Telomere dysfunction: a new player in radiation sensitivity

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ABSTRACT

Human individuals often exhibit important differences in their sensitivity to ionising radiation, the basis of which is now beginning to be understood. Extensive literature links impaired DNA repair with radiation sensitivity, this impairment being due to a lack of correct functioning in many proteins involved in DNA repair pathways and/or in DNA damage checkpoint responses. Given that ionising radiation is an important and widespread diagnostic and therapeutic tool, it is important to further investigate those factors and mechanisms that underlie individual radiosensitivity. Recently, evidence is accumulating that telomere function may well be involved in cellular and organism responses to ionising radiation, broadening still further the currently complex and challenging scenario.

INTRODUCTION

DNA double strand breaks (DSBs) are among the most dangerous lesions to mammalian cells exposed to ionising radiations because, although they are produced at a lower frequency compared with other DNA lesions, cellular repair mechanisms remove DNA DSBs less efficiently and with lower fidelity than other types of DNA damage (1,2). Most DNA double-stranded broken ends in mammalian cells are processed by the non-homologous end-joining (NHEJ) repair pathway. As expected, inactivation of the genes involved in NHEJ impairs DSB rejoining, causes increased sensitivity to ionising radiation, and predisposes to cancer (3-6). The NHEJ pathway of DSB repair does not require a homologous DNA template to initiate joining (7). When the number of breaks in a cell is high, this template-free mechanism can incorrectly join two broken DNA fragments giving rise to exchange-type chromosome aberrations. These aberrations are a potential cause of gene dose imbalances thus linking incorrect repair to carcinogenesis. When the NHEJ machinery fails to rejoin broken DNA fragments, the resulting situation is characterised by the presence of highly unstable open fragments, which are usually lethal to mammalian cells.

In contrast to broken chromosome ends, the ends of linear eukaryotic chromosomes are protected by telomeres, a complex of repetitive DNA sequences and proteins (8,9). Telomeres prevent the recognition of natural chromosome ends as DSBs, thus protecting them from degradation and recombination activities. In addition to their protective function, telomeres also facilitate the complete replication of chromosomes through elongation by telomerase. In the absence of a telomere maintenance system, human and mouse telomeres shorten 50-150 bp/end/cell division (10-12). Therefore, the

long-term proliferation of all eukaryotic cells requires a mechanism to counteract telomere attrition.

When telomeres lose their structure as a consequence of telomeric DNA erosion or defective telomeric proteins, their capping function is impaired and chromosome end fusions are formed. The covalent fusion of damaged telomeres requires the same factors as normal NHEJ (13,14) and gives rise to dicentric chromosomes that are unstable in mitosis. These types of aberrations can become fragmented and might generate genomic instability in proliferating cells by entering into bridge-fusion-breakage cycles when the two centromeres in fused chromatids are pulled to opposite poles at anaphase. Using an elegant model to investigate the instability following telomere loss in a marker chromosome, it has been demonstrated that a single telomere loss can result in cascades of instability affecting multiple chromosomes (15). The role of telomeres in preventing chromosome fusion has led to the proposal that loss of telomere function is responsible for generating chromosome instability in cancer (reviewed in 16-18).

Telomeric functions and DNA damage response proteins are closely related. In recent years, mounting evidence has shown that several factors are shared between the machineries for DNA damage response and telomere maintenance. Several proteins originally identified by their roles in DNA repair pathways, particularly those involved in NHEJ, are found in telomeres, participating in their protection and are necessary for capping the ends of mammalian chromosomes to avoid chromosome end fusions (19-21). Reciprocally, the human telomere-associated protein TRF2 is phosphorylated

and associates with genomic DSBs as an early response to DNA damage (22-23). Additionally, an apparent telomere dysfunction phenotype has been reported in cells from Ataxia telangiectasia patients (24) and in cell lines derived from other genomic instability and radiation sensitivity human syndromes (25,26). Given this close relationship between telomeres and DNA repair, here we explore recent work addressing the significance of correct telomere function in determining the cellular and the organismal responses to ionising radiation.

THE TELOMERE FUNCTIONAL STRUCTURE

Mammalian telomeric DNA comprises tandem arrays of duplex TTAGGG repeats with the G-rich 3'strand extending beyond its complement to form a single stranded overhang of about 100 bases long. These telomeric repeats are bound by numerous proteins that determine the structure of the telomere terminus and participate in the regulation of the telomere length maintenance. The physical structure of the telomere was revealed by electron microscopy to be a large duplex loop, the t-loop (27). In this configuration, the end of the telomere is tucked in: the long single stranded array of TTAGGG repeats at the 3' end loops back on itself and invades the double stranded telomeric DNA (figure 1). Telomeric repeat binding factor TRF2 binds the double-stranded telomeric tract (28) and is required to remodel linear DNA into t-loops in vitro (29). Inhibition of endogenous TRF2 binding by expressing a dominant negative form of TRF2 results in end-to-end fusion of chromosomes (28) that are formed by means of the NHEJ repair pathway (14) without attrition of telomeric repeats. This evidence suggests that one way in which chromosome ends are protected from fusion is by their t-loop structure: without an accessible end, the NHEJ

machinery will not be able to process the 3' overhang and ligate the two chromosome ends. An additional but not exclusive possibility for end joining protection has recently been distinguished. Karlseder and colleagues (30) unexpectedly found that TRF2 binds and restrains ATM activation at telomeres. The ATM kinase is an important transducer of the DNA damage signal (reviewed in 31) that mediates activation of DNA repair pathways and cell cycle checkpoints. The proposal that the telomeric protein TRF2 is an ATM inhibitor is attractive because it suggests that ATM activity could be precisely restrained at chromosome ends without affecting the DNA damage response elsewhere in the genome.

Despite the effort to hide telomeres from the DNA damage repair system, several proteins involved in DNA damage response pathways are localized at functional telomeres, and contribute to t-loop formation and maintenance. Among them, some NHEJ proteins are required to generate t-loops in vivo. In fact, chromosome end fusions, a prominent marker of telomere dysfunction, have been identified in cells derived from mice deficient in DNA-PKcs, Ku70 or Ku86 (32-36). These proteins are integral components of the NHEJ pathway in higher eukaryotes and are involved in early steps of this repair pathway (DSB recognition and stabilization).

Besides their end protection function, telomeres also provide a substrate for chromosome length maintenance. The most versatile and widely used method of telomere maintenance is based on telomerase, a two-component ribonucleoprotein enzyme that contains a reverse transcriptase, TERT, and a template RNA component, TERC. The 3' G-rich overhang is the substrate for this

enzyme, which is able to elongate telomeres by adding new TTAGGG repeats onto pre-existing ones. Human telomerase is regulated during development: telomerase expression is dramatically reduced in many somatic cells during embryonic development. Most somatic human tissues and primary cells possess low or undetectable telomerase activity, and telomeres shorten with each division in vivo and in vitro. When telomeres shorten below a critical length, they lose their capacity to provide an adequate cap to the chromosome end, and such shortened telomeres can signal to cells to cease proliferation, in a process called replicative senescence. In contrast, highly proliferative cells in humans, such as those found in ovaries and testes present robust telomerase activity (37-40). Moreover, in order to proliferate indefinitely, cancer cells require a telomere maintenance system which, in the majority of cases is provided by upregulation of *hTERT*.

In addition to the telomerase developmental regulation mentioned above, telomere length regulation also involves the accessibility of individual telomeres to telomerase. In cells with active telomerase, telomere length is in homeostasis, and telomeres are maintained within a narrow size distribution. Teixeira and colleagues (41) recently showed that short telomeres are elongated more frequently than long telomeres, arguing that telomeres switch between two states, one that allows telomere extension and one that does not. It was suggested that the main control at individual telomeres was exerted by the human double stranded telomeric repeat binding factor 1 TRF1 (42) together with the telomeric binding protein POT1 (Protection Of Telomeres 1), which binds and coats the single-stranded DNA at the 3' end (43). POT1, which is recruited by TRF1, is involved in the regulation of telomerase activity at

individual chromosome ends because the 3'end cannot simultaneously bind the protein and the telomerase RNA template. A recent X-ray structure study shows that depending on its location relative to the DNA 3' end, human POT1 can either repress or permit telomerase access (44,45). When hPOT1 binds the very end of the chromosome, the action of telomerase is inhibited. On the contrary, when hPOT1 is bound at a position one telomeric repeat before the 3' end, the telomere is extended.

TELOMERE SHORTENING AND RADIATION SENSITIVITY

Cloning of the mouse telomerase RNA gene, *mTERC*, allowed the generation of telomerase knockout mice deleted for this RNA gene (11). The inbreed strains of *Mus musculus* frequently used for laboratory experiments possess long telomere tracks (40-60 kb of TTAGGG repeats). TERC deficiency leads to viable and fertile animals that suffer progressive telomere attrition with age and in successive mouse inbreed generations. These animals have been propagated using inbreeding crosses for up to six generations. Aged and late generation animals exhibit a wide range of defects, including chromosome end fusions, genomic instability and depletion of highly proliferative tissues (11,46). In this model it is possible to compare the behaviour of cells possessing long telomeres and functional telomerase (mTERC$^{+/+}$), long telomeres and no telomerase activity (young and early generation mTERC$^{-/-}$), and short telomeres and no telomerase activity (aged and late generation mTERC$^{-/-}$). The generation of these telomerase-deficient animals allowed the assessment of the influence of telomere shortening and telomerase deficiency on the outcome of radiation exposure. Two studies carried out simultaneously in two independent laboratories revealed that telomere length importantly affects radiation

sensitivity (47,48). Late generation mTERC$^{-/-}$ mice showed increased mortality when exposed to single acute or fractionated doses of γ-rays. Spleen, bone marrow and small intestine suffered cell depletion in moribund irradiated mTERC$^{-/-}$ animals and apoptosis increased in splenocytes and small intestine of late generation mTERC$^{-/-}$ mice after irradiation (47,48). Cytogenetic analysis revealed higher levels of chromosome aberrations in late generation mTERC$^{-/-}$ than in wild-type primary embryonic fibroblasts exposed to ionising radiation (47,48). Similar increased sensitivity was also reported for doxorubicin, a DSBs-inducing chemical agent (49). Most importantly, as the increased sensitivity to radiation and DSB-inducing chemicals was not observed in early generation mTERC$^{-/-}$ mice, the effects were attributed to telomere shortening rather than telomerase deficiency *per se*. To check whether DNA repair pathways were impaired in telomerase-deficient mice with short telomeres, NHEJ repair efficiency was assayed in the context of V(D)J recombination. It was concluded that NHEJ, the major mechanism involved in repairing DSBs in mammals, was intact in mTERC$^{-/-}$ mice and was not influenced by the functional status of telomeres or by the absence of telomerase (47,48). Similarly, homologous recombination repair was intact in this animal model (47). Thus, the reasons for the increased sensitivity of telomerase-deficient mice with short telomeres remained elusive for some years.

The basis for the synergy between telomere shortening and radiation sensitivity was examined by conducting exhaustive cytogenetic analyses on mTERC$^{-/-}$ primary embryonic fibroblasts using combined telomeric PNA FISH and M-FISH techniques (50). In this study, rather than measuring telomere dysfunction as a reduction of the average telomere length, the individual chromosomes with

227 critically shortened telomeres in each embryo were identified. This was done by
228 measuring the frequency of signal-free ends for each terminus after FISH with
229 telomeric probes. Using this approach, it was concluded that in the irradiated
230 mTERC$^{-/-}$ embryonic fibroblasts, the chromosomes most frequently involved in
231 aberrations coincided with the set of chromosomes with the shortest telomeres.
232 However, the aberrations involving these dysfunctional telomeres were not only
233 end-to-end fusions but also rearrangements resulting from eroded telomeres
234 joined to radiation-induced broken chromosomes. On the basis of the results
235 obtained by Latre and colleagues (50), the following picture was put forward to
236 explain the increased radiation sensitivity of animals with short telomeres: In
237 irradiated cells having normal telomere length, DSBs are the only substrate for
238 end joining reactions and chromosomal rearrangements can only be formed
239 by the joining of two broken ends (DSB-DSB rearrangements). In contrast, in
240 irradiated cells with critically shortened telomeres two types of substrates are
241 available for end joining reactions, uncapped telomeres and DSBs. As a
242 consequence of this, three types of rearrangements can be formed: DSB-DSB,
243 telomere-telomere and telomere-DSB rearrangements (figures 2A and B). Thus,
244 acting as an additional choice for joining, critically short telomeres decrease
245 the fidelity of repairing DSBs induced by radiation all over the genome (figure
246 2C). In other words, the increased sensitivity of cells and animals with
247 dysfunctional telomeres to DSB inducing agents is not a direct consequence of
248 a defect in DNA DSB repair pathways but rather of uncapped telomeres
249 interfering with the proper repair of radiation-induced DSBs.
250
251 The emerging notion that critically short telomeres act as if they were true DSB
252 and activate the DNA damage response was reinforced by studies

demonstrating that shortened telomeres in senescent human fibroblasts are sensed as if they were DSBs. Using a chromatin immnunoprecipitation methodology, d'Adda di Fagagna and co-workers (51) demonstrated that DNA damage response factors form foci on telomeres in senescent human cells. These DNA damage response factors include the phosphorylated histone H2AX (γH2AX), one of the earliest modifications that occur at sites of double-stranded DNA breaks, and other proteins such as 53BP1, MDC1 and NBS1, which are involved in DNA repair as well as in DNA damage checkpoints. On the basis of these findings, d'Adda di Fagagna and co-workers (51) proposed that telomere-initiated senescence reflects a cell-cycle arrest enforced when the DNA damage checkpoint pathway is activated by critically short telomeres. More specifically, Zou and colleagues (52) identified the shortest telomeres in normal BJ foreskin fibroblasts arrested at senescence as those lacking a detectable telomeric FISH signal and demonstrated that probes adjacent to the shortest ends co-localized with γ-H2AX/53BP1 staining. A parallel situation was also observed in T cells and fibroblasts derived from mTERC$^{-/-}$ mice, where it was shown that an important fraction of signal-free chromosome ends in late generation mTERC$^{-/-}$ mice showed co-localization with γH2AX (53). Therefore, it can be concluded that short telomeres activate a DNA damage checkpoint response that is characteristic of cells bearing DNA DSBs. In this context, it is not surprising that uncapped ends due to telomere shortening are not only sensed as, but also act as, if they were true DSBs thus joining to spontaneous and radiation-induced DSBs.

LOSS OF TELOMERIC PROTEINS AND RADIATION SENSITIVITY

279 Telomere function not only depends on telomeric DNA length but also on
280 proteins and factors required for the formation of a functional t-loop structure.
281 Among them, DNA-PKcs is not only involved in constructing functional
282 telomeres but also in the NHEJ pathway. Deficiency in this protein impairs DSB
283 repair and promotes chromosome end fusions with telomeric DNA sequences
284 at junction points, indicating that telomere dysfunction in this environment is not
285 due to erosion of telomeric DNA but rather to the impairment of the telomere
286 structure (32,35,36). Using chromosome orientation FISH (CO-FISH) that allows
287 distinction between telomere-telomere fusions (with two blocks of telomere
288 chromatin at the point of fusion) and telomere-DSB fusions (with only a single
289 block of telomeric sequence at the fusion point), Bailey and co-workers (54)
290 demonstrated the formation of telomere-DSB rearrangements in irradiated cells
291 derived from the *scid* mice. The high frequency of these events in transformed
292 *scid* fibroblasts, which bear a leaky mutation in DNA-PKcs, indicates that
293 telomere dysfunction makes a strong, and previously unsuspected contribution
294 to the characteristic radiation sensitivity associated with DNA-PKcs deficiency
295 (54). A similar although less pronounced situation was observed in primary cells
296 of mice with a null mutation for DNA-PKcs (55). Telomere-DSB fusions with
297 interstitial telomeric signals at the junction point were observed in primary
298 embryonic fibroblasts derived from this DNA-PKcs-deficient mice model,
299 reinforcing the idea that not only eroded but also structurally dysfunctional
300 telomeres can join radiation or spontaneous broken chromosome ends (figure
301 3). It is likely that the reported differences in the extent to which telomere-DSB
302 rearrangements are formed after radiation exposure in DNA-PKcs deficient
303 mice depend on different rates of telomere dysfunction between the two cell
304 types (i.e. transformed scid fibroblasts and primary knockout fibroblasts). This

could reflect differences in telomere metabolism and telomere structure between primary and immortalized cells as recently suggested by Rebuzzini et al (56).

A similar situation was also observed in cells expressing a TRF2 dominant-negative allele, which lacks both the N- and the C-terminal domains and removes endogenous TRF2 from telomeres. Bailey and colleagues (54) demonstrated that telomeres uncapped through TRF2 dominant-negative expression also fuse to DNA double-stranded broken ends forming telomere-DSB chromosome rearrangements. Thus, telomere-DSB fusion is a general property of dysfunctional telomeres and is not linked exclusively to the DNA-PKcs- or telomerase-deficient mouse phenotypes. The notion that the DNA repair machinery of mammalian cells acts on dysfunctional telomeres as if they were true DSBs is reinforced by a study carried out by Takai et al (57). After TRF2 inhibition, through RNAi or a dominant negative TRF2 allele, dysfunctional telomeres become associated with DNA damage response factors, such as 53BP1, γ-H2AX, Mre11 and Rad17 (57).

RELEVANCE FOR RADIATION PROTECTION

The models presented above may explain how radiation sensitivity is linked to telomere dysfunction due to lack of telomeric proteins or to excessive telomeric DNA erosion. From the perspective of radiation protection, the former may contribute together with DNA repair defects to the increased radiation sensitivity of several genomic instability syndromes, while the later could be relevant to the general population. Many in vivo studies support the consensus that telomere length decreases progressively with age in most human somatic

cells due to lack of, or insufficient telomerase activity (58). This inverse correlation between telomere length and age has been reported for skin samples and normal skin fibroblasts (59,60), peripheral blood leukocytes (60), normal colorectal tissue (61), liver (62) and pancreas (63) among other tissues and cell types. Most of these tissues and organs are mitotically active, indicating an outcome of cell divisions on telomere shortening during ageing. The human cell responds to short telomeres just before they acquire a critical length by undergoing cell cycle arrest and/or apoptosis. This growth arrest, termed replicative senescence, requires activation of the $p16^{INK4a}$-Rb or p53-p21 pathways (64-66) and occurs in vitro with increasing population doublings and in vivo with increasing age (67-69). Senescent cells may accumulate with age owing to many factors. These include replicative exhaustion (telomere-dependent senescence) (70-72), DNA damage from endogenous or exogenous sources (73), and inappropriate inactivation of mitogenic pathways (74-76). Thus, the senescence response very likely provides an effective tumour suppressor mechanism whereby cells that possess the potential for neoplastic transformation, enter a state in which they are incapable of further dividing (77).

Accumulation of somatic mutations has long been considered a major cause of cancer. A large body of evidence argues that DNA damage mutations accumulate with age in mammals (78). Moreover, an increase in chromosomal aberrations with age has been observed above the age of 50 in humans. Finally, evidence is accumulating that age-dependent epigenetic alterations of chromatin, such as DNA methylation or histone modifications, are involved in the development of cancer in elderly people as they may change the

357 expression of cancer associated genes (79). All of which points to an overall
358 deterioration in genome integrity with age that might disable the senescence
359 checkpoint, allowing unrestrained growth and subsequent telomere attrition.
360
361 The ability of different cell types to spontaneously overcome senescence differs
362 by several orders of magnitude. In skin fibroblasts, the frequency of
363 spontaneous emergence is $<10^{-9}$ while in mammary fibroblasts it is 10^{-7} (80).
364 Normal human mammary epithelial cells (HMEC) derived from mammary
365 cosmetic reductions can emerge from the first growth plateau at 10^{-4}-10^{-5} (80),
366 two to four orders of magnitude more frequently than fibroblasts. HMECs at this
367 second proliferation phase have lost expression of p16 due to promoter
368 hypermethylation. During this extended growth phase telomere lengths
369 become extremely short and cells exhibit highly unstable genomes (80). In
370 striking parallel with the telomerase knockout model, the chromosome arms
371 carrying the shortest telomeres in HMECs emerging from senescence are
372 specific for each donor and coincide with those involved in telomere-telomere
373 fusions (81), thus indicating that telomere dysfunction drives genomic instability
374 in HMECs.
375
376 From the radiation protection perspective, it may not be surprising that, in a
377 similar way to the situation observed in mTERC-deficient mice, uncapped
378 telomeres in human epithelial cells might join radiation induced DNA DSBs thus
379 increasing radiation sensitivity of cells with dysfunctional telomeres. In fact,
380 HMECs from late population doublings are more sensitive to radiation exposures
381 than those derived from early population doublings (Soler et al, unpublished
382 results). To add further relevance to these results, silencing of $p16^{INK4a}$ has been

observed as a common event in normal breast specimens (82). These cells were found in discrete foci in a substantial fraction of women with no indication of or predisposition for breast cancer (82). Therefore, it remains to be determined whether loss of cell cycle checkpoints in human cells with age renders accumulation of short telomeres susceptible to join radiation induced DSBs in tissues of elderly people. If so, it could have consequences on radiation sensitivity associated with age in humans because radiation exposure may more readily compromise their normal tissue function.

CONCLUSIONS

An increase in somatic mutations and chromosome aberrations has been documented in aged cells of both humans and mice (78, 83-86). This increase presumably relates to cumulative lifetime exposure to DNA damaging agents, as well as to an accumulation of mutations resulting from proof-reading and mismatch errors during DNA replication. This process might be exacerbated by a diminished efficiency of NHEJ repair pathways with age (87). Besides the above-mentioned elements, telomere function now emerges as an additional factor potentially contributing to increasing the sensitivity of aged human cells to endogenous and exogenous DNA damaging agents. Since eroded or structurally impaired telomeres are sensed as and act as DNA DSBs they can interact with themselves or with existing chromosome DSBs in the cell, thus sharply increasing the possibility of mis-rejoining and revealing itself as an important factor that definitely contributes to genomic instability. The basis underlying radiation sensitivity, although still not clearly elucidated, has traditionally been linked to impaired DNA damage responses. In the light of the

409 recent works it is clearly shown that radiation sensitivity is not only linked to
410 malfunction or lack of DNA repair proteins but also to malfunction or lack of
411 proteins involved in the correct metabolism and capping of telomeres or to a
412 combination of both. In this new scenario the connexion between telomere
413 function and metabolism and DNA repair mechanisms emerges as an
414 important notion, where telomere impairment strongly contributes to the
415 radiation-sensitive phenotype. The fact that telomere dysfunction can also be
416 produced by the sole act of cell ageing might give radiation sensitivity a
417 temporal aspect: shortened telomeres in aged cells may potentially increase
418 radiation sensitivity in elderly organisms.

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FIGURE LEGENDS

Figure 1. A and B: Proteins that directly bind the telomeric DNA include the TTAGGG Repeat Factors 1 and 2 and POT1. TRF1 and TRF2 bind the double-stranded telomeric DNA as homodimers. POT1 (Protection of Telomeres 1) is known to bind the single-stranded telomeric DNA and plays an important role in regulating access of telomerase to telomeres (see The Telomere Functional Structure section). This figure also shows some DNA-repair proteins that do not directly bind to telomere strands but which are known to interact with TRF1 or TRF2. Some of the DNA repair proteins found at the telomere repeat binding factor 1 (TRF1) (**A**) and 2 (TRF2) (**B**) complexes are shown: Ku86, a repair protein belonging to the NHEJ repair pathway, interacts with TRF1 and, in turn, is able to directly interact with DNA-PKcs (the catalytic subunit of the DNA Protein Kinase), thus locating certain components of the NHEJ machinery at the telomere. Lack of these proteins generates telomere dysfunction and telomere fusions (see Loss of Telomeric Proteins and Radiation Sensitivity section). Other proteins involved in DNA repair such as ATM (Ataxia Telangiectasia Mutated), WRN (Werner protein) and the MRN complex (formed by MRE11, Rad50 and NBS1) are known to interact with the TRF2 homodimer. Lack of any of these proteins produces a telomere dysfunction phenotype (see The Telomere Functional Structure section). **C**: Telomere in t-loop conformation. The double-stranded telomeric DNA is sheltered with the TRF1 and 2 homodimers. The single-stranded array of TTAGGG repeats at the 3' end (in blue) invades the double stranded telomeric DNA, thus forcing the double strand to loop back on itself, forming the T-loop. At the point of invasion, TRF2 binds the double stranded tract and helps to stabilize the loop. The single-stranded G-rich 3' end

remains protected inside the loop structure. POT1 protein binds and coats the displaced G-rich telomeric single strand as well as the G-rich 3' ending invader strand.

Figure 2. A. Representative examples of the three types of chromosome rearrangements observed in irradiated telomerase-deficient mouse embryonic fibroblasts. In the top image, a reciprocal translocation between chromosomes 6 and 16 is shown. These two rearranged chromosomes derive from two breaks followed by misjoining of the broken ends. The image in the middle shows a telomere-telomere fusion between chromosomes 4 and 12, both of them with uncapped telomeres. No breaks occurred to produce this aberration. The lower images correspond to two rearrangements of the telomere-DSB type derived from a single break in chromosome 4. On the right, the broken chromosome 4 centric fragment joined to an uncapped chromosome 12 with eroded telomeres giving rise to a dicentric chromosome (4;12). On the left the broken chromosome 4 acentric fragment joined to an uncapped chromosome 19, producing a translocation. **B.** Diagram showing the frequencies of radiation-induced chromosome aberrations in telomerase-deficient and wild type primary embryonic fibroblasts. Total frequency of aberrations is higher in the mTerc$^{-/-}$ fibroblasts, the difference being due to telomere-DSB rearrangements; frequencies of DSB-DSB rearrangements are similar in the two environments and telomere-telomere fusions were also observed with the same frequency in non-irradiate mTerc$^{-/-}$ fibroblasts. **C.** Model explaining the increased radiation sensitivity of cells with short dysfunctional telomeres. Given a radiation-induced break in a chromosome, the probabilities of correct rejoining are dramatically different in cells with normal and with critically shortened telomeres. In the

former, the broken chromosome can only join to another broken end, with high probabilities of reconstituting the original chromosome if the number of induced DNA breaks was low. On the contrary, in cells with short telomeres, there may be several unprotected chromosome ends to which the broken end can join, thus making the restoration of the original chromosome much more unlikely.

Figure 3. Different types of chromosomal rearrangements in irradiated DNA-PKcs-defective/deficient cells. **A**. **DSB-DSB rearrangements**: SKY image shows several exchange-type rearrangements: arrowheads point to conventional dicentrics and translocations which follow the typical two-colour pattern. **B**. **Telomere-DSB rearrangements**: SKY-FISH image of a rearranged chromosome: on the left, the stained pattern of the chromosome; in the middle the reversed DAPI image; and the pseudocoloured image on the right. The SKY analysis reveals that chromosome 19 (green in the pseudocolour image) is translocated with chromosome 7 (brown) at the centromere level. The same rearranged chromosome can be observed inside the circle after telomeric FISH labelling. Three pairs of telomeres can be observed in this chromosome: the broken centromere of chromosome 7 has fused with the centromeric telomeres of chromosome 19. Because the telomeric signals are bright and clear, this DSB-to-telomere fusion cannot be attributed to telomere shortening but rather to telomere dysfunction. **C. Telomere-telomere fusions**: Telomeric FISH image where two chromosomes have fused at the telomere level (*). Note that FISH signals from dysfunctional fused telomeres are brighter than those not fused, thus reinforcing the idea that none of the telomeres involved in the fusion have suffered attrition.