce1 induced DSBs were less repaired by end joining suggesting that DSBs processing is different from the rest of the genome [228]. In agreement with previous data, Isce1 induced chromosomal rearrangements were different between telomeres and interstitial sequences demonstrating that DSB was not repaired near the telomeres [229, 230].

The understanding of the interaction between telomeres and DSBs was extended to study the behaviour of dysfunctional telomeres. Dysfunctional or eroded telomeres are sensed as true DSBs according to the presence of DNA damage response proteins at telomeres in senescent cells or Shelterin deficient cells. No evidence is actually available for the presence of radioinduced breaks at telomeres.. Furthermore in irradiated cells, eroded telomeres are used as inappropriate substrates to NHEJ and joined with radiation induced broken ends. Three types of chromosomal rearrangements are subsequently formed: telomere-telomere, telomere-DSB, DSB-DSB. These kinds of rearrangements were also described in the absence of telomere capping. Hence, the disruption of DNA-PKcs or TRF2 promotes end fusions in mammalian cells, and dysfunctional telomeres could join to radiation induced breaks [231, 232]. Together those data suggest that uncapped or short telomeres act as DSBs interfering with the correct rejoining of broken ends and illustrate the interplay between telomere and DNA repair.

3.1.4 Damaged telomeres and telomerase

Telomerase is suspected to have a role in the repair of some DNA damage, particularly double-strand breaks [233]. This theory is supported by the fact that a very short template homology is necessary for the telomerase to act, inducing a low specificity necessity for its recruitment [234]. Moreover, double-strand breaks are accepted as the most important lesion subsequent to ionizing radiation, and many in vivo and cell line based reports describe an increase of telomerase activity after exposure to ionizing radiation [235-241]. It is proposed that telomerase could play a role in radiation induced damage response. The consequences of mutations in the telomerase complex genes (DKC1, TR, TERT) result in a loss of telomere maintenance and telomere shortening. However, nothing is known on the role of mutated telomerase in DNA damage repair. In the mean time, a lower repair mechanism of double-strand breaks has been reported in mice lacking telomerase activity with shorter telomeres [242]. However, the underlying mechanism is still not well understood. Indeed, telomerase would lengthen preferentially the shortest telomeres [243]. The proposed theory states that irradiated cells undergo an extensive senescence process and that telomerase could lengthen telomeres in those cells, which in turn appears to be important in post-irradiated survival and damage processing.

3.2 Oxidative stress and telomere erosion

Two kinds of telomere shortening exist in cells. The first one is replicative erosion. Telomeres limit the number of cell cycles and act as a mitotic clock [244]. The second one is induced by exogene or endogen factors that could also modulate the replicative life span. In addition to constant replicative shortening, erosion can also depend on stress. Several conditions can induce premature senescence as radiation [245], treatment with oxygen species [246, 247], culture in chronic mild hyperoxia [248], or oncogenes [249]. Stress dependant shortening associated with replicative attrition led at least to senescence. Thus, human cells MRC5/WI38 exhibit a high telomere shortening and a reduced life span under hyperoxia conditions [250]. Accelerated telomere shortening could be compensated by antioxidative treatment. Treatment with ascorbic acid [251] or radical scavengers like phenyl butylnitrone [252] reduced the telomere shortening rate and prolonged the life span. Therefore, antioxidant enzymes (glutathione peroxidase or superoxide dismutase) play an important role in preventing rapid ROS-dependant shortening [253]. How could oxidative stress influence telomere shortening? Telomeres are preferential targets for acute oxidative damage [254, 255]. The DNA repeats GGG are highly sensitive to oxygen species and generated lesions such as 8oxoG, the main substrate of the Base Excision Repair (BER) pathway. Consequently SSBs were accumulated at telomeres due to low efficient repair of SSB compared to non-transcribed sequences like minisatellites [256-258]. Indeed the access to lesions at telomeres was restricted by the telosome, and DNA repair enzymes were less efficient at this zone than at the bulk genome. In this context, persistent SSB would interfere with the replication fork leading to telomere loss. In fact, lesions in C-rich strand (template for leading strand) induce stalled replication fork and 5′ end would be degraded leading to shortening of telomere. Impaired BER repair at telomeres elicit stress dependant shortening [258].

3.3 Telomere length and radiosensitivity/radioresistance

A link between radiosensitivity and telomere maintenance is detectable in yeast and Caenorhabditis elegans, as several proteins are involved both in telomere length regulation and radiosensitivity [259, 260]. More recently, studies in mice have provided a mammalian precedent. Several mouse models, including mice deficient in Ku, DNA-PKcs, PARP, and ATM, all

of which are radiosensitive *in vivo*, also show clear telomere alterations [261]. A number of lines of evidence indicate that abnormalities in telomere length or maintenance associate with radiosensitivity at the cellular or whole organism level. Late generation (G3/G4) of mTERC$^{-/-}$ mice cells exhibit shorter telomeres and higher sensitivity to radiation exposure than wild type cells [239, 242]. Moreover, an inverse correlation between telomere length and chromosome radiosensitivity was observed in murine lymphoma cells (L5178Y-S and L5178Y cells). In humans, the relationship between telomere length and radiation sensitivity is complex and short telomeres do not always associate with radiosensitivity [233, 262, 263], although human genetic diseases characterized by clinical radiosensitivity, such as ataxia-telangiectasia (AT) and Nijmegen breakage syndrome (NBS), show alterations in telomere maintenance, [185]. In the context of breast cancer McIlrath and coworkers have observed a correlation between telomere length and in vitro G_2 chromosomal radiosensitivity in lymphocytes from breast cancer patients [233]. Moreover a recent report demonstrates that telomere length modulates chromosome *in vitro* radiosensitivity in healthy individuals [264].

In conclusion, deficiencies or defects in a range of proteins that associate with telomeres and affect their function also have roles in DNA repair or damage processing and hence confer both telomere dysfunction and radiosensitivity [261]. This common involvement of proteins in both DNA repair and telomere maintenance has led to the proposal that telomere maintenance is an integral part of DNA damage response [146, 265].

Several data indicate that telomere maintenance mechanisms appear to be a promising target for radiosensitization and elucidation of the molecular mechanisms are necessary to improve therapeutic regulation of radiotherapy in human tumor cells. However, additional investigation have to be performed to assess whether or not telomere length acts as biomarker of individual chromosome instability upon IR

Rationale and aim of the project

Ionizing radiations are a well known genotoxic agents, widely studied for the great impact of their applications (*i.e.*, radiotherapy and hadrontherapy) and effects (*i.e.*, exposure risk for astronauts in space missions).
In order to study the biological effects of high-LET radiations, several endpoints have been evaluated both in rodent- and in human-irradiated cells, including chromosomal aberrations [266-268], micronuclei (MN) [269], chromosomal non-disjunction [270], mutations [271, 272], DNA fragmentation [273, 274], clonogenic survival [275-277], and cell cycle effects [278]. However, aspects related to telomere length modulation and telomere metabolism have been so far poorly investigated both in primary and in immortalized cells exposed to low- and high-LET radiations.

The aim of the first part of the study was to analyze the DNA-damage and the genotoxic effects of low-energy protons (high-LET radiation), and X-rays (low-LET radiation) in human primary fibroblasts. Results obtained indicated that the DNA damage induced by high-LET radiation appear to be less amenable to be repaired than low-LET induced damage. This phenomenon seems to be related to the ‘complexity’ of the damage induced by high-LET radiations, resulting in more severe biological consequences.

After confirming the greater biological effectiveness of high-LET radiations compared to low-LET ones, we focused our attention on studying telomere metabolism within 24 hours from the exposure to both types of radiations. Interestingly, data obtained showed a different kinetics of telomere length modulation in cells exposed to low- or high-LET radiations. Moreover, the phenomenon observed appeared to be conserved both in primary and in immortalized cell lines. Interestingly, exposure of human primary fibroblasts to high-LET radiation determined a telomere elongation respect to untreated cells, whereas no telomere length modulation was observed in low-LET treated fibroblasts. In order to investigate the molecular mechanism underlying the observed elongation, the expression levels of the telomerase (*i.e.,* hTERT) and its enzymatic activity were evaluated. Several works pointed out the possibility that the telomerase acts as a telomere repair protein by adding *de novo* telomeric repeats, such hypothesis being supported by the evidence that the expression and the activity of this enzyme is radio-induced [238, 241, 279, 280]. Indeed, results obtained

excluded the involvement of the telomerase in the observed telomere lengthening induced by high-LET radiation, thus supporting the activation of a telomerase-independent mechanism. Some mammalian cells lacking in any telomerase activity are able to maintain the length of their telomeres for many population doublings (PDs) [281-284]. This indicated the existence of one or more non-telomerase mechanism(s) for telomere maintenance, further termed Alternative Lengthening of Telomeres (ALT) [285]. To date, clear evidences of the existence of an ALT activity has been demonstrated only in human tumours and immortalized cell lines, and in telomerase-null mouse cell lines [281-283, 286]. To analyze whether a recombinational mechanism could be responsible for the high-LET-induced telomere lengthening observed in human primary fibroblasts, two types of experiments were performed. On one side, the incidence of recombinational events at telomeres (T-SCE) was measured, and on the other side the colocalization of telomeres and PML bodies (that are considered as an hallmark of cells with activated ALT pathway [159], was analyzed. Strikingly, our results indicated that the DNA damage induced by high-LET radiation is somehow able to induce telomere lengthening through the transient activation of an ALT recombinational pathway.

Recent reports demonstrated that NBS1 is essential for the correct functioning of the ALT pathway [190, 287]. *NBS1* gene, mutated in the NBS human chromosome instability disorder [288, 289], encodes for the NBS1 protein, a central player in the response to the ionizing radiation-induced DNA damage [288], as well as in the homologous recombination repair [290]. In order to confirm the high-LET-induced recombinational ALT pathway, telomere length was evaluated in Lymphoblastoid Cell Lines (LCLs) heterozygous ($NBS1^{+/-}$) and homozygous ($NBS1^{-/-}$) for a mutation of the *NBS1* gene, as well as in normal cells ($NBS1^{+/+}$). Remarkably, a telomere elongation was observed in $NBS1^{+/+}$ and $NBS1^{+/-}$ cells, but not in $NBS1^{-/-}$ones after exposure to high-LET radiations. These data evidenced that the process of telomere lengthening induced by high-LET radiation is NBS1-dependent, thus supporting the hypothesis that telomere elongation is mediated by recombinational mechanisms.

Beside the analysis performed at 24 hours, telomere length modulation was followed up to 15 days from the irradiation of both human primary fibroblasts and LCLs. Dynamics of telomere lengths modulation appeared to be different after low- and high-LET irradiation. Our data showed that the telomere lengthening observed in high-LET-treated cells seems to be

maintained at 3-4 days, as well as 15 days after exposure. Interestingly, the time-course of the low-LET radiation-induced telomere length modulation appeared to be more complex than the high-LET one. In fact, after 3-4 days telomere erosion was reported, whereas after 15 days from the exposure a telomere lengthening was observed in primary as well as in immortalized cell lines.

To explain the time course of low-LET-induced telomere length modulation we have hypothesized that a direct correlation between telomere length and radioresistance/radiosensitivity could account for this phenomenon. To test our hypothesis, we decided to perform experiments in TK6 lymphoblast cells, since they represent a good and widely used radiobiological cellular model. Data obtained brought us to suggest a model: the radioresistance of cells with longer telomeres drives a selection process that led to an increased telomere length in clones survived to low-LET radiation exposure. A direct correlation between telomere length and radisensitivity/radiresistance has already been proposed in some published reports [233, 239, 242] and imply that telomeres length measurement could be potentially used as a tool to predict clinical radiation response in radiotherapy.

Results

1 DNA damage induced by high-LET radiation is repaired less efficiently than low-LET one.

1.1 Kinetics of DSBs repair is slower in high-LET treated samples than in low-LET ones

In Fig. 8, representative images of γ-H2AX foci induction at several harvesting times from irradiation of HFFF2 fibroblasts with 1 Gy of either X-rays or 28.5 keV/µm protons are shown.
In Fig. 9 are reported the kinetics and yield of γ-H2AX foci induction in cells irradiated with graded doses of radiation and harvested up to 24 hours. Consistent with data obtained with other sources of high-LET radiations [291], the number of γ-H2AX foci induced by protons shortly after irradiation, that is 30 minutes, was significantly lower than for X-rays at each tested dose (A, B and C) (Student's *t*-test $p<0.0001$). In addition, foci were more persistent in proton-irradiated samples than in X-rays, this was particularly visible for the 4 hours harvesting point at both 1 and 2 Gy (B and C): around 49% of the initial number of foci persisted for protons versus 22-30% for X-rays. Furthermore, at 24 hours from protons, we found 1.1, 1.2, and 1.7 of residual foci per cell, at 0.5, 1 and 2 Gy, respectively, which correspond to 9.4, 6.4 and 5.7% of the number induced at 30 min. Such percentage, when scored in X-ray-treated cells, corresponded to 0.45, 1.4 and 2.2%, of those induced at 30 min.

1.2 Yield of micronucleus induction is LET- dependent

The induction of micronuclei in binucleated-cells is reported in Fig. 10. As can be easily seen, 28.5 keV/µm protons were far more efficient than X-rays, at least in the range of 0.25-1.5Gy. At the highest dose, the frequency of MN deeply declined for protons, probably as results of cell cycle delay, whereas for X-rays, a linear trend was observed up to 2 Gy.

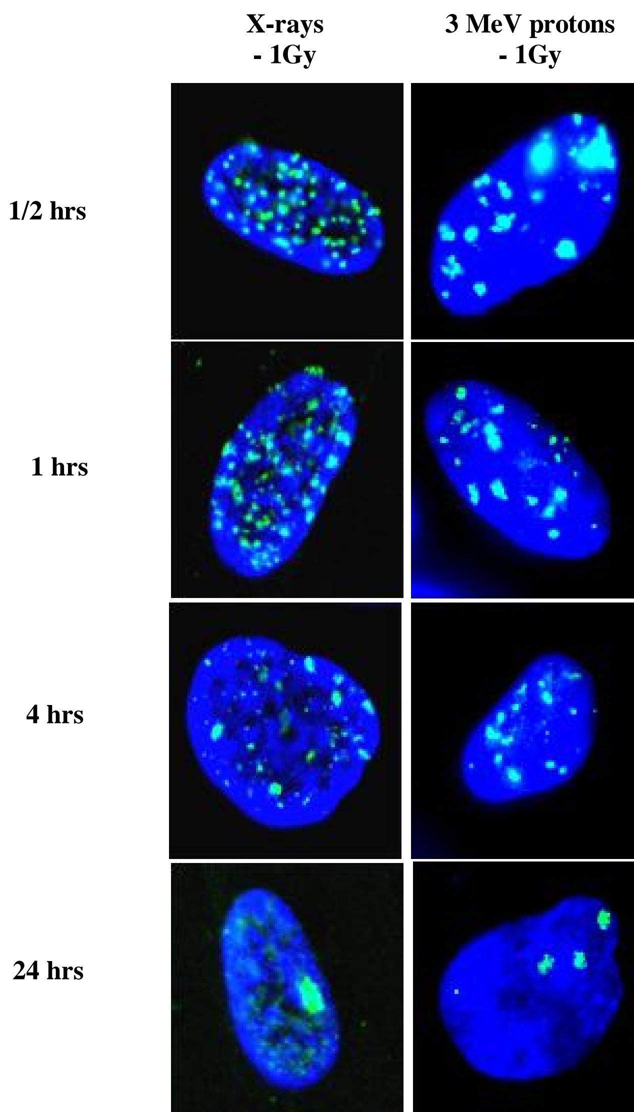

Fig. 8 - An example of γ-H2AX foci induced by 1 Gy of either X-rays or 3 MeV protons and harvested at different time. γ-H2AX was stained with FITC (green signals) whereas DNA was counterstained with DAPI.

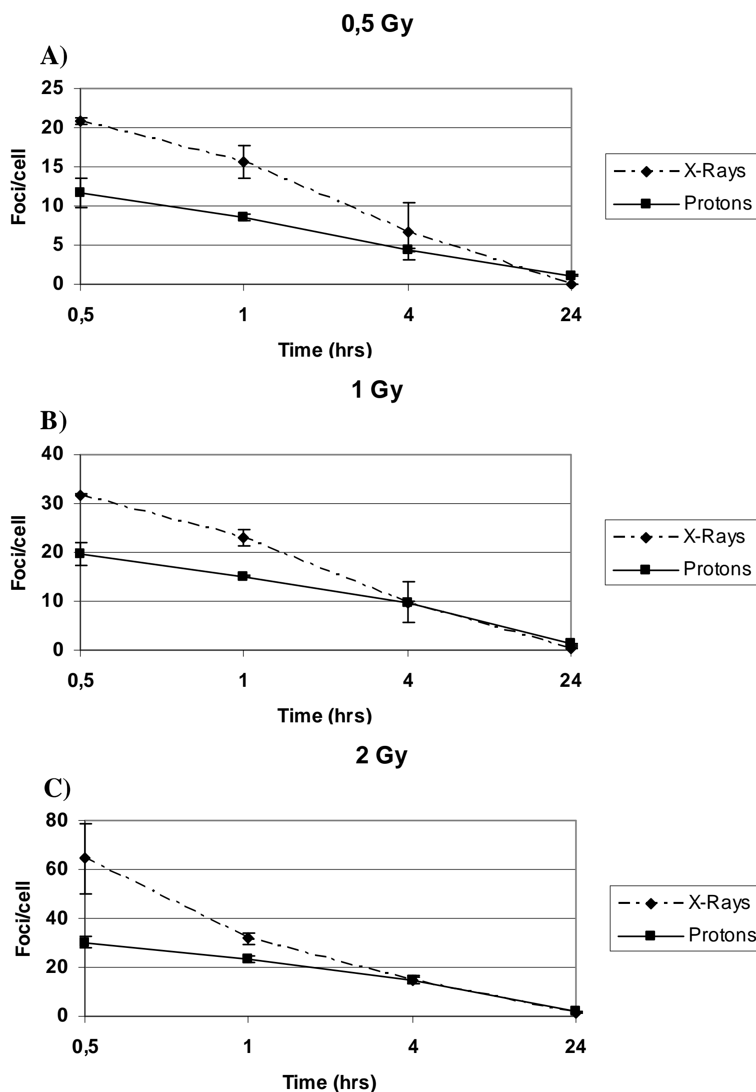

Fig. 9 - Kinetics of γ-H2AX foci induction in HFFF2 human fibroblasts after exposure to A) 0.5 , B) 1, and C) 2 Gy of X-rays or 3 MeV protons. Values observed in sham treated cultures were subtracted from treated samples. Error bar denotes standard deviations.

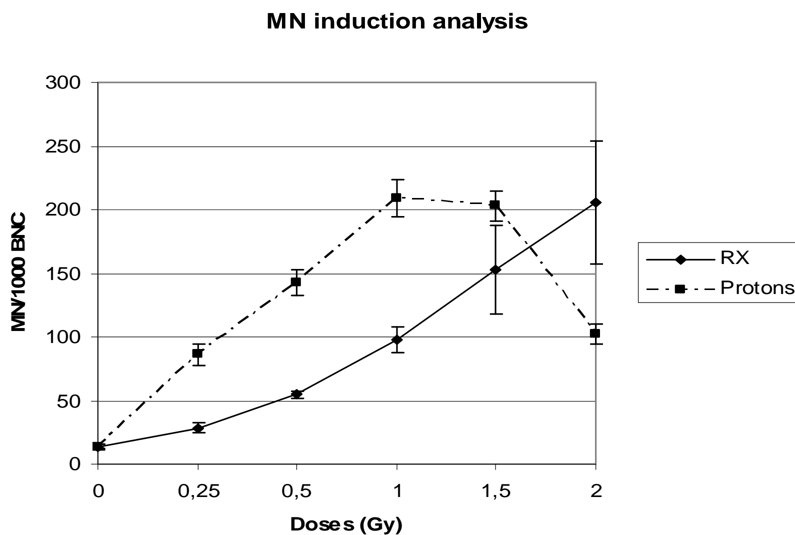

Fig. 10 - Frequency of MN in cytokinesis-blocked binucleated cells (BNC) after exposure to graded doses of X-rays or 3 MeV protons. The error bar denotes standard error of the mean.

2 High-LET radiation induce telomere telomere lengthening through a recombination mediated mechanism shortly after irradiation

2.1 High-LET radiation induce telomere elongation in the first 24 hours after irradiation

Telomere length analysis in HFFF2 human fibroblasts exposed to low- (X rays and 62 MeV protons, 1-2 keV/µm) and high- (3 MeV protons, 28.5 keV/µm) LET radiations and harvested 24 hours later show a different pattern of telomere lengths. In particular, no differences were observed in telomere length after 4 Gy of low-LET irradiation compared with non-irradiated cells, whereas after the same dose of high-LET irradiation a significant increase of telomere length was observed (Fig. 11). Same data are presented in Fig. 13 as distributions of telomere length.

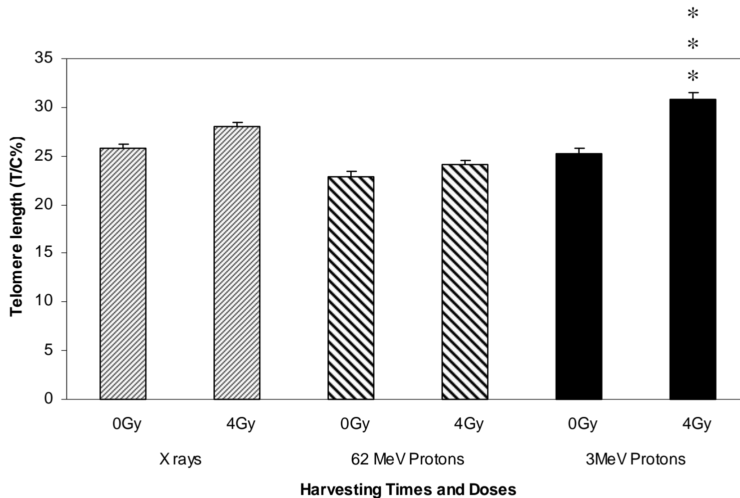

Fig. 11 - Mean telomere length expressed in T/C% (Telomere fluorescence/Centromere 2 fluorescence percentage) in X-rays, 62 MeV protons and 3 MeV protons irradiated samples. Error bars represent standard error (Mann-Whitney test, *** = $P < 0.0001$).

In Fig. 12 are shown the percentage increase of telomere length in treated versus untreated samples for all the tested radiation. After 24 hours, both X-rays and 62 MeV protons did not induce a significant telomere lengthening (with a percentage increase of 8.3% and 5.2%, respectively, if compared to the untreated control). On the other hand 3 MeV protons induced a significant telomere length increase of 22%.

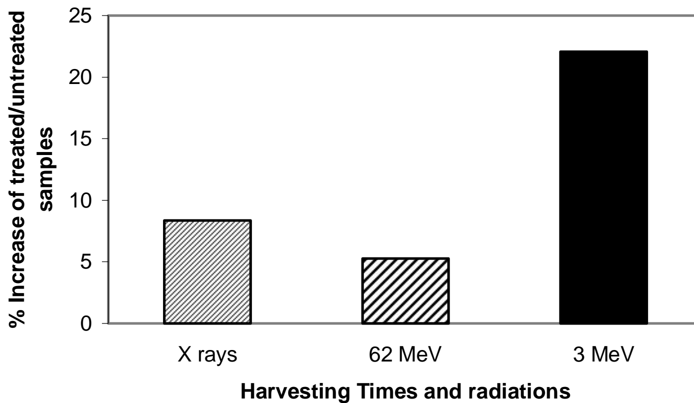

Fig. 12 - Increases of telomere length in treated versus untreated samples expressed as percentage. Striped columns referred to exposure to low-LET radiations while black column represent the increase after high-LET radiation exposure.

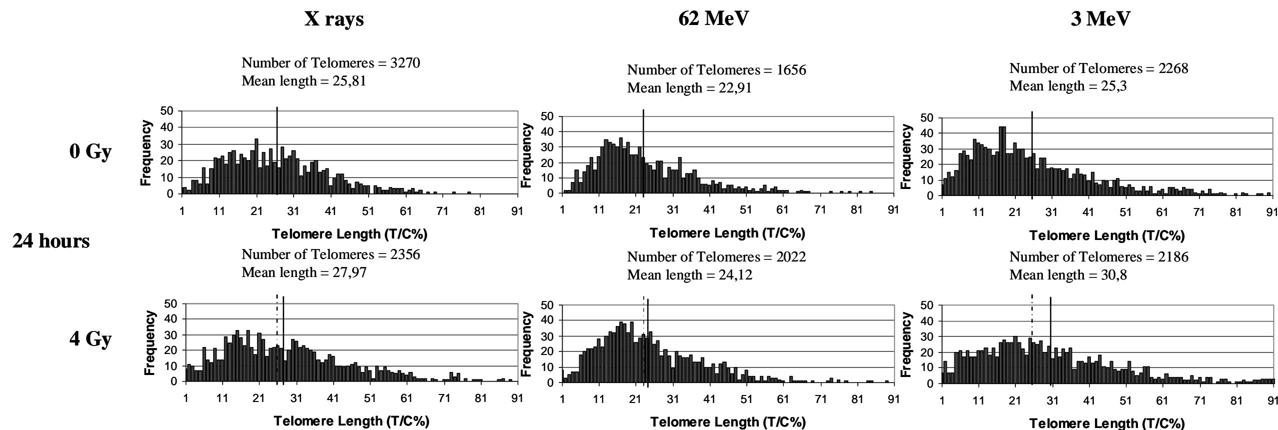

Fig. 13 - Telomere length distributions in HFFF2 treated with 4 Gy of X-rays, 62 MeV protons and 3 MeV protons after 24 hours from treatment. Solid lines represent the mean telomere length of each sample while dashed lines represent mean telomere length of untreated samples transposed on treated samples. Note that the gap between solid and dashed lines is from 3 to 5 fold greater in 3 MeV protons compared to X-rays and 62 MeV protons treated samples.

2.2 No telomerase induction was observed in primary fibroblast exposed to low- and high-LET radiations

Results from RQ-TRAP assay showed neither telomerase activation nor telomerase modulation after X-rays or 3 MeV protons irradiation indicating that telomere elongation appears to follow a telomerase-independent mechanism (Fig. 14).

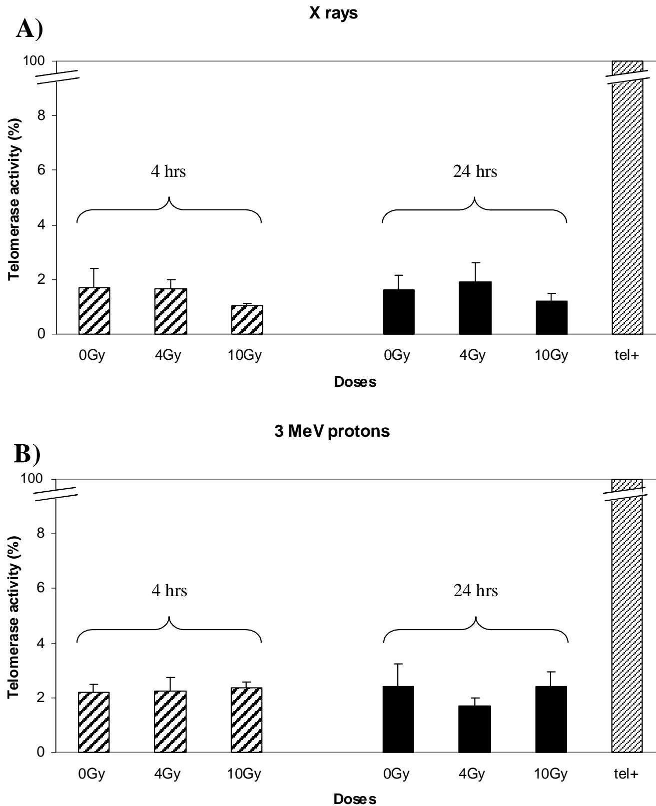

Fig. 14 - Telomerase activity after A) X-rays or B) proton irradiation evaluated by RQ-Trap Assay. Striped columns referred to telomerase activity evaluated at 4 hours from exposure while black columns referred to telomerase activity after 24 hours from exposure. Errors bars denote standard deviations.

2.3 Increased T-SCE frequency in high-LET treated primary fibroblasts

To evaluate whether a recombinational mechanism could be responsible of telomere lengthening in high-LET-treated samples, we performed a Chromosome Orientation FISH technique (CO-FISH), which allows the detection of telomere recombination events between sister chromatids exchange (Fig. 15A and 15B). CO-FISH showed that 3 MeV protons-treated cells display a higher T-SCE frequency if compared with untreated controls and with low-LET treated samples after 24 hours from treatment (Fig. 16). In particular we observed that high-LET radiation induce a three fold increase of T-SCE per chromosome (1.32% in untreated sample versus 3.8 in 3 MeV protons treated samples).

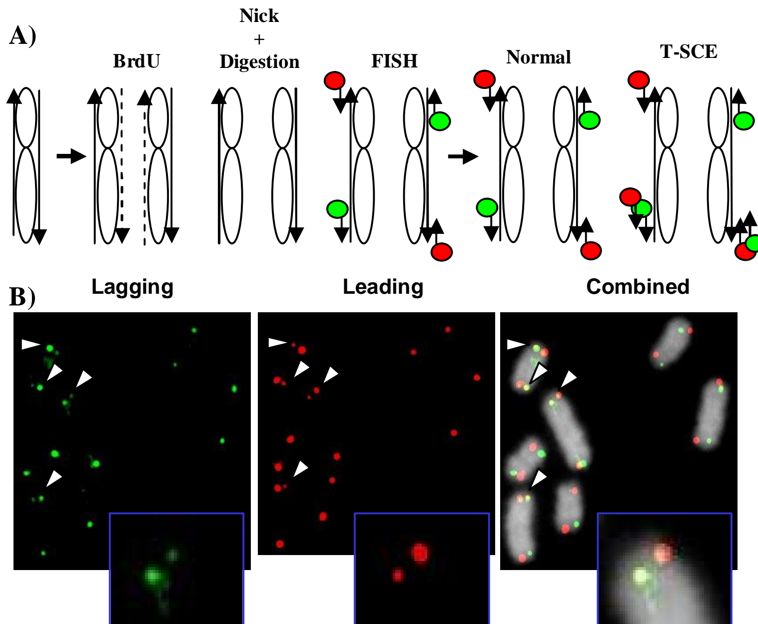

Fig. 15 – A) Schematic representation of the CO-FISH technique used to label telomeres produced by lagging strand and leading strand DNA synthesis. A sister chromatid exchange within telomeric DNA (T-SCE) will lead to combined green and red fluorescence. B) Representative CO-FISH images of chromosomes hybridized against the leading and the lagging telomere.

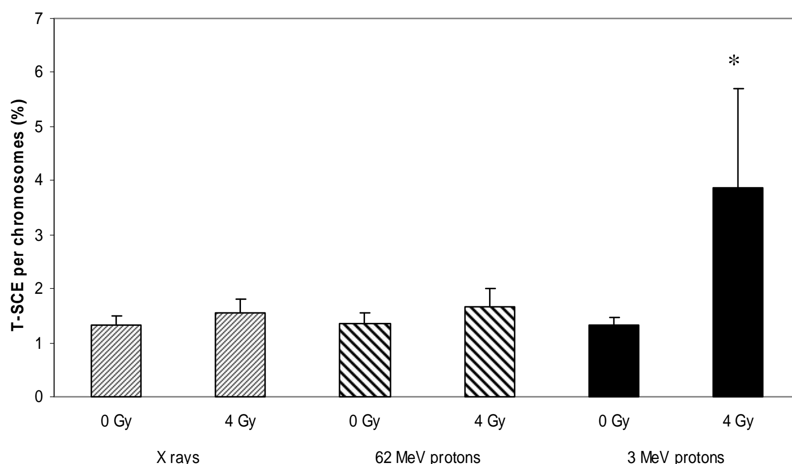

Fig. 16 – Quantification of T-SCE in low-and high-LET-treated HFFF2 evaluated at 24 hours from exposure. Only T-SCE events observed with both leading- and lagging-strand probes simultaneously were considered positive. Error bars denotes standard deviations (χ^2 test, * = $P < 0.0001$)

2.4 High-LET radiations induce transient colocalization of telomere DNA and PML protein

Another landmark of cells with increased telomere recombination is the presence of the so-called ALT-associated promyelocytic leukemia (PML) bodies (APBs), characterized by colocalization of telomeres and PML protein (Fig. 17). 3 MeV proton-irradiated cells showed a significant increase in the frequency of cells with APBs (Fig. 18A) and in the number of colocalization events per cell [292], after 6 hours from the treatment but not after 24 hours. Low-LET radiation did not induce colocalization of telomeres and PML proteins neither at 6 nor at 24 hours from irradiation (Fig. 18B).

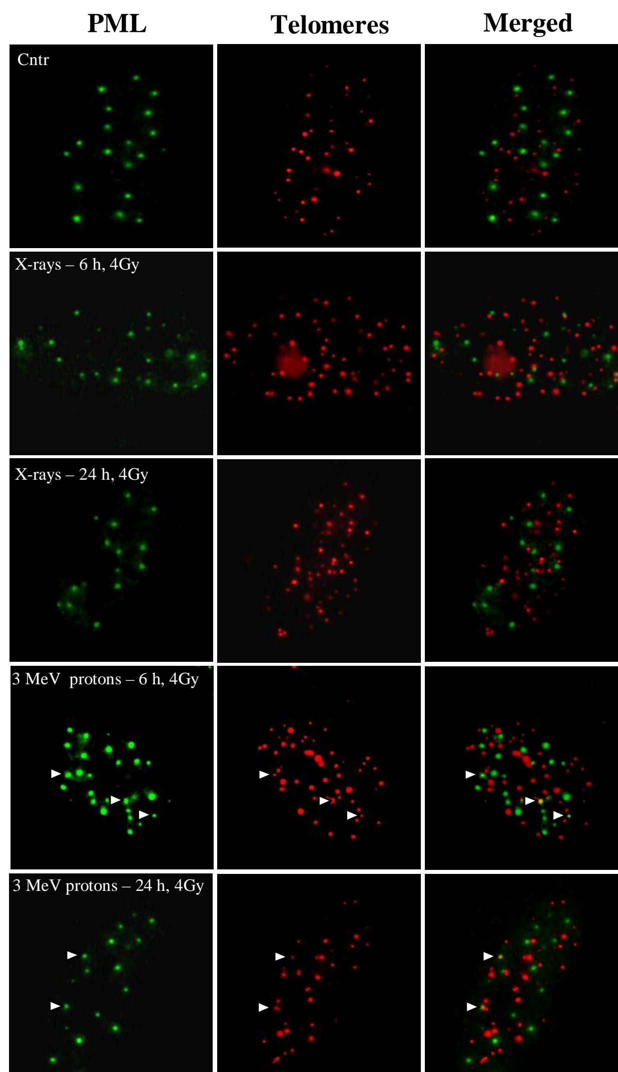

Fig. 17 – Representative images showing either PML fluorescence (green) and telomere fluorescence (red) or combined fluorescence (yellow) in X-rays and 3 MeV proton-treated cells harvested 6 and 24 hours after the exposure.

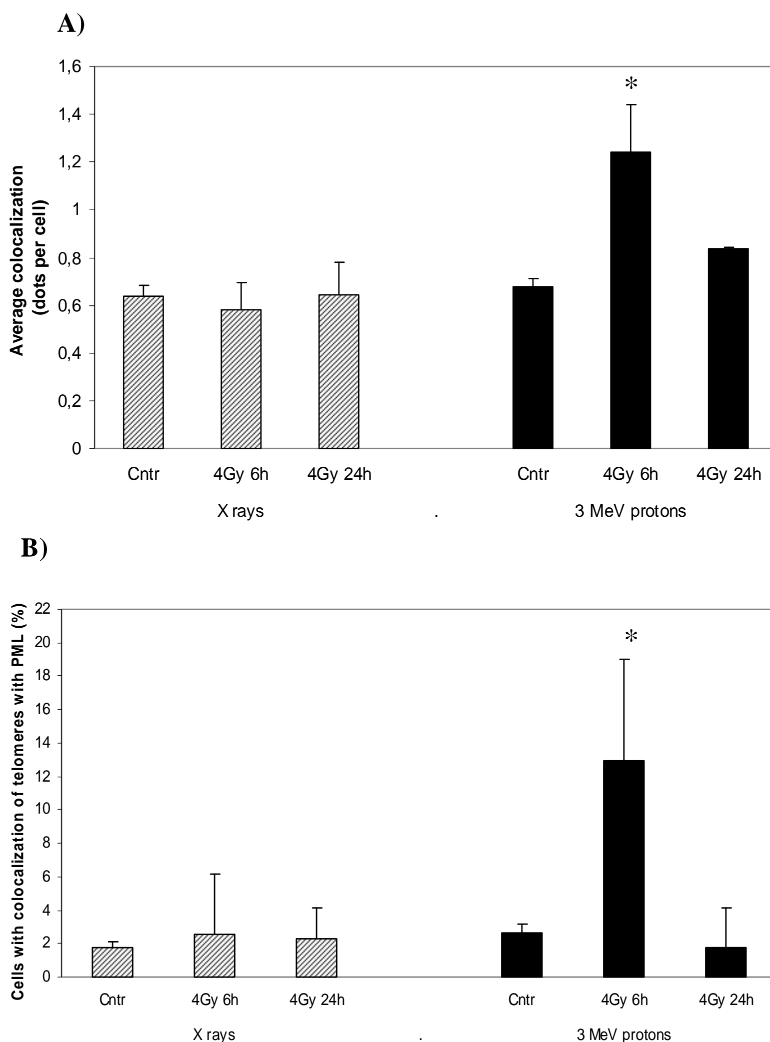

Fig. 18 – In the upper histogram (A) is reported the number of APBs per counted in every nucleus. In the lower histogram (B) is reported the percentage of cells showing colocalization of telomeres with PML proteins. A cell was considered positive when showed 3 or more colocalization events (χ^2 test, * = $P < 0.0001$).

3 Cells defective in Homologous Recombination repair do not show telomere elongation after high-LET irradiation

In Fig. 19 are shown the means of telomere lengths of $NBS1^{+/+}$, $NBS1^{+/-}$ and $NBS1^{-/-}$ cells after 24 hours from the exposure to graded doses of X-rays (0-4 Gy). No significant modulation of telomere length was observed. Interestingly, we found a shorter telomere length value in $NBS1^{-/-}$ untreated samples, confirming data from literature that report shorter telomere in patients affected by NBS [185]. In Fig. 13 are reported data obtained after treatment with carbon ions (39 keV/um). Our results showed that after 24 hours from the exposure a telomere lengthening was observed in LCLs established from $NBS1^{+/+}$ and $NBS1^{+/-}$ individuals, but not in LCLs established from the $NBS1^{-/-}$ patient.

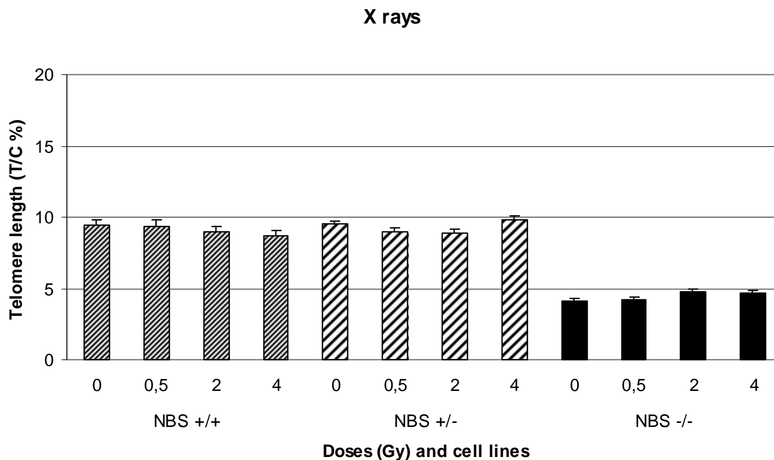

Fig. 19– Mean telomere length expressed in T/C% in Normal, $NBS1^{+/-}$ and $NBS1^{-/-}$ LCLs after exposure to graded doses of X-rays. A non-parametric 'Mann-Whitney' test with "two-tails" was used to calculate the statistical significance of the observed differences. Bars represent standard errors.

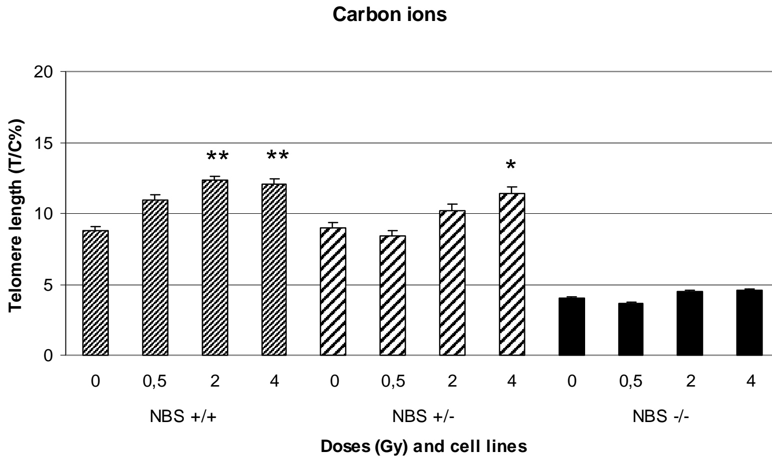

Fig. 20 Mean telomere length expressed in T/C% in NBS1$^{+/+}$, NBS1$^{+/-}$ and NBS1$^{-/-}$ LCLs after exposure to graded doses of carbon ions. For the non parametric Mann-Whitney test differences are considered significant for $P<0.05$ (*), highly significant for $P<0.001$ (**). Bars represent standard errors.

3.1 Neither low-LET nor high-LET radiations induce telomerase activity in lymphoblastoid cell lines

The time course of telomerase activity following irradiation with 2 and 4 Gy of X-rays and carbon ions were compared by RQ-TRAP assay. In Fig. 21 is reported the telomerase activity after X-ray and carbon ions exposure evaluated at 4 and 24 hours. As shown there is no significant induction or repression of telomerase activity in normal, NBS1$^{+/-}$ and NBS1$^{-/-}$ cell lines.

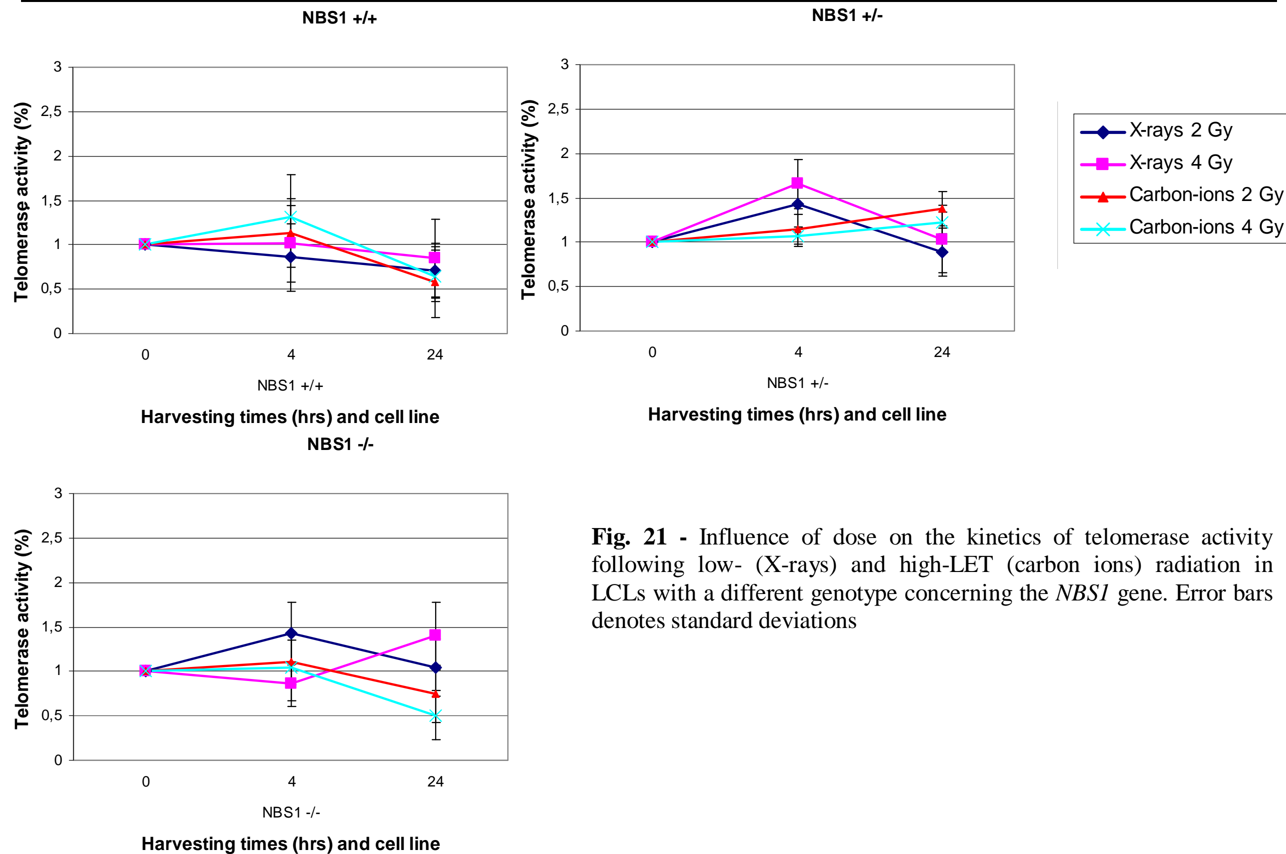

Fig. 21 - Influence of dose on the kinetics of telomerase activity following low- (X-rays) and high-LET (carbon ions) radiation in LCLs with a different genotype concerning the *NBS1* gene. Error bars denotes standard deviations

4 Telomere length modulation in human cells exposed to low- and high-LET radiation and followed up to 15 days

4.1 Different telomere length modulation dynamics in human cells exposed to low- and high-LET radiations

Beside the analysis at 24 hours from exposure other experiments with different harvesting times (3-4 days and 15 days) have been performed in HFFF2 cells.
The results obtained are shown in Fig. 22. High-LET radiations (3 MeV protons) induce a significant increase of telomere length at 24 hours, as well as at 4 and 15 days from exposure with an increase of 22, 20 and 37% respectively. Interestingly a different situation was obtained in X-ray-treated samples.

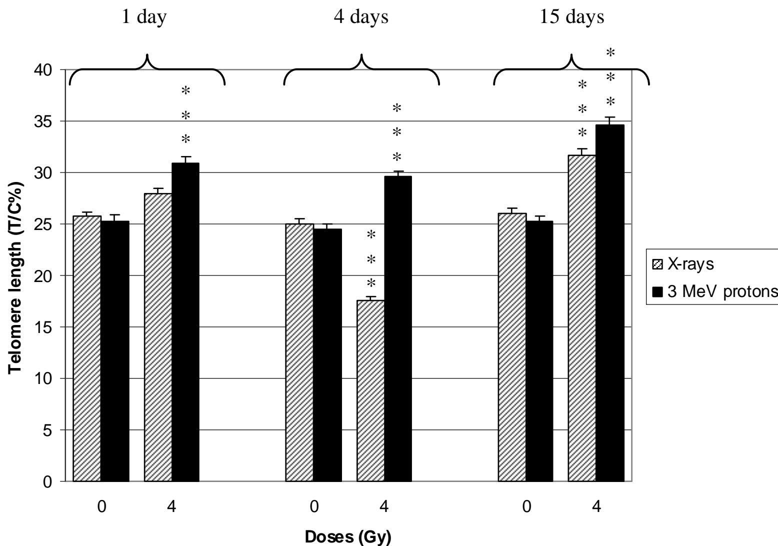

Fig. 22 - Mean telomere length expressed as T/C% in HFFF2 human primary fibroblasts exposed to 4 Gy of low- and high-LET radiations and harvested at 24 hours, 4 days and 15 days from exposure (Mann-Whitney test, *** = $P < 0.0001$).

Despite the lack in length modulation observed at 24 hours from exposure it was detected a telomere length decrease of 29% after 4 days and a telomere elongation (22%) after 15 days.

Similar results have been obtained in normal LCLs exposed to graded doses of low- (X-rays) and high-LET (carbon ions) radiations. In Fig. 23 are shown data obtained after X-rays (striped columns) and carbon ions (black columns) treatment.

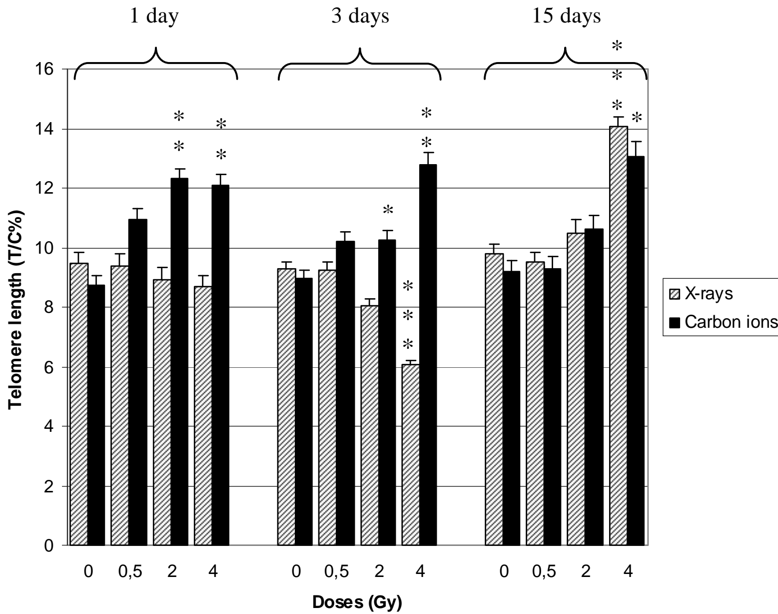

Fig. 23 - Mean telomere length expressed as T/C% in LCLs exposed to graded doses (0.5, 2, 4 Gy) of low- and high-LET radiations and harvested at 24 hours, 3 days and 15 days from exposure. Bars represent standard errors (Mann-Whitney test, * = P < 0.05 ** = P < 0.001 *** = P < 0.0001).

5 TK6 lymphoblasts which survived X-ray-irradiation display telomeres longer than untreated cells

5.1 TK6 cells show telomere erosion after 3 days from X-rays exposure

To characterize telomere lengths in the TK6 population at cellular level telomeric Q-FISH experiments were performed on 48 metaphases for a total of 8832 telomere signals in 3 independent experiments. Results obtained are shown in Fig. 24A. The mean telomere length observed in TK6 cells was 11.53 T/C%. Interestingly we have found a great variation in telomere length among the metaphases analyzed, in particular such length ranged between 3.33 and 23.06 T/C%.

In Fig. 24 B are shown telomere length data obtained from the analysis of 20 TK6 metaphases exposed to 4 Gy of X-rays and harvested 3 days later, compared to control metaphases. A significant decrease in telomere length, from 10.8 to 6.9% T/C%, was observed.

5.2 Determination of cellular survival in TK6 whole population

TK6 cells were exposed to graded doses of X-rays (0.5-4 Gy). Results obtained indicate that the surviving fraction after 0.5, 1, 2 and 4 Gy was respectively of 78%, 20%, 3% and 0,2%. We determined the D_0 value which is defined as the dose required to reduce the number of surviving cells to 37% of their former value using data from an exponential survival curve. The D_0 value was 0.63 Gy.

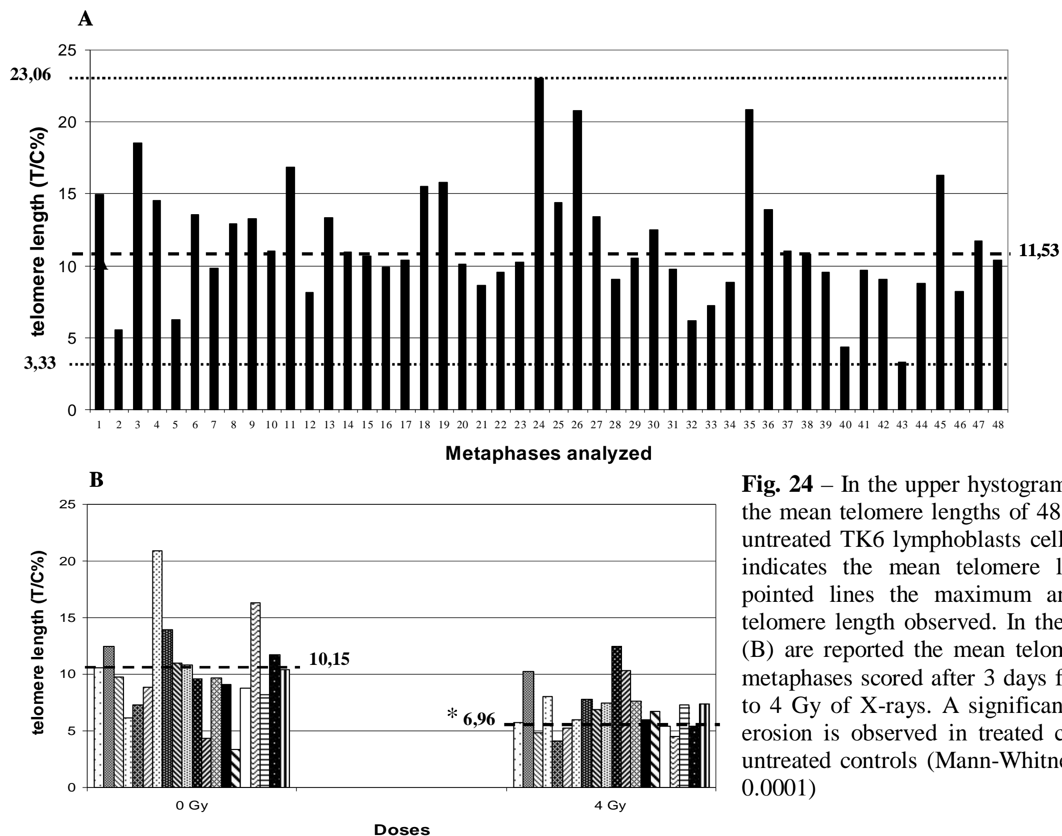

Fig. 24 – In the upper hystogram (A) are reported the mean telomere lengths of 48 metaphases from untreated TK6 lymphoblasts cell line. Dotted line indicates the mean telomere length value and pointed lines the maximum and the minimum telomere length observed. In the lower histogram (B) are reported the mean telomere length of 20 metaphases scored after 3 days from the exposure to 4 Gy of X-rays. A significant telomere length erosion is observed in treated cells compared to untreated controls (Mann-Whitney test, ***= $P < 0.0001$)

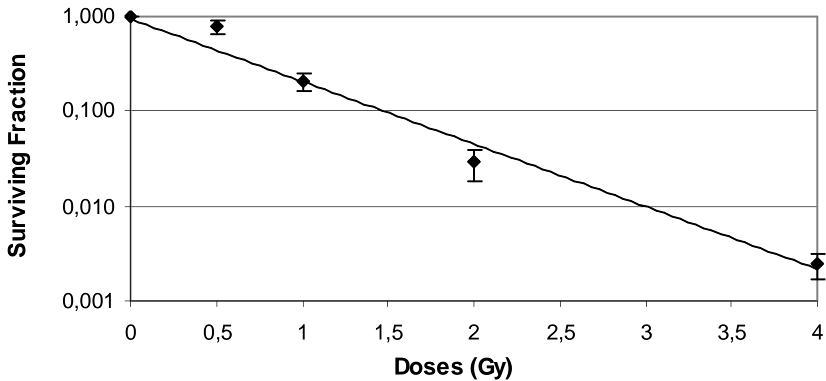

Fig. 25 - Cell killing as a function of the dose of X-rays for TK6 cells. Data points represent the mean of 4 independent experiments. Bars represent standard deviation of the mean.

5.3 TK6 survived clones show telomere longer than untreated controls

Cells which survived to 4 Gy of X-rays were grown as individual clones and telomere lengths were analyzed 20 days later by means of Q-FISH. As shown in Fig. 26 the mean telomere length obtained from the analysis of 9 clones surviving 4 Gy was 13.5 T/C% and was significantly higher than the mean telomere length measured in clones established from untreated cells (9.8 T/C%). The percentage increase of treated versus untreated samples was 37%. Twenty metaphases for each clone were scored, which correspond to 3720 telomere signals analyzed.

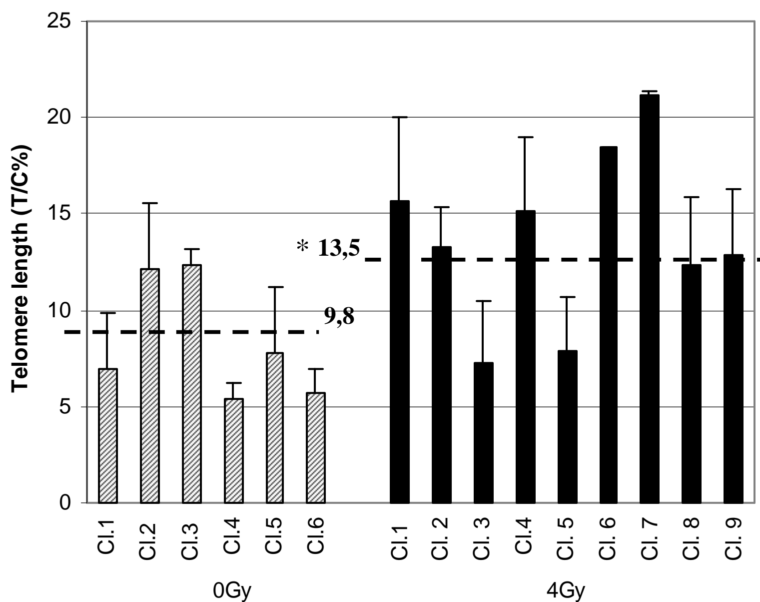

Fig. 26 - Telomere length expressed as T/C% in 4 Gy survived clones compared to clones grown from untreated cells. Dotted lines represent the mean telomere length of untreated and 4 Gy treated clones (Mann Whitney test, * = $P < 0.05$).

Discussion

1 DNA damage induced by high-LET radiation is repaired less efficiently than that produced by low-LET one

In the first part of this study, the genotoxic effects of low- and high-LET radiation have been investigated. First, we evaluated whether cellular differences in the capability to handle lesions characterized by different complexity may be reflected in the amount and kinetics of disappearance of γ-H2AX-induced foci. We found that high-LET which corresponds to 28.5 keV/μm LET radiations (3 MeV protons, 0.8 MeV on the cell surface), induced a lower number of foci per Gy, compared with low-LET ones (X-rays). This result is consistent with recent data [291, 293], and may be ascribed to the induction of multiple DSBs within radiation tracks. Furthermore, after proton irradiation, we detected a higher fraction of γ-H2AX-remaining foci, particularly in the first hours after exposure, indicating the persistence of a subset of unrepaired DNA lesions. Interestingly, in PFGE experiments, though doses were far higher, a slower kinetics of DSB rejoining was detected shortly after irradiation in V79 cells exposed to protons of the same LET [277]. Overall, these data seem to indicate that protons induce a subclass of DNA lesions less prone to be repaired by the fast component of the DNA repair processes. After 4 hours, the slow repair continued in proton-treated cells, and at 24 hours the yield of γ-H2AX-induced foci was comparable between the two types of radiations used. However, taking into account the differences in the initial yield of γ-H2AX foci, as measured 30 minutes after irradiation, a higher number of unrejoined DSBs was detected after proton irradiation. This result may indicate that a subclass of DNA lesions characterized by high complexity is left unrepaired up to 24 hours after high-LET irradiation. A longer persistence of γ-H2AX foci has been also reported in MRC-5 fibroblasts exposed to 39 keV/μm carbon ions [291]. Consistent with a delayed DSBs repair and with the clastogenic effect of protons detected in Cl-1 Chinese hamster cells [269], we found a LET-dependence in the induction of MN in cytochalasin-arrested binucleated cells, with the effectiveness at least two times greater in 3 MeV protons- compared to X-ray-treated samples. The

MN assay discriminated between the two types of radiations at doses as low as 0.25 Gy. Conversely, at the highest dose, the frequency of scored MN deeply decreased to values lower than X-rays, probably as an effect of a higher G_2-phase block elicited in proton damaged cells [278]. This is supported by the observation that at 24 hours from proton exposure the mitotic index dropped to 0.6% (data not shown), whereas the fraction of G_2-phase arrested cells was higher than in untreated samples, as shown by the cytofluorimetric analysis (data not shown; see fig. 1 and 2 in supplementary data). These results confirm the findings that as a function of dose and radiation quality, heavily damaged cells do not divide and will be not scored for MN, as recently reported by Wu and coworkers [294]. In conclusion, a delayed kinetic of DSBs repair and a higher induction of MN was observed in proton-irradiated samples.

2 High-LET radiation induce telomere lengthening through a recombination mediated mechanism shortly after irradiation

The results so far collected in human primary fibroblasts pointed out a clear effect of the radiation quality on the endpoint analyzed, which is the variation in telomere length observed at 24 hours from irradiation. No induction of telomere abnormality was observed after 4 Gy of X-ray and 62 MeV proton irradiation, whereas a telomere elongation was detected for proton beams with energy of 3 MeV. Interestingly, these results have been confirmed also in murine embryo fibroblasts (MEFs) (data not shown; see fig. 3 in supplementary data).
Since telomere length modulation is associated to the different type of radiation used, it is conceivable that elongation occurring after high-LET radiation is linked to the type of lesion inflicted to DNA.
Interestingly, from a mechanicistic point of view, it was shown that the upregulation of telomerase activity occurred *in vitro* in X-rays exposed human and murine cells [279, 280, 295]. Hence, it is conceivable to speculate that telomere elongation occurred as result of chromosome healing mediated by radiation-activated telomerase. A possible proposed mechanism for telomere elongation is that when telomeres reach a minimum critical length, telomerase catalitic subunit (*hTERT*) gene transcription and telomerase activity are induced [296]. With the aim to ascertain the involvement of telomerase in telomere elongation observed in

human primary fibroblasts, *hTERT* gene expression and telomerase activity were evaluated. Data obtained from gene expression analysis (data not shown) and from RQ-TRAP assay, demonstrated that the enzyme was not induced by both X-rays and protons, for doses as high as 10 Gy. These results suggest that what we observed in terms of telomere elongation occurrs as result of a telomerase-independent mechanism.
More recently several reports have shown an alternative mechanism of telomere lengthening (ALT) based on the recombination between telomere sequences [159]. Typically, ALT cells survive cellular crisis by gaining the ability to maintain telomere length through recombination-based mechanisms. It has been hypothesized that if telomerase is not activated, telomere dysfunction permits inappropriate telomere recombination, and hence telomere length maintenance [297].
Since it has been demonstrated that increased T-SCEs are a hallmark of ALT-positive cells [178], we have performed a CO-FISH analysis with the aim to detect telomere recombination events between sister telomeres, thus allowing to evaluate the possible activation of the ALT pathway. Notably, our data show that high-LET-irradiated samples displayed a higher frequency of T-SCEs per chromosome (3.9 %) compared to those scored in untreated controls (1.3 %), thus indicating a possible relationship between telomere elongation and telomere recombination. Interestingly, the percentage of T-SCEs per chromosome was not different between sham treated and irradiated-samples for Low-LET X-rays or 62 MeV protons.
An additional hallmark of ALT-positive cells is the presence of specific PML bodies, called APBs, that contain telomeric DNA, telomere-associated proteins and recombination-associated proteins, such as NBS1 [159]. In order to better understand whether the mechanism of high-LET radiation induced-telomere elongation was based on an ALT mechanism, immuno-FISH experiments were performed using telomere probes and an antibody directed against the PML protein. Confirming our hypothesis of a radiation induced activation of the ALT pathway, 3 MeV protons-irradiated HFFF2 cells showed a significant increase in the frequency of cells with APBs and in the number of colocalization events per cell, when compared to untreated controls. Immuno-FISH data suggest that such an ALT pathway activation is transient, since colocalization of telomeric DNA and the PML proteins occurred at 6 hours, but not at 24 hours from irradiation. On the other hand, X-ray irradiation did not induce a significant variation neither in the frequency of cells with APBs nor in the number of colocalization events per cell. These findings indicate that telomere elongation observed shortly after 3 MeV proton irradiation could be due to an ALT mechanism based on

recombination between telomere sequences. Despite data in literature have shown that mechanism underlying T-SCEs are different and independent from those of SCE that occur in other genomic locations [176], it is interesting to note that a relationship between high-LET radiations and SCEs induction was reported in hamster and human cell lines [298, 299], and that such relationship appear to be mediated by homologous recombination [300].
In this context, we speculate that the different response in telomere elongation, as detected at 24 hours from irradiation, could be related to the quality of radiation used and to the “complexity” of the DNA damage: a more complex damage, as that obtained after high-LET radiation, could be responsible for a transient activation of ALT pathway, thus resulting in telomere lengthening.

3 Cells defective in Homologous Recombination repair do not show telomere elongation after high-LET irradiation

In humans, mutations in the components of the MRN complex are hypomorphic: the encoded proteins are usually truncated and exhibit reduced function. NBS is caused by hypomorphic mutations in *NBS1,* which in most cases produce a truncated 70kDa NBS1 protein [288, 301]. Cells established from NBS patients exhibit chromosomal instability, hypersensitivity to ionizing radiation, abnormal S-phase checkpoints [302], accelerated rate of telomere shortening and reduced capacity for homologous recombination [185, 303].
To elucidate whether proteins involved in HR may have a role in the high-LET induced telomere lengthening, experiments on LCLs having a different genotype with respect to the *NBS1* gene ($NBS1^{+/+}$, $NBS1^{+/-}$, $NBS1^{-/-}$) have been performed. The experiments were carried out by evaluating telomere length modulation induced by carbon ions (39 keV/µm) and X-rays.
Although the cellular model (suspension growing cells) imposed to use a different source of high-LET radiation (carbon ions instead of the previously used 3 MeV protons), it was very interesting to note that similarly to what observed in HFFF2) telomere lengthening was observed also after carbon ions exposure of $NBS1^{+/+}$ LCLs, but not after X-ray irradiation. This further support_that: *i)* high-LET radiations, regardless of cellular type, are in general able to induce telomere lengthening, probably

in relation to the complexity of the induced DNA damage; *ii)* telomere lengthening has been detected in telomerase positive LCLs, thus rising the possibility that temporarily both ALT pathway and telomerase activation can coexist [304]. To rule out that the lengthening was due to an induction of telomerase activity, RQ-TRAP assay experiments were carried out. Data obtained showed that the telomerase activity was not induced in any of the cell lines analysed. This demonstrate that in this case as well as in primary fibroblasts the telomere elongation was telomerase-independent.
As previously shown, NBS1 was found to be co-localized with PML, as well as to be associated with a nuclear dot-associated PML-binding protein, SP100 [305]. Moreover, NBS1 is required for the recruitment and/or the assembly of a subgroup of proteins into APBs, including MRE11, RAD50, and BRCA1 [287]. As demonstrated by Zhong and coworkers [190] depletion of NBS1, with or without depletion of other members of the MRN complex, results in the inhibition of ALT-mediated telomere maintenance. Such reported results rose the question whether or not NBS1$^{-/-}$ cells could elongate their telomeres by the activation of a radiation-induced ALT pathway. Our results did not show any modulation of telomere length in carbon ions treated-NBS1$^{-/-}$ LCLs, whereas telomere elongation was reported in NBS1$^{+/-}$ cells. These data support the notion that the process involved in telomere lengthening is an ALT-like pathway, transiently activated after high-LET irradiation. Moreover, the response observed in the heterozygote demonstrated that the “gene dose effect” does not affect the induction of such phenomenon.

4 Telomere length modulation in human cells exposed to low- and high-LET radiation followed up to 15 days

The exposure of cells to radiations of different quality, determines a different response in term of telomere length modulation not only at the 24 hours time-point, but also within the first 15 days. In fact, the telomere elongation observed in high-LET radiation-treated cells (primary fibroblasts as well as LCLs MEFs) was maintained in the first 15 days after exposure. Such data support our idea that the recombinational pathway is activated in the first hours after the irradiation leading to a telomere lengthening, and that such elongation is just maintained during the life-span of the cell.
On the other hand, low-LET radiation induced an effect, in terms of telomere length modulation, starting from the 3rd - 4th day after exposure. In

fact, our data show that X-rays induced a telomere erosion after 3 or 4 days (depending on the cell line analyzed), and subsequently a telomere elongation after 15 days.
It is possible to speculate that the different modulation of telomere lengths in the first 15 days from the exposure to high-and low-LET radiation, is strictly related to the quality of the DNA induced-damage. In this scenario, complex DNA damage induced by high-LET radiation could be able to activate precociously the recombinational mechanism, leading to telomere elongation, as discussed above. Conversely, the greater indirect effects of low-LET radiation compared to high-LET one, could generate an acute oxidative stress shortly after irradiation, thus leading to telomere erosion in the first 72-96 hours. In fact it is known that oxidative damage, is repaired less efficiently in telomeric DNA than elsewhere in the chromosome, and oxidative stress accelerates telomere attrition [306]. The subsequent telomere lengthening observed after 15 days from exposure, could be the consequence either of a late ALT pathway activation or simply of a process of positive selection which favor cells with long telomeres.
However, we can not rule out the possibility that both the above mentioned processes may cooperate to determine the telomere elongation observed.

5 TK6 lymphoblasts which survived to X-ray-irradiation display telomeres longer than untreated cells

Radiotherapy is still considered mandatory as treatment for many types of cancer. However, many patients develop recurrences, though the mechanisms behind resistance to radiation treatment remain largely elusive. Hence there is a need for a better understanding of the biological mechanism underlying of radioresistance. Various factors of extra- as well as intra-nuclear origin have been identified as responsible for influencing radiation responsiveness of cells. Among them, in the last years telomeres and telomere dysfunction have been proposed as new player in radiation sensitivity [307].
To better understand whether telomere length variations observed after low-LET exposure could be determined by a selection in favour of cells with long telomeres respect to those with short telomeres, experiments on TK6 cells exposed to X-rays were carried out. TK6 cells are a widely used and easy to handle radiobiological cellular model. Interestingly, in a recent publication it was reported that TK6 cells exposed to 4 Gy of X-rays

showed telomerase induction and telomere lengthening, as detected by means of Telomere Restriction Fragments (TRF) experiments [308] The author proposed that telomerase upregulation could account for the telomere lengthening observed 15 days after irradiation. Our results, instead, exclude the involvement of telomerase in the elongation process (data not shown; see fig. 4 in supplementary data). Moreover, data obtained showed that after 4 Gy of X-rays the low percentage of TK6 lymphoblasts which survived (surviving fraction of 0.03%), grown as independent clones, displayed a mean of telomere length significant higher than clones grown from untreated cells. This led us to speculate that cells with longer telomeres behave as radioresistant ones and hence are selected by irradiation, expanding in the growing population. For low-LET irradiation such selection event seems to take place at 72-96 hours from exposure. This hypothesis could be supported by the observation that 24 hours after X-ray irradiation, neither in TK6 nor in normal human HFFF2 fibroblasts and lymphoblastoids cells, telomere attrition was detected; contrastingly, a significant shortening was observed at 72-96 hours from exposure. Data so far collected in our laboratory concerning primary as well in immortalized cell lines support the hypothesis that low-LET irradiation induce a primary effect of telomere attrition in the first 72-96 hours. Telomere attrition maybe associated with the activation of an apoptotic pathway and cell arrest [309], leading to the death of those cells that display short telomeres. Between the 3rd-4th day and the 15th day, we observed that cells survived to the irradiation, grown as individual clones, divided to reconstitute a population displaying a higher mean telomere length. Our selective process hypothesis is borne by the evidence that untreated TK6 cells showed a great variation in the telomere length among the metaphases analyzed. In fact, the heterogeneity of the telomere lengths observed in the whole TK6 untreated population, represent the background necessary condition for the occurrence of a selective process.

Recently, several studies have uncovered an inverse relationship between telomere length and radiation sensitivity in late generation telomerase deficient $hTR^{-/-}$ mice [239], in human lymphocytes and in human tumoral cell lines [264, 310, 311]. However data reported in literature are still contradictory and some authors have not found such relationship [262, 263, 312]. In this context, our results support the notion that telomere length play a relevant role in the radioresistance phenomenon. Telomere maintenance mechanisms therefore appear to be a promising target for radiosensitization. Elucidation of the molecular mechanisms may improve therapeutic regulation of radiotherapy in human tumor cells.

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