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Role of PML in telomere metabolism of normal and cancer cells.

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GLOSSARY

ALT: Alternative Lengthening of Telomeres
APB: ALT associated PML body
APL: Acute Promyelocytic Leukemia
ATM: Ataxia Telangiectasia Mutated
ATR: Ataxia Telangiectasia and Rad3 related
BLM :Bloom's syndrome protein
BSA: Bovine serum albumine
CBP: CREB-binding protein
CDKs: Cyclin-Dependent Kinases
ChK: Checkpoint Kinase
CK2: Casein Kinase-2
CMV: Cyto Megalo Virus
DAPI: 4',6-diamidino-2-phenylindole
DBMA: 7,12-dimethylbenzanthracene
DC: Dyskeratosis congenital
DDR: DNA damage response
DMEM: Dulbecco's Modified Eagle Medium
DNA-PK: DNA dependent Protein Kinase
DNA-Pkcs: DNA dependent Protein Kinase catalytic subunity
DNMT: DNA methyltransferase
DSB: DNA Double Strand Break
ECTR: Extra-Chromosomal Telomeric Repeats
ERCC1: Excision Repair Cross-Complementing 1
ERK: Extracellular signal-Regulated Kinase
FBS: Fetal Bovine Serum
FISH: Fluorescence In Situ Hybridization
G4: G-quadruplex
HDAC: Histone Deacetylases
HP1: Heterochromatin Protein 1
HR: Homologous Recombination
KO: Knock Out
MEFs: Mouse Embryonic Fibroblasts
MRE11 mRNA Meiotic Recombination 11 messenger RNA
MRN complex: Mre11-RAD50-NBS1 complex
NB: Nuclear Body

NBS1: Nijmegen Breakage Syndrome 1
NHEJ: Non-Homologous End Joining
NLS : Nuclear Localization Signal
ORC: Origin Recognition Complex
PARP : Poly-ADP Ribose Polymerase
PIKK : Phosphatidil Inositol 3- Kinase-like Kinase
PML : Promyelocytic Leukaemia protein
PML NBs: PML Nuclear Bodies
PNA: Peptide Nucleic Acid
POT1: Protection Of Telomeres 1
RA: Retinoic Acid
RAP1: Repressor/Activator Protein 1
RAR: Retinoic Acid Receptor
RB: Retinoblastoma protein
RBCC: RING finger, B boxes, Coiled-coil
RHPS4: 3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl] acridinium methosulfate
RNP: RiboNucleoProtein
RPA: Replication Protein A
RT: Reverse Transcriptase
SAHFs: Senescence Associated Heterochromatin Foci
SCID mice: Severe Combined Immunodeficient mice
shRNA: short hairpin RNA
SMC: Structural Maintenance of Chromosomes
SUMO: Small Ubiquitin-like Modifier
TEBP: Telomere End Binding Protein
TER: Telomerase RNA subunity
Terc: Telomerase RNA Component
TERT: Telomerase Reverse Transcriptase
TIFs: Telomere Dysfunction- Induced Foci
TIN2: TRF1-Interacting Nuclear factor 1
TPA: 12-O-tetradecanoylphorbol-13-acetate
TPE: Telomere Position Effect
TPP1: (PIP1/PTOP/TINT1)
TRF: Telomere Repeat binding Factors

WRN: Werner's syndrome protein
WT: Wild Type
XPF:RNA-Polymerase σ Factor

SUMMARY

Telomeres are specialized structures that cap chromosomes ends, protecting them from degradation and processing by the DNA-repair machinery. In normal cells, telomeres become progressively shorter during replication, and this leads to replicative senescence. The majority of human tumors (approximately 90%) express the ribonucleic complex telomerase, that prevents this continuous DNA loss, thus endowing cells with immortal growth properties. In the remaining 10% of tumors telomerase is not expressed, and telomeric DNA is preserved through alternative mechanisms (Alternative Lengthening of Telomeres, ALT). An hallmark of all ALT tumor cell lines is the presence of nuclear structures that localize exclusively at telomeres (ALT-associated PML nuclear bodies, APBs). Here we show that PML (ProMyelocitic Leukemia protein), a nuclear protein that is an essential component of PML nuclear bodies (PML-NBs), colocalizes with telomeres not only in telomerase-positive tumor cell lines, but also in normal cells, although at few telomeres. Interestingly, in normal cells telomere-specific DNA-damage induced recruitment of PML to damaged telomeres. Furthermore, PML depletion led to the formation of Telomere Dysfunction-Induced Foci (TIFs) and consequent growth inhibition in normal and ALT cell lines. Preliminary cytogenetic characterization of PML-depleted fibroblasts revealed diffused genomic defects, suggesting that PML is a key regulator of telomere maintenance and whole genomic stability. Unpublished data from our lab have shown that DNA-PKcs, an enzyme involved in DNA repair and telomere stability, is part of the PML-macromolecular protein complex. To investigate the mechanism involvement of PML activity in telomere maintenance we studied by confocal microscopy its interaction with the active phosporylated form of DNA-PKcs in ALT cell lines and in normal cells. As expected, not only the two proteins interacted at telomeres, but PML was essential for the correct localization of DNA-PKcs at the chromosome ends. Additionally, telomeric-specific damage in normal cells increased the interaction between DNA-PKcs and PML. Altogether these findings shed light on a

novel role for PML in the regulation of telomere metabolism, and are the basis for future investigations on its role in both aging and cancer.

INTRODUCTION

1. Telomeres

The evolution of linear genomes required the development of mechanisms that could protect the chromosome ends from genomic instability and DNA loss. Chromosomes ends, in fact, are subjected to enzymatic attacks by the systems that cells developed to detect and repair DNA breaks. To this end, specialized structures named telomeres (from the Greek word 'end part') have evolved at the chromosome ends of linear genomes to cap and protect these regions.

Moreover, when scientists began to understand the mechanism of DNA replication, they predicted that the lagging strand of linear chromosomes copied by the semi-conservative replication machinery could not be fully replicated. As it turned out, this can be explained by what James Watson in 1972 named the "end replication problem" (Watson, 1972). The DNA polymerase works in a 5' to 3' direction and replicates the leading strand completely. On the contrary, the lagging strand is replicated in short fragments, called Okazaki fragments, by using RNA primers as templates. These RNA primers are then converted to DNA, with the exception of the last RNA primer, at the 5' end of the lagging strand. Thus, a section of DNA is lost during each cycle of replication. The extent of this loss is estimated to be around 50-150 bp (Makarov et al., 1997) of DNA at each round of cell cycle. In 1973, A.M. Olovnikov postulated the existence of 'telogenes', sequences of DNA that carry no genetic information located at the ends of the DNA molecules, that would shorten during each mitotic arrest cycle until they reached a critical level, that resulted in the arrest of cell division (Olovnikov, 1973). Subsequently, Elizabeth Blackburn discovered in Tetrahymena that telomeres consist of a simple repeated DNA sequence (Blackburn and Gall, 1978), and, together with Jack Szostak, was able to demonstrate that these telomeric repeats protect the chromosomes from degradation

(Szostak and Blackburn, 1982). For this discovery they received the 2009 Nobel Prize in Physiology or Medicine.

Telomere erosion is considered to be a mitotic clock, as it regulates the number of divisions that a given cell can undergo before entering a senescent state (Smith and Whitney, 1980).

1.1. Telomere Structure

1.1.1 The telomere sequence.

Telomeres consist of G-rich DNA repeats (irregular TGGG repeats in yeast, regular TTAGGG repeats in vertebrates) capped by complexes of specialized proteins (de Lange et al., 1990).

The length of these telomeres varies amongst different organisms and even in the same organisms between cells with different origin. For example, yeast telomeres are about 300 bp long, whereas in human cells the telomere length spans between 3 and 20 kb, mice have long telomeres that can reach up to 150 kb (Moyzis et al., 1988).

Telomeres in vertebrates end in a 3' overhang of the G-rich strand (G-strand overhang) (Wright et al., 1997), that spans between 50 and 400 nt and can fold back and invade the double-stranded region of the telomere, generating a looped structure known as the telomere loop or T-loop (Griffith et al., 1999) (Fig.1) that protects chromosome ends. It still has to be demonstrated whether T-loops are present at all chromosome ends, whether their function regards solely chromosome protection, or whether they have a role in regulating other features of telomeres, like the recruitment of the telomere-specific reverse transcriptase enzyme, known as telomerase (see following paragraphs). T-loops have been found at telomeres of humans, mice, trypanosomes and plants. The 3' G-rich overhang can also fold into a 4-stranded DNA structure, termed G-quadruplex (G4) (Williamson, 1994). G4

formation has been shown to inhibit telomere elongation in vitro, by inhibiting the activity of the telomerase enzyme (Han and Hurley, 2000).

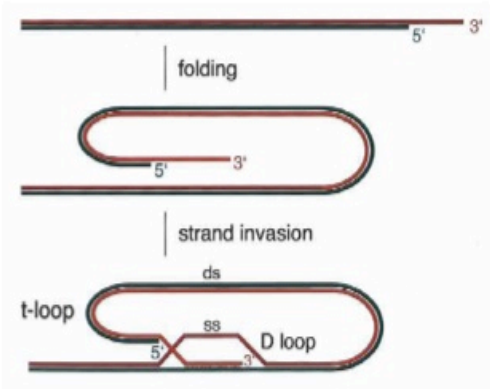

Figure 1. The T-loop structure. The 3' overhang is strand-invaded into the adjacent duplex telomeric repeat array, forming a T-loop. (de Lange 2005)

Adjacent to the human terminal (TTAGGG)n repeat is a complex region of segmentally duplicated DNA tracts, generally referred to as subtelomeric DNA repeats. This class of low-copy DNA repeats is characterized by very high sequence similarity (90% to 99.5%) between duplicated tracts, and very large and heterogeneous duplicated segments (1 kb to > 200 kb). Some of the segmental duplications are unique to subtelomeric repeat regions, some are shared with a subset of pericentromeric repeat regions, and some are shared with one or several interstitial chromosomal loci.

1.1.2. The shelterin complex.

Telomeric repeat sequences are recognized by a specific set of sequence-and structure-specific DNA-binding factors.

For example, the shelterin complex, composed of six proteins, is considered to be strictly telomere specific and necessary to protect human telomeres (de Lange, 2005): the shelterin components, in fact, are specific for telomere DNA, they localize at chromosome ends and are expressed throughout the cell cycle.

Two shelterin subunits, the Telomere Repeat binding Factors TRF1 and TRF2, bind with high specificity the double-stranded TTAGGG repeats of the telomere, with their SANT/Myb-type DNA-binding domain (Court et al., 2005) (Fig.2). These proteins form homodimers and higher order oligomers. TRF1 can bind neighbouring telomeric sequences, but can also bridge two different DNA molecules or distant regions of the same telomere (Griffith et al., 1998). The amount of TRF1 in telomeres can be linked to the telomere length and this protein is considered the main regulator of telomere length (Smogorzewska et al., 2000). Accordingly, a dominant negative mutant of TRF1 causes telomere elongation in telomerase-positive cells, while TRF1 overexpression causes gradual shortening of telomeres (Karlseder et al., 2003).

Structural differences between TRF1 and TRF2 are responsible for their different functions. While TRF1 is mainly involved in telomere length control, TRF2 is involved in telomere protection (Blasco, 2005a; van Steensel et al., 1998). In particular, TRF2 stabilizes the G-strand overhang and promotes telomere looping (Stansel et al., 2001). Inhibition of TRF2 function results in telomere fusions, as consequence of the covalent bond between G-strands of different chromosomes and is dependent on non-homologous end-joining (NHEJ)(Smogorzewska et al., 2002) TRF2 also localizes at double-stranded breaks of non-telomeric DNA, suggesting a more general role for this protein in DNA damage response (Bradshaw et al., 2005).

A third shelterin protein, POT1 (Protection Of Telomeres 1), directly recognizes TTAGGG repeats, but specifically binds to single-stranded telomere overhangs (Lei et al., 2004) (Fig.2). POT1 is considered a chromosome end-capping protein. Becuase POT1 localizes just two nucleotide from the end of the duplex

telomeric DNA in the 3' overhang, it is reasonable to assume that this protein may protect the 5' end of telomeric DNA. Loss of the G-overhang causes a reduction in the binding of POT1, and leads to aberrant regulation of telomere length. Depending on its localization on the single-stranded overhang, POT1 can either facilitate or inhibit the telomerase activity (Lei et al., 2005). POT1 deficiency causes the activation of a DNA damage checkpoint and deregulated episodes of homologous recombination at the telomere (Wu et al., 2006).

TRF1, TRF2 and POT1 are interconnected by three additional shelterin proteins, TIN2, TPP1 and RAP1 (Fig.2). TIN2 (TRF1-Interacting Nuclear factor 2) provides a scaffold for the binding of other telomeric proteins. TIN2 is though to recruit TRF1 and TRF2 and stabilize TRF2 on telomeres. TIN2 also interacts with TPP1 (PIP1/PTOP/TINT1) that links the TRF1-TIN2-TRF2 complex with
POT1 (Liu et al., 2004; Ye et al., 2004). These functions make TIN2 a key regulator in keeping telomeric proteins together, and, because of its function in mediating the TRF1 complex, they identify it as negative regulator of telomere length.

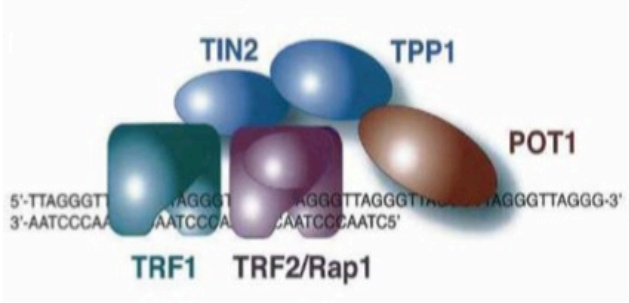

Figure 2. The shelterin complex. TRF1 and TRF2 bind the double-stranded repeats of the telomere. POT1 binds to single-stranded telomere overhang. TIN2, TPP1 and RAP1 are scaffold proteins that interconnect TRF1, TRF2 and POT1. (de Lange 2005)

TPP1 recruits POT1 to the TIN2/TRF1 complex. Its structure reveals two protein-protein interacting domains, a central region that has affinity for POT1, and a C-terminus for the binding to

TIN2. TPP1 acts as a negative regulator of telomere length, and its depletion causes telomere elongation (Ye and de Lange, 2004).

Rap1 (Repressor/Activator Protein) is a telomere specific protein that acts as negative regulator of telomere length. Rap1 is connected to the telomere complex through protein-protein interaction with TRF2 (Li and de Lange, 2003; O'Connor et al., 2004). Rap1 has been isolated in a complex with TRF2 and other proteins involved in homologous and in non-homologous recombination (RAD50, MRE11, Ku70/86 and PARP)(O'Connor et al., 2004).

1.1.3. Other telomeric proteins.

In addition to shelterin, other factors interact dynamically with mammalian telomeres and influence the stability of chromosome ends. Many of these proteins are involved in DNA recombination and repair. The DNA repair factors associated with telomeres are members of the MRN complex [namely MRE11 (Meiotic Recombination 11), RAD50 and NBS1], involved in DNA double-strand breaks repair, DNA-PK (DNA dependent Protein Kinase, that comprises a catalytic subunit DNA-PKcs and the regulatory subunits Ku70 and Ku80), ATM (Ataxia-Telangiectasia Mutated) and ATR (ATM and Rad3-related). DNA processing enzymes at telomeres are the RecQ-like helicases WRN (Werner's syndrome protein) and BLM (Bloom's syndrome protein), ERCC1/XPF (Excision Repair Cross-Complementing 1/ RNA-Polymerase σ Factor) and the 5' exonuclease Apollo. Tankyrase 1 and 2 and PARP (Poly ADP-Ribose Polymerase) also associate with telomeres (de Lange, 2005; Verdun and Karlseder, 2007)(Fig.3). Proteins that are part of the shelterin complex like TRF1, TRF2 and POT1, regulate the access and the activity of the different enzymes mentioned above. For example, TRF2 and POT1 modulate the recruitment of WRN and BLM, required for an efficient telomere replication. TRF2 can sequester ERCC1/XPF in

an inactive form, and following TRF2 inhibition, these enzymes are involved in the removal of the G-tail (Zhu et al., 2003).

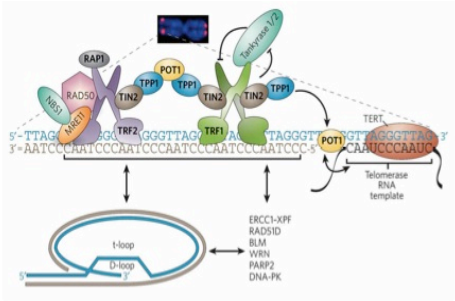

Figure 3. The mammalian telomeric complex. Specific protein complexes bind to the double- and single-stranded telomeric DNA. The component of the shelterin complex are shown in bold text. Other factors that can interact with telomeres are listened. Bidirectional arrows indicate interactions. (Verdun and Karlseder 2007)

Figure 3. The mammalian telomeric complex. Specific protein complexes bind to the double- and single- stranded telomeric DNA. The component of the shelterin complex are shown in bold text. Other factors that can interact with telomeres are listened. Bidirectional arrows indicate interactions. (Verdun and Karlseder 2007)

1.2. Replication of telomeres.

A major question in telomere biology regards their replication, especially concerning their replication origins. In yeast, the origin of replication is not within the telomeric repeats, but in subtelomeric regions (Wellinger et al., 1993). In mammals, although their sequence is not encoding for any protein, we cannot exclude the possibility that telomeres can be used as replication origins. In particular, TRF2 could contribute to the formation of origins within telomeres, because of its extra-telomeric role in the initiation at the oriP origin of the Epstein-Barr DNA virus (Deng et al., 2002). Recent findings report the possibility of a direct recruitment of the Origin Recognition Complex (ORC) by TRF2 and subsequent TRF2-mediated opening of the double helix

(Amiard et al., 2007). In yeast, the replication of telomeres occurs in late S phase; conversely, Verdun and Karlseder have shown that mammalian telomeres replicate in two moments during cell cycle: in S phase and in G2-M phase (Verdun and Karlseder, 2006). It has been postulated that the late replication of the telomeric regions might have a role in coordinating cell-cycle events. Replication of telomeres is a complex process, due to the G-rich and repetitive nature of telomeric DNA; this property of telomeres, in fact, allows for the formation of secondary structures, such as G-quadruplexes and T-loops or other structures that have only been observed in vitro (triple helices, four-way junctions and D-loops). These structures hinder the progression of the replication fork, which consequently stops or stalls at telomeres. When this happens, the cell activates an ATM/ATR-dependent DNA-damage response, mediated by the MRE11-RAD50-NBS1 complex, which is involved in the initial recognition of double-strand DNA breaks. The single-stranded DNA-binding protein RPA (Replication Protein A) also appears at telomeres in S phase (Verdun and Karlseder, 2006). This and other telomeric proteins are fundamental for an efficient telomere synthesis, because they mediate the recruitment of helicases and possibly other co-factors that are capable of removing or remodelling the telomeric structures that can impair fork progression. Accordingly, in fission yeast, the homologues of TRF1 and TRF2, Taz1 are crucial for the efficient replication of telomeres . In human cells, the RecQ-like helicases WRN and BLM are important during replication of telomeres. WRN localizes to telomeres in S phase and is involved in removing the secondary structures that can be formed in the G-rich strand (Crabbe et al., 2004). The fine recruitment of WRN and BLM is performed by TRF2 and POT1. When the fork approaches the T-loop, there is an accumulation of superhelical stress that counteracts the action of topoisomerases. In this scenario, the binding of TRF2 to positive supercoils is energetically favoured, so that this protein is enriched around the fork and acts as a topological stress sensor (Amiard et al., 2007). The high

concentration of TRF2 favours the opening of the T-loop by activating the RecQ-helicases. TRF2 can therefore promote the T-loop formation or resolution, depending on the different subcomplexes in which it takes part during the cell cycle.

After telomere replication, the telomere overhangs are generated. For the leading telomere, the product of the replication is either blunt or 5' protruding, which implies the existence of a 5' resection activity to convert it into a 3' overhang. On the contrary, the newly generated lagging-strand carries a short 3' overhang, resulting from the removal of the most distal RNA primer used for the synthesis of Okazaki fragments. It is not clear whether this primer is placed at the very end of the chromosome or a few bases from the end, allowing a longer overhang than the length of the RNA fragment (Fig.4).

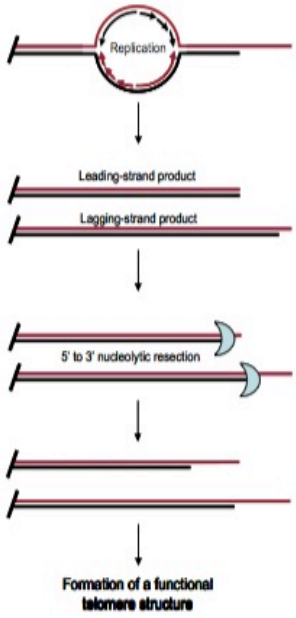

Figure 4. End replication and processing. Semi-conservative replication of telomeres generates a blunt- ended leading-strand product and a lagging-strand product with a short overhang. Nucleolytic digestion in the 5' to 3' direction then generates G overhang.

Whether a 5' resection activity also processes the lagging telomere is still unknown. Currently, no candidates for overhang-generating nucleases have been identified, but the 5' resection activity seems to involve the MRE11-RAD50-NBS1 complex. In Mre11-defective yeast strains, in fact, the length of the G-tail is reduced (Larrivee et al., 2004). However, G-tails are still present in cells that express nuclease-dead alleles of Mre11, suggesting that the nuclease activities of MRE11 are not strictly necessary for telomeric 5' resection.

1.3. Telomere elongation and Telomerase.

Telomeric DNA inexorably shortens during replication until it reaches a critical length, thus triggering cell cycle arrest and onset of senescence. As exception to this mechansism, germ cells, some populations of stem cells and cancer cells, activate compensating mechanisms to preserve genome integrity and telomere functions and continue to divide. These mechanisms can involve recombination events, but they are more commonly based on the extension of the 3' ends by telomerase. Telomerases are specialized reverse transcriptase (RT) complexes that catalyze the de novo extension reaction at the absolute ends of chromosomes. Active telomerases have been detected in extracts of human cells in early embryogenesis, in the germline, in a subset of epithelial and lymphoid progenitors and in almost all types of cancer (Collins and Mitchell, 2002; Forsyth et al., 2002). Inactivation of telomerases in human somatic cells has been proposed to be required for quiescence, differentiation and cell death and it is supposed to be a tumour-suppressor mechanism (Sharpless and DePinho, 2004; Shay and Wright, 2005; Wong and Collins, 2003).

The catalytic function of telomerases requires the activity of a RNA subunit, called TER, and the protein telomerase reverse

transcriptase, TERT. In fact, TER contains aninternal RNA molecule that is used as a template for the synthesis of telomeric repeats (Yu et al., 1990). The human TER primary transcript is synthesized by RNA polymerase II, and assembled to form a RNA motif (H/ACA motif), which contains conserved sequences and hairpins. H/ACA proteins assemble with this motif, to generate a stable ribonucleoprotein (RNP) (Mitchell et al., 1999) (Fig.5).

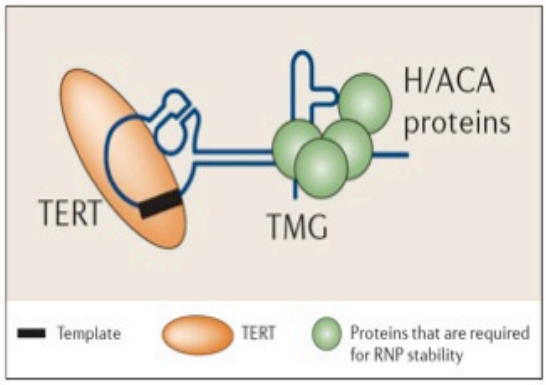

Figure 5. Telomerase holoenzyme. Ribonucleoprotein (RNP) consists of TER transcript assembled with H/ACA proteins, and contains a RNA molecule that can be used as a template. TERT binds the RNP to form the holoenzyme. (Collins 2006)

TERT harbours a central region that shares homology with the active-site motifs of the viral RT enzymes. TERT binds the RNP to form the telomerase holoenzyme (Fig.5). Additional holoenzyme proteins are required to endow the enzyme with the ability to function on chromosome substrates. TERT, TER and single-stranded DNA form a network of protein-nucleic acid interactions that orchestrate the proper positioning of the template and primer in the active site. After the addition of several repeated DNA sequences, the last event of the 'telomerase cycle' is the dissociation of the telomerase-RNA-telomeric-DNA hybrid (Collins, 2006) (Fig.6).

The interaction between telomerase and telomeres is a finely regulated mechanism, that ensures that telomerases acts on short

telomeres, but not on those that do not require telomere elongation, thus maintaining the telomere at an appropriate length. The accessibility of the G-overhang to telomerase is probably not dependent solely on the length of the telomere itself, but also on the phase of the cell cycle (Shore and Bianchi, 2009). The replication reaction opens the telomere to enzymes and proteins that generate a structure that allows for telomerase-mediated elongation. In budding yeast, telomerase activity is restricted to the S phase and is triggered by DNA replication (Marcand et al., 2000).

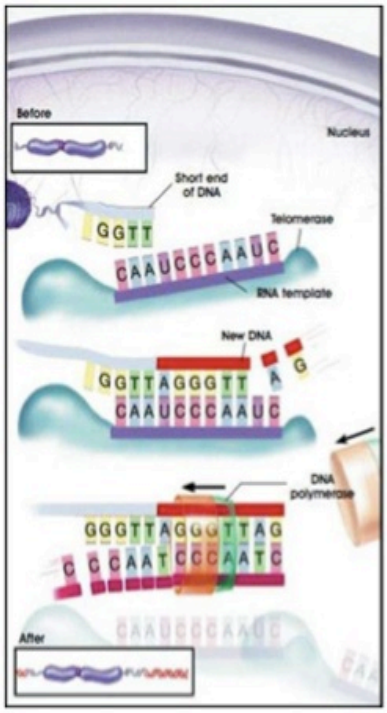

Figure 6. Telomerase extends the length of a telomere. Telomerase is an enzyme that lengthens telomeres by adding on repeating sequences of DNA. Telomerase binds to the ends of the telomere via an RNA template that is used for the attachment of a new strand of DNA. Telomerase add several repeated DNA sequences then releases and a second enzyme, DNA Polymerase, attaches the complementary strand of DNA completing the double stranded extension of the chromosome ends.

As for elongation of short telomers in several systems, the existence of a mechanism that senses the length of telomeric DNA and reduces the telomerase-dependent extension when telomeres exceed a certain length has been proposed. In human cells this negative feedback mechanism involves TRF1, which controls telomere length by inhibiting the activity of telomerases at individual telomeres (van Steensel and de Lange, 1997). Accordingly, overexpression of TRF1 leads to telomere shortening, whereas expression of a dominant-negative allele leads to telomere elongation. The T-loop structures also block telomerase access and the shelterin complexes control telomere length by regulating the transition from a closed to an opened T-loop conformation.

1.4. Alternative Lengthening of Telomeres mechanisms (ALT).

Although telomerase activity appears to be the most prevalent mechanism used by tumours to circumvent the telomere length-dependent barrier to proliferation, this enzyme is not activated in a subset of tumours and in some tumour-derived cell lines or in vitro immortalized cell lines (Bryan et al., 1995; Reddel et al., 1997). To prevent telomere shortening, however, these cells adopt a mechanism termed Alternative Lengthening of Telomeres (ALT), which relies on recombination (Henson et al., 2002).

Evidence for ALT activity has also been found in the tissues of late generation telomerase-null mice (Herrera et al., 2000). The ALT mechanism has precise features. A characteristic of ALT cells is the high heterogeneity of their telomere length, with repeats ranging in size from >20Kb to <5Kb. Visualization of telomeres by fluorescence in situ hybridization (FISH) shows that the heterogeneity in telomere length in ALT cell populations can be explained by the heterogeneity that exists within individual cells.

In fact, whereas some chromosome ends have no detectable telomeric sequence while others have very long telomere signals (Perrem et al., 2001). Another peculiar feature of ALT cells is the presence of ALT-associated PML bodies (APBs) (Yeager et al., 1999). PML (Promyelocytic Leukaemia Protein) is involved in many biological functions, and aggregates with other proteins in nuclear structures called PML nuclear bodies (PML NBs) (see introduction). APBs are PML NBs with ALT-specific contents. In addition to constitutive components of PML NBs, they also contain telomeric DNA, telomeric binding proteins (TRF1 and TRF2) and proteins involved in DNA recombination, replication and repair. These proteins include RAD51, RAD52 and RPA (Yeager et al., 1999), RAD51D (Tarsounas et al., 2004), BLM (Yankiwski et al., 2000), WRN (Johnson et al., 2001), Rap1 and BRCA1 (Wu et al., 2003), MRE11, RAD50 and NBS1 (Wu et al., 2000), ERCC1 and XPF (Zhu et al., 2003), hRAD1, hRAD9, hRAD17 and hHUS1 (Nabetani et al., 2004), Rif1 (Silverman et al., 2004) and hnRNP A2 (Moran-Jones et al., 2005). APBs appear as disc or ring shaped structures and they binding proteins that they contain is often greater than the amount present at individual telomeres. APBs were often detected by visualizing TRF1 or TRF2 within a PML NB (Fig.7), and the signal of TRF1 or TRF2 is larger and brighter than the signals from individual telomeres. They are present in only about 5% of interphase cells (Yeager et al., 1999) but the percentage increase consistently in late G(2)/M phase of the cell cycle (Grobelny et al., 2000).

The proportion of APB-positive cells in a given population can be increased from <5 to >50% by restriction of methionine (Jiang et al., 2007), an essential amino acid. Methionine restriction, in fact, causes cell-cycle arrest in G0/G1 phase. Methionine has a role as a methyl donor for methylation of DNA, RNA and proteins, so methionine restriction could reduce the levels of methylation at telomeric and subtelomeric regions, creating a favourable environment for the assembly of APBs.

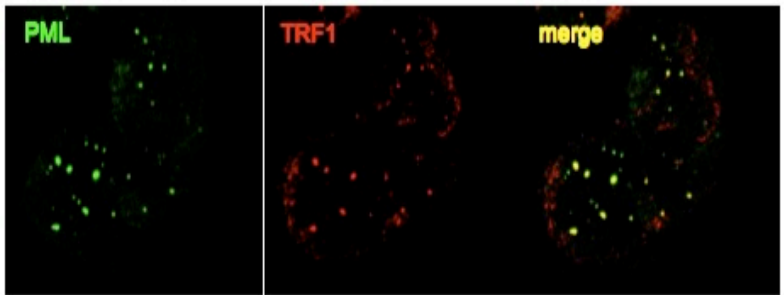

Figure 7. ALT-associated PML bodies (APBs). ALT cells contain a novel form of PML body in which PML co-localizes with telomeric DNA and telomeric binding proteins. Indirect immunofluorescence in U2OS ALT cells shows the co-localization of TRF1 (red) with PML protein (green) in large subnuclear structures.

Under these conditions, RNA interference of proteins of APBs has helped in establishing that PML, Sp100, TRF1, TRF2, TIN2, Rap1, MRE11, RAD50, NBS1 and 53BP1 are required for APBs formation (Jiang et al., 2007).

Since they are present only in ALT-postive cells, APBs can be used as marker for ALT, and it has been proposed that they might have a role in the ALT mechanism itself. One hypothesis is that APBs may focus, localize, or modify proteins that are required for the ALT mechanism. Another possibility is that they are involved in removing products of the ALT process, or that APBs have the function to repair telomeric DNA. However, a recently published work shows that APBs can be found in growth arrested/senescent cells and cells with "closed" telomeric chromatin, which is likely to repress recombination, suggesting that there is no simple correlation between the levels of ALT activity and the number of APBs or APB-positive cells. In this work, activation of p53 in p53-negative ALT cells induced up-regulation of p21 and caused senescence, accompanied by the increase of APB formation. Moreover, three members of the heterochromatin protein 1 (HP1) family, involved in chromatin silencing, have been found in APBs (Jiang et al., 2009). The SMC5/6 complex, member of the

Structural Maintenance of Chromosomes (SMC) and required for DNA damage repair (Lehmann et al., 1995), localizes to APBs. The SMC5/6 component MMS21, a small ubiquitin-like modifier (SUMO) ligase, is required for APB formation and is necessary and sufficient for the SUMOylation of several subunits of the shelterin complex. SUMOylation of shelterin may cause transient disassembly of the complex and provide a mechanism to facilitate telomere recombination in ALT cells by uncovering them. SMC5/6-knockdown affects telomere length and causes increase of chromosome free ends and end-to-end fusions (Potts and Yu, 2007).

Cellular immortalization depends on the aberrant expression of different genes, including repressors of any telomere maintenance mechanism (telomerase or ALT-mediated). Repressors of ALT activity are present in normal and some telomerase-positive cells, as demonstrated by the observation that ALT activity is repressed in some hybrid cells formed by fusing an ALT-positive cell and a telomerase-positive cell (or a normal cell) (Perrem et al., 1999). In contrast, ALT cells may contain a repressor of telomerase, because hybrids of ALT cells and some telomerase-positive cells show ALT activity and repression of telomerase (Katoh et al., 1998). The absence of telomerase activity from ALT cells correlates with lack of expression of the hTERT subunity, and sometimes hTER. This is associated with methylation of the hTERT CpG island (Dessain et al., 2000), and with promoter methylation in the hTERC gene, that encodes for hTER (Hoare et al., 2001). The expression of exogenous telomerase in ALT cells is usually compatible with ALT activity (Perrem et al., 2001).

However, in one study, the expression of telomerase in an ALT cell resulted in reduced ALT activity (Ford et al., 2001). One possibility is that ALT may be switched off in some cells, or that ALT and telomerase may compete for common molecular components, or for the access to the telomere. Another explanation is that in ALT cells events of telomere lengthening occur when telomeres shorten to a critical length, although with low frequency,

due to the presence of an exogenous telomerase that lengthens the shortest telomeres.

The telomere length in ALT cells changes in a dynamic way, with fluctuations occurring on individual telomeres during cellular proliferation. Telomeres undergo gradual shortening at a rate of 30-50 bp per cell division (which is similar to cells without any telomere maintenance mechanism), until they reach a critical threshold of 200 bp of telomeric repeats. When this happens, telomeres undergo a rapid and heterogeneous increase in length, sometimes of >23 kb. In some cells, telomere lengthening occurs in telomeres that did not appear to be critically short. This dynamic change in length is consistent with a mechanism mediated by recombination (Murnane et al., 1994). In support to this hypothesis, other organisms have shown to rely on the recombinational process for telomere maintenance, like the mosquito malarial vector, Anopheles gambiae, and some species of yeast (Nosek et al., 1998; Roth et al., 1997).

Several models have been postulated describing inter-telomeric recombination, or mechanism mediated by T-loops or circular and linear (ECTR) (Tokutake et al., 1998).

1.5. The epigenetic regulation of telomeres

Recent studies have shown that epigenetic regulation of the telomeric chromatin regulates telomere function and maintenance. Pioneer studies in yeast and Drosophila melanogaster revealed the presence of a repressive chromatin environment at telomeres, which is implicated in telomere homeostasis. Like yeast and fly telomeres, mammalian telomeres are now thought to exist mainly in an heterochromatic conformation.

The first evidence of a repressive environment at yeast telomeres came from the observation that telomeric chromatin can silence nearby genes when introduced into S. cerevisia

subtelomeric regions (Gottschling et al., 1990). This phenomenon is referred to as Telomere Position Effect (TPE). In budding yeast, TPE involves Rap1, a protein that binds the telomeric DNA duplex and recruits the Sir proteins to telomeres, spreading them to subtelomeric regions, where they interact with the amino-terminal tail of histones H3 and H4. The NAD-dependent histone deacetylase activity of Sir2 can deacetylate histones H3 and H4, with preference for acetylated lysine 16 of histone H4, and it is essential in promoting heterochromatin and the spreading of telomere silencing towards more centromeric regions (Hecht et al., 1995). In budding yeast, telomere silencing positively regulates telomere length, because several mutations that disrupt telomeric silencing also decrease the length of telomeres.

In fission yeast, the telomere structure is very similar to that of mammals. Clr4 (an orthologue of mammalian Suv39h HMTases) is involved in the methylation of H3K9, which creates a binding site for Swi6 (the orthologue of the D. melanogaster Heterochromatin Protein HP1) (Nakayama et al., 2001). Experiments performed in D. melanogaster show that the heterochromatin protein HP1 negatively regulates telomere elongation (Perrini et al., 2004).

Mammalian telomeres have the ability to silence subtelomeric genes through TPE and, similarly to yeast, TPE in human cells is influenced by telomere length, increasing its activity when telomeres are elongated (Baur et al., 2001; Koering et al., 2002). Unlike in yeast, in mammalians, not only subtelomeres but also telomeres contain nucleosomes, with an altered spacing compared with the non-telomeric chromatin (Tommerup et al., 1994). Many markers of heterochromatin have been identified in mammalian telomeres, like trimethylation of H3K9 and H4K20. The enzymes responsible of H3K9 methylation are SUV39H1 and SUV39H2 (Garcia-Cao et al., 2002), homologues of the yeast Clr4, whereas SUV4-20H1 carries out the methylation of H4K20 (Kourmouli et al., 2004). The retinoblastoma (RB) family of tumour suppressor proteins, in addition to their role as transcriptional repressors, also interacts with SUV420H1 and SUV420H2 to maintain

trimethylation of H4K20 (Gonzalo and Blasco, 2005). H3K9me provides a high-affinity-binding site for HP1, a repressive chromatin marker which is usually enriched in telomeres and subtelomeres (Fig.8). Mammalian telomeric and subtelomeric repeats are characterized by low levels of acetylated H3 (AcH3) andH4 (AcH4) (Benetti et al., 2007).

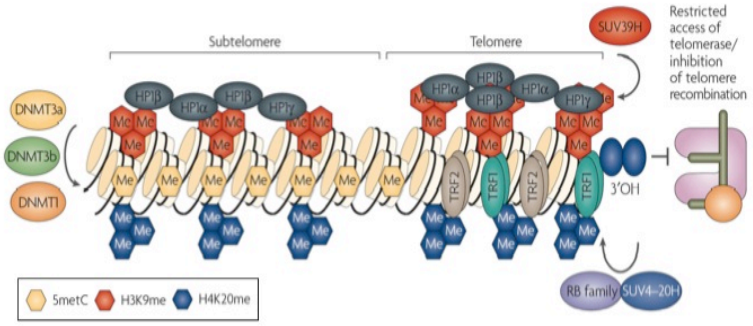

Figure 8. Epigenetic modifications at mammalian telomeric regions. Telomeric and subtelomeric regions are enriched in trimethylated H3K9 and H4K20, and HP1 isoforms. In addition, subtelomeric DNA is heavily methylated by the DNMT1, DNMT3a and DNMT3b enzymes. Both histone trimethylation and DNA methylation act as negative regulators of telomere length. DNA methylation also inhibits telomere recombination. (Blasco 2007)

In contrast to budding yeast, which lacks DNA methylation, in mice and humans the subtelomeric regions are methylated. Mammalian telomeres cannot be methylated because they lack CpG sequences, substrates for DNA methyltransferases (DNMTs). DNA methylation regulates the accessibility of DNA- binding factors and the remodelling of chromating, thereby exercising a key role in the regulation of transcription. DNA methylation at subtelomeres might also reinforce TPE.

Defects in histones and DNA modifications at telomeres correlate with loss of telomere-length control. Loss of SUV39H1 and SUV39H2 HMTases induce decreased levels of H3K9 trimethylation and telomere elongation (Garcia-Cao et al., 2004). Aberrant telomere elongation is also observed in cells that lack all

the members of the retinoblastoma family with consequent decreased levels of H4K20 trimethylation (Garcia-Cao et al., 2002). DNA methylation represents an additional way to control telomere length, and demethylation of subtelomeric regions results in dramatically elongated telomeres and increased homologous recombination (Gonzalo et al., 2006).

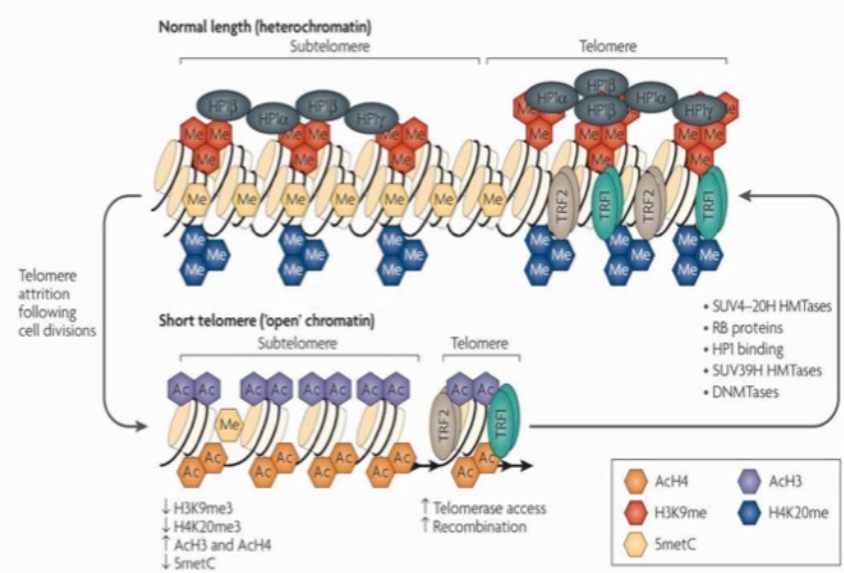

Figure 9. Model for the role of epigenetic modifications in telomere-length control. Telomeres and subtelomeres have a compacted and 'close' conformation. Telomere shortening leads to decrease of heterochromatic marks and increase of histones acetylation, resulting in a more 'open' chromatin state, which allows accessibility of telomerase and proteins involved in telomere recombination by ALT mechanism. Once telomeres are elongated, they can be newly assembled into heterochromatin. (Blasco 2007)

Shortening of telomeres that occurs during cell divisions and ageing results in a decreased density of heterochromatin marks, and an increased density at telomeric repeats and subtelomeres of marks that are characteristic of 'open' chromatin. This status could

favour the activation of telomere-elongation mechanisms, like telomerase and ALT (Fig.9).

In conclusion, histone and DNA methylation have an important role in regulating mammalian telomere length, and vice versa, telomere length dictates the assembly of heterochromatin domains at telomeres. This model could explain the link between cancer and aging. The shortening of telomeres that normally happens during aging, might results in epigenetic defects at telomeres, which in turn could favour the activation of the telomere maintenance machinery and allow cancer development.

1.6. Telomeres and DNA-damage response.

Various proteins involved in the DNA-damage response (DDR) physically associate with telomeres and play important roles in regulating normal telomeric functions. In fact, progressive attrition or uncapping of telomeres elicit a DNA-damageresponse as a result of the inability of the cell to distinguish dysfunctional telomeric ends from DNA double strand breaks (d'Adda di Fagagna et al., 2004).

Cells have evolved specific mechanisms to recognise sites of DNA damage and to promote the recruitment of DNA-repair proteins. When DNA damage is present, checkpointes are activated and the cell-cycle progression is slowed down or arrested to favour DNA damage repair . Once the DNA damage has been removed, cells resume their proliferation.

The inability to repair DNA damage leads to programmed cell death (apoptosis) or causes the entrance into permanent cell cycle arrest (senescence). Central in the DDR are two protein kinases of the PIKK (Phosphatidil Inositol 3-Kinase-like Kinase) family, ATM and ATR, with distinct but partially overlapping functions (Shiloh, 2003). These kinases are activated by DNA damage-binding proteins, like RPA or the MRE11-RAD50-NBS1 (MRN)

complexes (Uziel et al., 2003), which act as "sensors" of damage. Once activated, ATM and ATR phosphorylate a range of factors, including the checkpoint kinases ChK1 and ChK2, that then target various effectors involved in DNA repair and cell-cycle progression (Bartek et al., 2007).

Another target of PIKKs is the histone H2AX, which is phosphorylated (γ-H2AX) extensively in the chromatin flanking the sites of damage (Fernandez-Capetillo et al., 2004). γ-H2AX induces changes in the chromatin structure that facilitate the accumulation of DNA-repair andcheckpoint proteins, favouring the DDR (Fig.10).

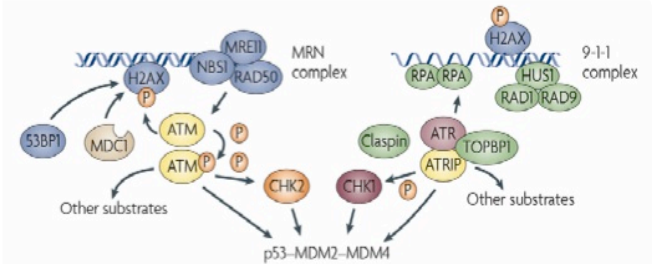

Figure 10. DNA damage response signaling pathway. Double-strand breaks are recognized by the MRN (Mre11-RAD50-NBS1) complex and lead to the activation of ATM. Activated ATM phosphorylates a range of substrates, including ChK2, p53 and H2AX. Other forms of DNA damage lead to the generation of single stranded regions that become coated with replication protein A (RPA). This attracts ATR, which phosphorylates downstream substrates, including ChK1, p53, MDM2. (Meek 2009)

DNA double-strand breaks (DSB) are the most deleterious among DNA modifications. DSBs are repaired by homologous recombination (HR, predominant in prokaryotes) or non-homologous end-joining (NHEJ, the major pathway in eukaryotes).

NHEJ is the predominant mechanism and can join any two exposed double-stranded DNA ends. Main components of NHEJ are the LigaseIV/XRCC4, Ku complex and DNA-dependent protein kinase catalytic subunit (DNA-PKcs). LigaseIV/XRCC4 is recruited to DNA ends via interaction with Ku and it is responsible

for NHEJ ligase activity (Grawunder et al., 1997). The Ku complex is an heterodimer comprising the Ku70 and Ku86 (also known as Ku80) subunits, that possesses high affinity for dsDNA ends, and is implicated in alignment and synapsis of DNA ends and recruitment of additional factors important for the processing of DNA breaks (Dynan and Yoo, 1998). DNA-PKcs is a member of the phosphoinositide-3-kinase-related (PIKK) family, which physically associates with the Ku heterodimer forming a catalytically active DNA-PK holoenzyme (Fig.11).

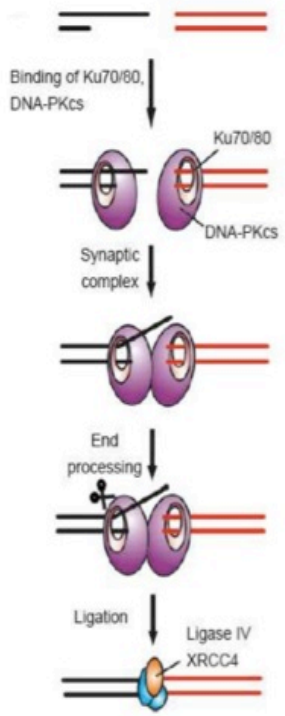

Figure 12. Simplified overview of non-homologous end-joining. NHEJ brings the ends of the broken DNA molecule together by the formation of a synaptic complex, consisting of two DNA ends, two Ku70/Ku80 and two DNA-PKcs molecules. Non compatible DNA ends are processed to form ligatable termini, followed by repair of the break by the LigaseIV/XRCC4 complex. (Weterings and Chen 2008)

The DNA-PK complex has a serine/threonine kinase activity. Ku recruits DNA-PKcs to a DSB, leading to a rapid activation of DNA-PK in response to DNA damage (Falck et al., 2005). The activation of this kinase appears to require a direct interaction of DNA-PKcs with free DNA. Little is known about DNA-PKcs function in DSB rejoining or its phosphorylation targets.

In vitro studies indicate that autophosphorylation of DNA-PKcs is critical for a correct NHEJ, confirming a role for DNA-PKcs in DNA ligation through remodelling of the DNA ends by DNA-PK complexes (Block et al., 2004a). DNA-PK phosphorylates histones H2AX and H1, and in this way it facilitates the NHEJ reaction by modifying the local chromatin environment to provide access to other DNA repair complexes at DSBs (Kysela et al., 2005). DNA-PK also recruits Artemis, a structure specific endonuaclease that mediates ligation of the ends (Ma et al., 2005).

1.6.1. DNA damage response proteins at functional telomeres.

As previously mentioned (see above paragraphs), DNA damage response factors associate with telomeres (MRN complex, DNA-PK, ATM and ATR). A role for these proteins at telomeres is confirmed by the observation that their inactivation leads to telomere dysfunctions. For example, inactivation of ATM causes telomere shortening (Metcalfe et al., 1996), and also RPA has been implicated in the control of telomere length because it facilitates the recruitment of ATR (Zou and Elledge, 2003). These data suggest that a PIKK-mediated checkpoint is necessary for normal telomere homeostasis. As reported in yeast, a role for the MRN complex in telomere maintenance may exist in mammals, as NBS1 associates with telomeres during S phase when telomeres are elongated and is required for effective telomere elongation by telomerase (Ranganathan et al., 2001). There are evidences that components of NHEJ also have a function in telomere maintenance in mammals. In fact, immunoprecipitation studies have revealed a physical association of Ku with telomeres (d'Adda di Fagagna et

al., 2001). Accordingly, inactivation of one allele of the Ku86 gene in human cells results in telomere shortening (Myung et al., 2004), while inactivation of both alleles leads to cell death (Li et al., 2002). One study shows that mice lacking Ku80 and embryonic stem cells lacking Ku70 have shorter telomeres, chromosome instability and fusions (d'Adda di Fagagna et al., 2001), however, another group reports contrasting data showing that inactivation of Ku80 does not lead to telomere shortening and that the chromosomal fusions retain telomeric DNA at fusion points (Samper et al., 2000).

The third subunit of the DNA-PK complex, DNA-PKcs, also contributes to the protection of telomeres. DNA-PKcs associates with telomeric DNA in human cells (d'Adda di Fagagna et al., 2001), and inhibition of the catalytic activity of DNA-PKcs through inactivation of one or both alleles, or by treatment with chemical inhibitors results in telomere shortening, genomic instability and telomere fusions (Bailey et al., 2004). Mouse embryo fibroblasts (MEFs) from DNA-PKcs-/- mice or cells from severe combined immunodeficient (SCID) mice, expressing a defective DNA-PKcs gene, show a significant increase in end-to-end fused chromosomes (Goytisolo et al., 2001), supporting the hypothesis that DNA-PKcs is involved in telomere capping. Since NHEJ is severely impaired in DNA-PKcs-/-, Ku70-/- and Ku80-/- mice, it will be interesting to determine which pathway is responsible for the observed fusions. It has also been shown that mice lacking DNA-PKcs and Terc undergo telomere loss with faster rates than mice lacking Terc alone (Espejel et al., 2002). This data support a role for DNA-PK in telomere homeostasis, both in end-capping and in the maintenance of telomere length.

A crucial question is which factor is phosphorylated by DNA-PKcs and what is the effect of this phosphorylation. A recent study shows that DNA-PK phosphorylates a telomere-associated protein, heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), and that this phosphorylation is triggered by the telomerase RNA component, hTER. hnRNP A1 binds telomeric DNA in a sequence

specific manner, and it seems to have a critical function in telomere maintenance. hnRNP A1 loss causes telomere shortening, and it has been postulated that it may contribute to telomere elongation. This data suggest that hTER-mediated stimulation of the kinase activity of DNA-PK towards telomere-associated proteins might play a role in telomere maintenance (Ting et al., 2009).

1.6.2. DNA damage response at dysfunctional telomeres.

When telomere function is disrupted, a DNA damage response is triggered, and because DNA repair activities can impair the integrity of chromosome ends extensive genome instability can arise. DNA damage response can be activated when telomere are rendered dysfunctional through replicative attrition of the telomeric DNA or by inhibition of shelterin (de Lange, 2005).

A suitable model for the study of the DNA-damage response to dysfunctional telomeres is the inhibition of TRF2, which causes the uncapping of telomeres. TRF2 is inhibited through RNA interference or by expressing a dominant-negative TRF2 allele that binds the endogenous TRF2 and forms an inactive heterodimer, unable to bind the DNA (de Lange, 2002; van Steensel et al., 1998). This allele, named TRF2$^{\Delta B \Delta M}$, blocks the accumulation of TRF2 on chromosome ends and effectively strips TRF2 and its interacting factors off the telomeres (Li et al., 2000; van Steensel et al., 1998).

Uncapped telomeres associate with DNA damage response factors such as 53BP1, γ-H2AX, Rad17, ATM and MRE11. These foci of DNA damage factors that co-localize with TRF1, are referred to as Telomere Dysfunction-Induced Foci (TIFs) (Takai et al., 2003) (Fig.12). TIFs are used as markers for telomere

dysfunction. The ATM kinase is the main transducer of the telomere damage signal.

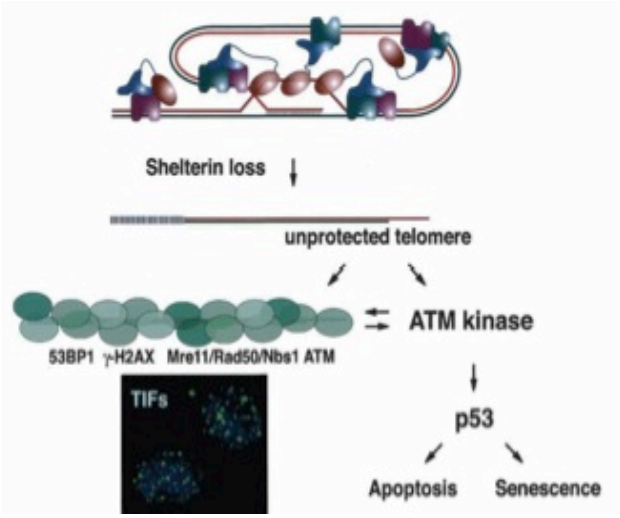

Figure 12. DNA damage response at dysfunctional telomeres. Inhibition of shelterin subunits causes loss of protection of telomeres, that become associated with DNA damage response factors, forming TIFs. Telomere damage activates ATM kinase, which leads to a p53-dependent G1/S arrest and can induce either apoptosis or senescence. (de Lange 2005)

However, telomere-directed senescence and TIFs formation is not abrogated in ATM-deficient cells, supporting the idea that dysfunctional telomeres might also induce an ATM-independent pathway (Takai et al., 2003). Recently published data postulate a mechanism in which TRF2 represses ATM kinase, while POT1 represses ATR kinase (Fig.13). This model may provide an explanation for how cells detect critically shortened telomeres: as the abundance of shelterin at telomeres depends on the length of the duplex telomeric repeats, short telomeres contain less TRF2 and POT1, leading to the expression of the ATM and ATR kinases and resulting in cell cycle arrest and DNA repair at telomeres (Denchi and de Lange, 2007). Inhibition of TRF2 leads to the formation of many fused telomeres, because of the rapid loss of the 3'overhang which mediates NHEJ (van Steensel et al., 1998) (Fig.14). Cells expressing $TRF2^{\Delta B \Delta M}$ show end-to-end fused

chromosomes, usually chromatid-type dicentrics, involving the leading strand (Bailey et al., 2001).

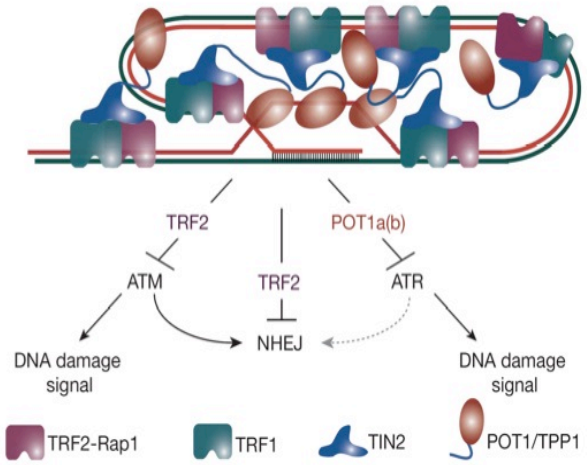

Figure 13. Model for repression of DNA damage response at telomeres. ATM and ATR are independently repressed by TRF2 and POT1. NHEJ is repressed by TRF2. NHEJ of telomeres lacking TRF2 is stimulated by either ATM or ATR signalling. (Denchi and de Lange 2007)

This observation indicates that in the absence of TRF2, cells fail to regenerate the 3' overhang and the ends created by leading strand synthesis remain blunt ended after DNA replication. Dicentric chromosomes are generally unstable in mitosis. The two centromeres of a dicentric chromosome can attach to opposite poles, forming an anaphase bridge, which requires the formation of breaks in the chromosomes to allow for cell division. Therefore, TRF2 inhibition generates genomic instability, with translocations, rearrangements and changes in chromosomes number (Smogorzewska et al., 2002).

The consequences of TRF2 inhibition and DNA damage response activation can be different depending on the cell type. In many cell types, including primary lymphocytes, TRF2 inhibition leads to immediate induction of apoptosis (Karlseder et al., 1999). Apoptosis is accompanied by the stabilization and activation of p53, resulting in expression of its downstream targets p21 and bax.

The ATM kinase is required for the initiation of this program and cell lines lacking this kinase escape apoptosis after TRF2 inhibition. In a subset of human cell types, especially in primary fibroblasts, TRF2 inhibition results in onset of senescence rather than apoptosis (Karlseder et al., 1999) (Fig.14).

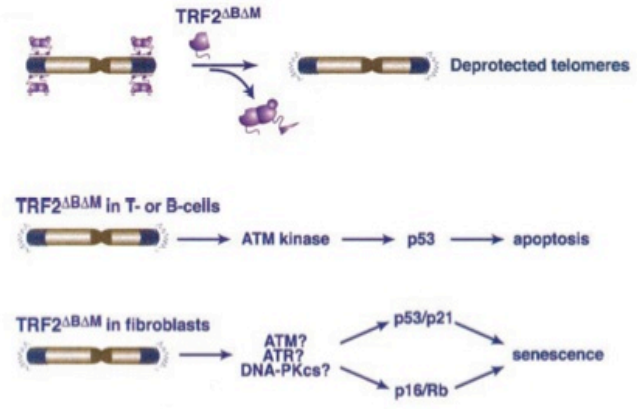

Figure 14. Cellular consequences of telomere deprotection due to inhibition of TRF2. TRF2ΔBΔM binds endogenous TRF2, forming a dimer unable to bind telomeres. Unprotected telomeres activate different pathways depending on the cell type. (de Lange 2002)

The activated pathways involve upregulation of p53 and its target p21, and repress Rb phosphorylation with a consequent induction of the CDK inhibitor, p16. Inhibition of p53 alone or pRb alone, fails to abrogate the cell cycle arrest induced by $TRF2^{\Delta B\Delta M}$, indicating that both tumour suppressors are capable of preventing entry into S phase when telomeres are damaged (Fig.14). Also in the senescent pathway the ATM kinase is involved in the signalling upstream of p53 and Rb. However, fibroblasts from ataxia telangestatica (A-T) patients, which are deficient for this kinase, are able to enter senescence after $TRF2^{\Delta B\Delta M}$ induction, probably implicating other kinases as ATR or DNA-PKcs (Fig.14).

1.7. Telomere, the molecular clock in aging and cancer.

The progressive shortening of telomeres is at the basis of cellular senescence, a mechanism involved in organism aging and protection against cancer. The mechanisms that can trigger senescence are the accumulation of DNA damage, the epigenetic deregulation of the INK4a/ARF locus which encodes the tumour suppressors p16INK4 and ARF (these proteins increase with aging and are a barrier to the outgrowth of cancer), and the shortening of telomeres (Collado et al., 2007) (Fig.15).

The contribution of telomere shortening in aging has been established following several observations. First of all, many studies have described an inverse correlation between telomere length and age in a variety of tissues (Cawthon et al., 2003; Ogami et al., 2004; Panossian et al., 2003). Secondly, factors that are considered to accelerate aging, such as stress, smoking and obesity, have been shown to negatively affect telomere length (Canela et al., 2007; Epel et al., 2004; Valdes et al., 2005).

It should also be taken into account that telomere shortening is accelerated by oxidative damage, which is also associated with aging (von Zglinicki and Martin-Ruiz, 2005). Therefore, telomere shortening could reflect not only the proliferative history of a cell, but also the accumulation of oxidative damage. In addition, several human premature aging syndromes are linked to mutations in telomerase or in proteins that directly affect telomere activity and result in accelerated rates of telomere shortening. These include dyskeratosis congenital (DC), aplastic anemia and idiophatic pulmonary fibrosis. Other premature aging syndromes are characterized by accelerated rate of telomere shortening, and are triggered by mutations in DNA repair proteins such as Nbs1 (Nijmegen breakage syndrome), Mre11 (ataxia telangiectasia-like disorder), WRN (Werner syndrome), BLM (Bloom syndrome), ATM (Ataxia telangiectasia) and proteins encoded by FANC genes (Fanconi anemia) (Blasco, 2005b).

Another demonstration that telomere shortening is one of the causes of cellular senescence comes from the observation that re-introduction of the TERT telomerase gene is sufficient to bypass replicative senescence and to confer immortal growth to a number of human primary cell lines (Bodnar et al., 1998).

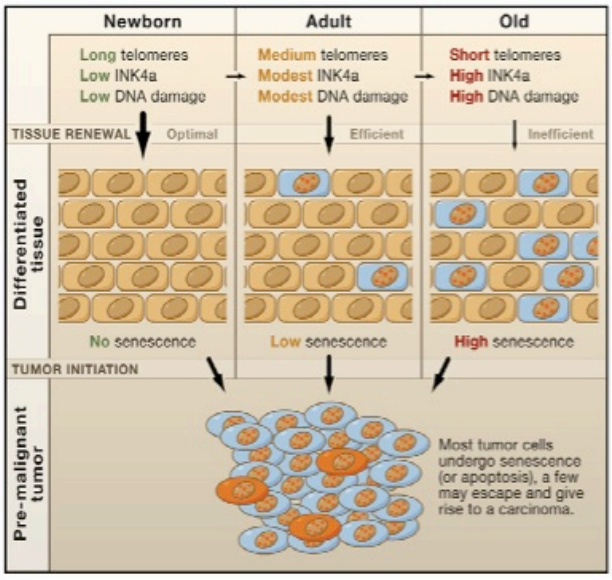

Figure 15. Multiple mechanisms that trigger senescence. Telomere shortening, derepression of the INK4a/ARF locus, and the accumulation of DNA damage operate during the lifespan of the organism. The final result is age-dependent loss of stem cell functionality and an age-dependent increase in the number of senescent cells in differentiated tissues (represented as blue cells). Tumours may arise at any point in life, but the stress signals characteristic of tumours will engage the senescence program. This tumor-specific senescence constitutes an important barrier to tumor progression. (Collado et al. 2007)

One of the best tools to study of the impact of short telomeres on aging is the telomerase-deficient mouse model. Terc-deficient mice were first generated by elimination of the mouse Terc gene (Blasco et al., 1997). Terc-/- mice show shortened lifespan (even at the first generation), infertility, heart failure, immunosenescence, decreased regeneration of the digestive system, the skin, and the hematopoietic system (Lee et al., 1998; Leri et al., 2003).

Dysfunctional telomeres in Terc-deficient mice are detected as damaged DNA, triggering cell cycle arrest and apoptosis. The pathologies developed by telomerase-null mice recapitulate human deseases associated with aging. Telomere-driven aging could be the consequence of the accumulation of senescent cells, or of the loss of stem cell function. For example, abundant senescence cells (as measured by senescence associated β-galactosidase activity, SA β-gal) have been observed in the liver of telomerase null mice (Satyanarayana et al., 2003).

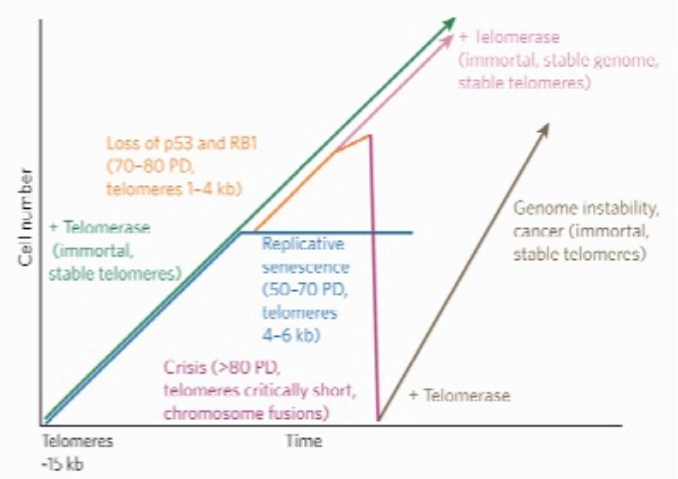

Figure 16. Telomere shortening, aging and cancer. Primary cells divide and telomeres shorten until they reach a critical length that triggers senescence (blue). Activation of telomerase before senescence allows cells to divide indefinitely and maintain a stable genome (green). If RB and p53 are suppressed, cells continue dividing (orange) arriving to telomeric crisis, characterized by a massive genomic instability and cell death (dark pink). If telomerase is activated before erosion is completed, this re-establishes telomere maintenance and genomic stability (light pink). Activation of telomerase after crisis allows transformed clones to become immortal and leads to cancer (brown). (Verdun and Karlseder 2007)

On the other hand, progressive telomere shortening in the context of Terc- deficient mice is responsible for the decreased functionality of stem cells, and is considered a barrier for stem cell proliferation, in turn limiting tissue regeneration (Flores et al., 2005). The telomere clock limits not only the proliferation of

normal noncancerous cells, but also the proliferation of those cells that are already primed toneoplastic transformation. For this reason, all human cancers have to acquire mechanisms that ensure telomere maintainance, like the expression of telomerase or ALT. This is why the Terc-deficient mouse model has also been used to investigate the role of telomeres and telomerase in tumorigenesis. In particular, Terc-/- mice are resistant to both induced and spontaneous tumorigenesis (Gonzalez-Suarez et al., 2000). Mice that are simultaneously deficient in telomerase and the tumour suppressor proteins p19ARF, p16, p21, APC, ATM, DNA-PKcs, Ku, PARP1 and PMS2 also show reduced tumorigenesis (Blasco, 2007). This indicates that short telomeres are a potent suppressor of tumorigenesis even in a tumour-prone genetic background, because they can induce cellular arrest and apoptosis. Two main exceptions to this general trend occur when lack of telomerase is combined with a deficiency in p53 or with overexpression of the telomere-binding protein TRF2 (Blanco et al., 2007). The barrier imposed by short telomeres is bypassed and cells proliferate in the presence of chromosomal aberrations, in a condition called "crisis". Crisis fuels the development of cancer (Artandi et al., 2000), because transformed clones can emerge mainly by activation of telomerase (Fig.16).

This observation emphasizes the dual role of telomerase: on one hand, it works as an oncogene, as reactivation of the enzyme in cells with critically short telomeres allows for genomically unstable and immortal clones to be established; on the other hand, it is a tumour suppressor, since activation of telomerase in cells that have not reached crisis prevents telomere-mediated genomic instability, which is priming to cancer development.

The balance between telomere length maintainance and preventing aberrant telomere aberration creates what we could refere to as "the Cancer/Ageing paradox": stem cells need telomere elongation to assure tissue self-renewing, meanwhile normal cells needs a molecular clock that ensure stop growing allowing tissue regeneration.

2. Promyelocytic Leukemia Protein (PML)

The study of chromosomal translocations associated with haemopoietic tumors has led to the identification of new genes putatively involved in neoplastic transformation (Sawyers et al., 1991).

The *PML* gene, also designated *myl*, was identified by cloning the breakpoint sites of the 15; 17 chromosome translocation associated with acute promyelocytic leukemia (APL). As a consequence of this balanced translocation, PML (Promyelocytic Leukemia Protein) fuses to Retinoic acid receptor α (RARα), leading to the production of two fusion genes that encode for PML-RARα and RARα-PML chimeric proteins. Both of them coexist in the leukemic cells, but to date work has focused on PML-RARα since it retains most of the functional domains of the parental proteins, while little is known about RARα-PML.

2.1. Acute Promyelocytic Leukemia (APL)

Acute Promyelocytic Leukemia represents the 5-10% of all the Acute Myeloid Leukemias (AML) cases. According to the cytomorphological French-American-British classification, it corresponds to the M3 subtype, in which leukemic blasts are arrested at a promyelocytic stage of differentiation.

The pathogenesis of acute myeloid leukemia, differently from other forms of cancer, is predominantly associated with chromosomal translocations that give rise to the formation of active fusion proteins. Specific subtypes of AML are associated with specific aberrations. Remarkably, one of the two components of the fusion protein is generally a transcription factor involved in the control of myeloid differentiation (Alcalay et al., 2001).

APL is associated with at least four different chromosomal translocations, responsible for the generation of four fusion proteins: PML-RARα t(15:17), observed in the vast majority of APL cases (95%), PLZF-RARα t(11;17) (4% of APL cases) and the less common, sporadically observed, NPM-RARα and NuMA-RARα fusion proteins (Fig.17).

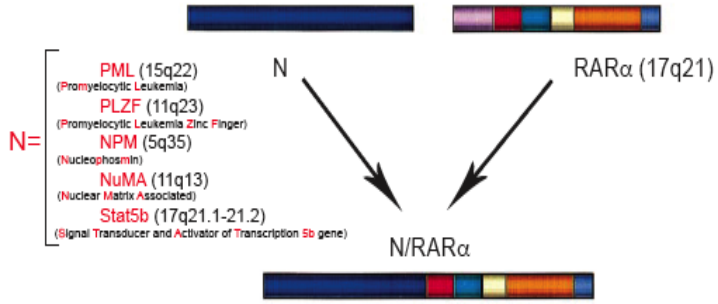

Figure17. Molecular Pathogenesis of APL. APL is a common form of acute myeloid leukemia (AML) that, moleculary, harbors a chromosomal translocation involving the RARa gene on choromosome 17. To date, five different fusion partners of RARa have been identified that include the promyelocytic gene (PML), the promyelocytic leukemia zinc finger gene (PLZF), the nucleophosmin gene (NPM), the nuclear mitotic apparatus gene (NuMA) and the signal transducer and activator of transcription 5b gene (Stat5b).

RARα (retinoic acid receptor α), the common denominator of all these translocations, is a member of the nuclear hormone receptor superfamily of transcription factors. Upon binding of the Retinoic Acids (RA), RARα transactivates target genes involved in hematopoietic differentiation while, in the absence of ligand, the transcription is repressed by the recruitment of histone deacetylases (HDACs), through a direct interaction between RARα and the co-repressors N-CoR and SMRT (Fig.17).

In APL, the fusion protein PML-RARα aberrantly binds to HDACs leading to repression of transcription and consequently to a block of differentiation (Grignani et al., 1998).The altered recruitment of HDAC is due to oligomerization of the fusion protein through the PML moiety (Minucci et al., 2001). Differently

from the normal counterpart RARα, the fusion protein doesn't release HDACs, even in the presence of physiological concentrations of retinoic acid. By contrast, high doses of RA cause dissociation of the PML-RARα/HDACs complex, restoring the normal differentiation pathway. The clinical treatment of APL patients with RA represents the first example of "differentiation therapy".

The block of differentiation is not the only feature of leukemia, which is also characterized by the abnormal proliferation of leukemic cells. Indeed, leukemic cells do not have cell cycle profiles different from that of their physiological counterparts, but rather they survive in conditions where a normal cell would undergo programmed cell death. PML-RARα associates physically with PML, thus potentially interfering with its function. This hypothesis was further corroborated by experiments carried out in mice. Transgenic mice expressing PML-RARα develop leukemia with APL features when expressed in the myeloid compartment, while dominant negative RARα mutant, that do not interfere with PML function, do not, underscoring the importance in leukemogenesis of the functional disruption of PML. A possible explanation could be that, as previously mentioned, PML is required for the pathological requirement of HDACs, but it is also possible that the leukemic fusion protein, acting as a dominant negative, could interfere with the growth suppressive activity of PML, lending the leukemic blasts a marked survival advantage.

2.2. PML Locus and Alternative Spliced PML Variants.

The PML genomic locus spans approximately 35 Kb and is subdivided in nine exons. Due to alternative splicing of the gene transcript, which always involves coding exons, PML exists in a number of different isoform(Gupta et al., 1981) (Fagioli et al., 1992) (Fig.19). The exons implicated in the alternative splicing fall into two groups: central exons (4, 5 and 6) and 3' exons (7, 8 and

9). All transcriptional variants share the N-terminal region, but differ in their central and C-terminal portions. The N-terminal common region to all PML isoforms (amino-acids 1-394) contains the region homologous to the DNA-binding finger domain of numerous transcriptional factors (de The et al., 1991; Kakizuka et al., 1991). Amino acids 228- 394 form part of another region that has the characteristic of a α-helix. All PML isoforms seem to have the potential to act as transcription factor and to form homo- or heterodimers. The adjacent region of a108 amino acids constitutes the variable central region for the alternative splicing of exons 4, 5 and 6 and are also part of the α-helix. The different length of the α-helix among isoforms could influence the binding properties of the dimerization interface of the proteins. The next region corresponds to the serine/proline-rich domain retained in all PML isoforms. The C-terminus which follows differs in multiple isoforms and it' s variably encoded by exons 7, 8 and 9 (Fagioli et al., 1992) (Fig.18).

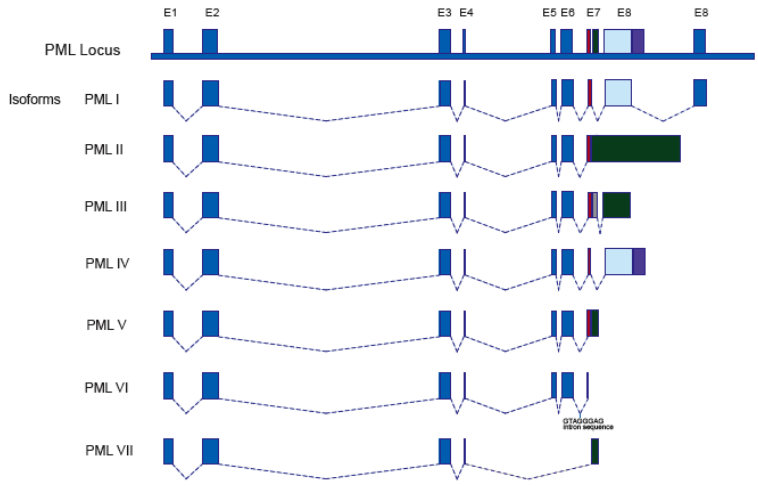

Figure.18. Exon assembly of PML isoforms.

On the basis of the sequence differences of the carboxy-termini, PML isoforms can be divided into seven groups, designed as PMLI-VII (Jensen et al., 2001). A further division into sub-groups a/b/c reflects the alternative splicing of the central exons. Therefore all PML isoforms share the same N-terminal part (amino-acids 1-552), but differ in the C-terminus portion, except for the shortest one, PML VII. PML VII is also the only isoform that doesn't contain a nuclear localization signal (NLS). All PML isoforms are almost equally expressed in the different cell lines (Fagioli et al., 1992).

The function of the complex splicing pattern of PML is not known. It is possible, however that the different PML isoforms could provide different surfaces on which protein interactions may occur, thus affecting NB composition and function.

2.3. PML Protein Structure

PML is an ubiquitary expressed protein that belongs to the TRIM family, characterized by the presence of a TRIpartite Motif (TRIM). The TRIM is composed of three cysteine-rich zinc-binding domains, a RING-finger and two B-boxes (B1 and B2), followed by a α-helical coiled-coil (CC) region (Reymond et al., 2001) (Fig.19). PML, as mostly of the TRIM family members, forms high molecular weight complexes *in vivo* as a consequence of self-association properties of the coiled-coil region. The coiled-coil region is responsible for the formation of stable PML homodimers and for the PML/PML-RARα heterodimerization (Grignani et al., 1998), whereas the RING-finger domain is involved in protein-protein interaction (Borden et al., 1995). The TRIM motif is essential for correct localization of PML localization and can by itself recapitulate the growth suppressor activity of the protein (Fagioli et al., 1998).

In addition to splicing, PML is also subjected to post-translational modifications that may increase the level of

complexity, either by directly affecting protein-protein interactions or by interfering with the ability of PML to form NBs. In fact, PML sequence contains three SUMO-1 modification sites (lysine 65 in the RING finger, lys 160 in the B1 box and lys 490 in the NLS); they are located within the common region shared by all PML isoforms, suggesting a relevant role for this covalent modification. PML is also a phosphoprotein. It is present within the cell in both the phosphorylated and the unphosphorylated form. The treatment with alkaline phosphates, in fact, abolished the slow-migrating band of PML in a Western Blot analysis. Moreover experiments of ^{32}P incorporation, followed by complete hydrolysis and thin layer chromatography demonstrated that PML is phosphorylated at both tyrosines and serines, but not at threonine residues (Chang et al., 1995). Recently it has been described a PML post translation regulatory mechanism depending by the serine/threonine casein kinase 2 (CK2) activity.

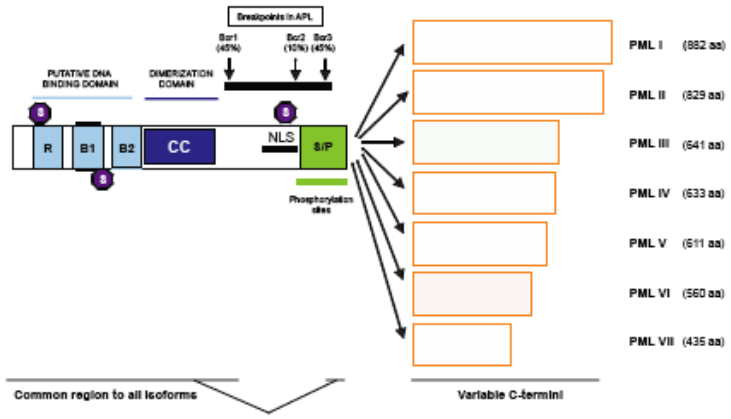

Figure 19. PML Protein Structure. All PML isoforms share the N-terminal region which contains the RING finger domain, two B boxes and a predicted a-helical Coiled Coil domain (RBCC motif). The seven different C-termini derive from an alternative use of 3' exons, resulting in different size of the isoforms. All isoforms contain three characterized SUMOylation sites (S) (amino acid position 65 in the RING finger, 160 in the B1-Box and 490 in the NLS) and a nuclear localization signal (NLS).

CK2-mediated PML phosphorylation at the serine 517 of the serine/proline-rich domain is critical in the PML ubiquitylation and degradation upon cellular stress (Scaglioni et al., 2006).

In APL the PML locus on chromosome 15 can be alternatively disrupted in three different sites (breakpoint cluster regions-bcr): bcr3 into the third intron, bcr1 into the seventh intron and bcr2 into exon 6. The resulting PML-RARα proteins contain the N-terminal part and the TRIM domain, but invariably lose the C-terminal domain. In this part resides a potential phosphorylation site with a serine/proline-rich region that could be involved in the regulation of the protein. Therefore the PML-RARα chimeric protein would be differently regulated from the normal PML protein and this could contribute to the pathogenesis of APL.

2.4. PML and stress response pathways.

In normal mammalian cells PML is primarily localized in multiprotein nuclear complexes referred as PML Nuclear Bodies (NBs). They are discrete nuclear sub domains associated to the nuclear matrix. By immunofluorescence, NBs appear as spheres of various sizes (from 0.2 to 1μm), in number of 10-20 per nucleus and present in almost every type of cell studied (Fig.20). Electron microscopy analysis revealed the doughnut shape of these structures and the localization in the interchromosomal spaces.

These structures were originally identified as autoantigens in patients affected by primary biliary cirrhosis. Subsequently they have been fascinating researchers for a long time because of their disruption in the leukemic blasts of APL. The expression of the PML-RARα fusion protein causes the delocalization of PML NBs into a multitude of small punctate structures described as microspeckles. The treatment with retinoic acids leads to clinical remission correlated with NBs reformation. These findings

strongly suggest that the integrity of PML NBs could be critical for normal cellular functions.

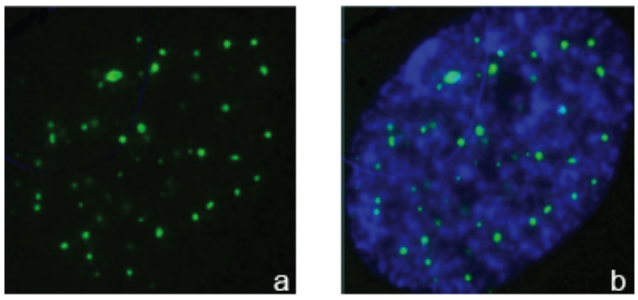

Figure 20. PML Nuclear Bodies. PML is a part of a multiprotein complex called PML Nuclear Bodies, ND10 or PODs (PML oncogenic domains). It is a heterogeneous group of multi-protein complexes which localizes specifically in the nucleus with a typical micro-speckled pattern. a) Immunofluorescence of PML in a fibroblast cell line (WI38) with an antibody (PG-M3) which recognizes all PML isoforms. b) DAPI coloration to show the nuclear protein localization**.**

In addition to PML, a high number of proteins involved in a variety of different cellular functions are represented in the Nuclear Bodies and including Sp100 (Maul et al., 1995), Blm (Ishov et al., 1999), p53 and Rb (Alcalay et al., 1998), (Ferbeyre et al., 2000), (Fogal et al., 2000), (Pearson et al., 2000). DNA damage repair genes, such as Mre11 and NBS (Lombard and Guarente, 2000) and transactivators such as CBP/p300 (Boisvert et al., 2001). In many cases, colocalization of the mentioned NBs components with PML is not complete, further underlying the dynamic nature of these nuclear sub domains.

A number of tumors and tumor-derived cell lines are characterized by a novel form of PML NBs, termed ALT-associated PML Bodies (APBs), in which PML localize at the telomere together with others telomere binding proteins to maintain telomerase length (see below for details).

Genetic evidences indicate that the structure of PML-NBs depends on the presence of PML (Ishov et al., 1999). In cells derived from PML knock-out mice, PML NBs do not exist and all protein normally targeted to these domains are nuclear diffuse or

concentrate in distinct domains. The product of the BLM gene, for instance, which is inactivated in the Bloom syndrome, is normally found to co-localize with PML in the nuclear bodies. By contrast, in PML null cells BLM is no longer found in these structures (Zhong et al., 1999).

The PML SUMOylation is also required for PML-NB-formation (Ishov et al., 1999), (Wang et al., 1998b), (Zhong et al., 2000), and, in fact, many of the proteins found in the PML NBs are SUMOylated (Seeler and Dejean, 2003). Recently it has been demonstrated that a SUMO binding site in PML, independent of its sumoylation sites, and the PML Ring domain, is necessary for PML-NB formation. The sumoylated PML through the sumo binding motif could constitutes the first step for subsequent recruitment of sumoylated proteins and proteins containing sumo binding motifs to the PML NBs (Shen et al., 2006).

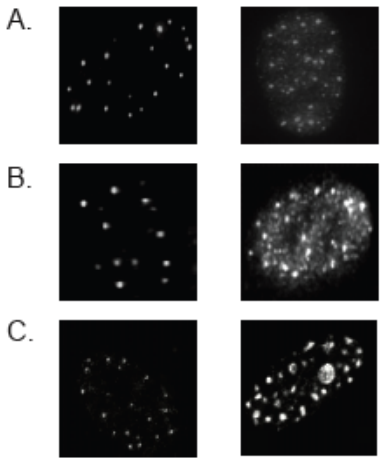

Figure 21. PML Nuclear Bodies respond to many cellular stress. A. UVC treatment induces a reorganization of PML NBs into a micro speckled patterns. B. PML NBs are modified upon ionizing radiation. An overall increase in the number of PML NBs are observed by indirect immunofluorescence of endogenous PML in WI38 after irradiation and recovered at 37C for 12h before fixation and anti PML immonostaining.C.RASV12 expression increases the number and size of the PML NBs and these changes are accompanied by increase in PML protein levels. Indirect immonofluorescence of PML in WI38 cell line infected with the empty vector or RASV12 4 days after selection.

The morphology of PML NBs is altered by environmental stresses and by a variety of viruses. Heat shock (Maul et al., 1995), heavy metals such as Cadmium (Nefkens et al., 2003), gamma and ultraviolet radiations and oncogenes, such as Ras (Pearson et al., 2000), cause PML-NBs to disperse (Fig.21).

PML NBs are also affected by infections of the cell with various DNA or RNA viruses and are disrupted during the early phases of the infection. Additionally PML is up regulated by interferon, an antiviral agent. An increased PML expression is also observed in inflammatory tissues and is associated with inflammatory disorders such as hepatitis. This suggests that PML and PML NBs could be involved in the cellular defense pathway against viral infections. Notably PML ko mice are highly susceptible to infections.

Additionally, it was recently shown that, in hypoxia conditions, the mammalian target of rapamycin (mTOR) localizes into the NBs as well (Bernardi et al., 2006). Indeed, normally, mTOR shuttles between the cytoplasm and the nucleus. Upon hypoxia and hypoxia-mimetic agents, mTOR localizes mainly in the nucleus, and, in particular at the NBs level. PML would act as a negative regulator of mTOR itself, leading to a decrease of the transcription factor HIF-1α (hypoxia-inducible factor 1α) synthesis and consequently to a block of neovascolarization.

Accordingly, in PML ko cells show a defect in mTOR nuclear accumulation and PML -/- tumors have increased angiogenesis.

In conclusion, the presence of proteins involved in DNA repair and cell cycle control (Negorev and Maul, 2001) (Hofmann and Will, 2003) within the nuclear bodies and the dynamic response of the latter to a variety of stresses imply that PML NBs may exert multiple roles in the cellular stress response.

Several models have been proposed for the function of PML bodies. In the first model, PML NBs have no direct function but represent aggregates of excess nucleoplasmic protein (Negorev and Maul, 2001). In a second model, the bodies may function as sites of post-translational modification and degradation of proteins. In a third model, PML NBs may function as sites of specific nuclear

events, such as transcriptional regulation, DNA replication and viral defence. Nevertheless, despite the large number of studies carried out, the physiological role of NBs within the cell is still under debate.

A cytoplasmic PML isoform, in which a nuclear export sequence (NES) in exon 9 is retained, is also generated as a consequence of the alternative splicing. This isoform can shuttle between nucleus and cytoplasm and it's not localized at NBs. In a recent work, this cytoplasmic PML protein has been implicated in the modulation of TGF-β signaling (Lin et al., 2004).

2.5. PML Function

The study of mice in which PML has been inactivated by homologous recombination has led to the conclusion that the PML gene is not required for viability, at least in mouse. PML ko mice develop normally and do not get spontaneous cancers. However these animals are more sensitive to tumor promoting agents. When exposed to the tumor initiator dimethylbenzanthracene (DMBA) followed by treatment with the tumor promoter 12-o-tetradecanoylphorbol-13-acetate (TPA), PML null mice developed skin papillomas at a much higher frequency than wild type controls. Moreover, the injection of DMBA into the salivary glands, a procedure that normally produces sarcomas and fibrosarcomas in wild type animals, in PML-/- mice induced more tumors with a different spectrum: T and B lymphomas and malignant fibrohistiocytomas (Wang et al., 1998a). PML can therefore antagonize the initiation, promotion and progression of tumors of different histological origins, behaving *in vivo* as a tumor suppressor. Consistent with these findings, the over expression of PML inhibits the transformation of rat embryo fibroblasts induced by Ha-Ras expression in combination with oncogenic mutants of p53 or c-Myc (Mu et al., 1994) and a dramatic drop in the capacity of the cell lines to form colonies in a typical colony formation

assay (Fagioli et al., 1998). PML can exert its growth suppression activity either through the induction of apoptosis or replicative senescence.

2.5.1. PML and p53-dependent apoptosis.

The first evidence that PML is involved in the apoptotic pathway p53-dependent comes from experiments carried out in PML-/- mice. These animals are resistant to lethal effects of ionizing radiation (Wang et al., 1998b). Accordingly PML null splenocytes are resistant to γ-irradiation induced apoptosis (Guo et al., 2000). In these cells DNA damage induced apoptosis depends entirely on the presence of normal p53 function. P53-/- cells, in fact, are completely insensitive to irradiation, while PML-/- cells display an intermediate phenotype, suggesting that PML may be required only for a proper p53 pro-apoptotic function. Upon γ-irradiation p53 becomes stabilized and activated by post-translational modifications that take place at several residues. The DNA damage checkpoint kinase Chk2 is responsible for serine 20 modification. PML recruits Chk2 into PML NBs and enhances p53 phosphorylation (Yang et al., 2002). Similarly, the homeodomain-interacting protein kinase-2 (HIPK2) has been found to colocalize with PML within the nuclear bodies (Hofmann et al., 2002) (D'Orazi et al., 2002). HIPK2 is responsible for phosphorylating p53 on ser 46 upon ultraviolet irradiation, an event that has been linked to induction of apoptosis (Oda et al., 2000). A further enzyme affecting p53 activity and localizing to NBs is the herpesvirus associated ubiquitin-specific protease. A fraction of the main p53 regulator MDM2 was also found to be associated with PML NBs, in particular after the inhibition of the nuclear export (Lain et al., 1999). Recently it was shown that PML and MDM2 interact (Wei et al., 2003) and that upon DNA damage PML protects p53 from MDM2-mediated ubiquitination and degradation (Louria-Hayon et al., 2003). In addition following UV irradiation

the two different complexes PML/p53 and PML/MDM2 take place, leading to p53 stabilization (Kurki et al., 2003).

2.5.2. PML in p53-Independent Apoptosis.

Splenocytes and hepatocytes from PML-/- mice present defect in apoptosis induced by Fas and TNF. These two agents induce extrinsic apoptotic pathways activation considered p53-independent (Wang et al., 1998b). Since the death receptors are located at the plasma membrane and PML NBs reside into the nucleus, a protein involved in the transduction of the signal is necessary. DAXX is the best candidate for this role. DAXX was originally cloned as a Fas-interacting protein and found to act as a positive mediator of Fas and TNF induced apoptosis.

Two possible models explaining the cooperation between PML and DAXX in regulating apoptosis exist (Bernardi and Pandolfi, 2003). In the first one, upon Fas-ligand binding, DAXX moves to the nucleus where it directly interacts with PML. DAXX is a transcriptional repressor and PML is thought to antagonize its transcriptional function by titrating it in the PML NBs. Therefore DAXX cannot suppress anymore the transcription of anti-apoptotic genes and cell undergoes apoptosis. The second model proposes that the PML/DAXX complex is released from the nuclear bodies upon PML desumoylation by Supr-1 or upon DAXX phosphorylation by the homeodomain-interacting protein kinase 1 (HIPK-1). The PML/DAXX complex could have transcriptional functions and therefore could activate pro-apoptotic genes. The precise mechanism, however, is still unknown.

2.5.3. PML and Senescence.

The replicative senescence described for the first time in the 1961 by Hayflick and Moorhead is an irreversible state of cell proliferation arrest triggered by telomere attrition. An identical endpoint can be produced in response to oxidative stress, DNA

damage, chromatin remodeling and intense mitogenic signaling. These non-telomeric stimuli induce senescence, defined "premature" only after a few cell divisions (Serrano and Blasco, 2001).

The Rb and p53 tumor suppressor are important regulators of senescence. Senescence is accompanied by increased levels of p53 and hypophosphorylated Rb (Atadja et al., 1995) (Shay et al., 1991) and enforced expression of either proteins induces senescence in some cell types (Ferbeyre et al., 2002). The p53 is a transcriptional regulator controlling a wide range of genes that cause cell-cycle arrest, like p21, and its transcriptional activity and stability increased in senescent cells (Itahana et al., 2001). The p53 can be activated by a variety of signals, some of which produce specific p53 post-translational modifications such as acetylation and phosphorylation (Appella and Anderson, 2001). A link between premature senescence, PML NBs and p53 has been established. In primary fibroblasts PML overexpression induces premature senescence (Fig.22) in a p53-dependent manner, since p53 null mouse embryo fibroblasts are protected from this effect.

PML itself is up regulated during replicative or Ras-induced senescence (Pearson et al., 2000). The resulting increase in PML protein levels leads to a concomitant increase in number and size of PML NBs (Fig.21c). The expression of the activated form of Ras (Ras^{VAL12}) induces also the relocalization of p53 within the PML NBs and promotes its acetylation at lysine 382. Acetylation is essential for p53 biological function and is profoundly impaired in PML-/- MEFs. Although PML does not possess intrinsic acetyltransferase activity, it directly interacts with the acetyltransferase CBP/p300, recruiting it to the nuclear bodies. Therefore PML, p53 and CBP/p300 form a tricomplex that localize to PML NBs, favoring in this way p53 acetylation by CBP/p300. In conclusion, PML is required for p53 acetylation and senescence upon oncogene expression. However the conclusive evidence that PML, like p53, is essential for the induction of cellular senescence upon oncogenic transformation comes from the observation that in

PML null cells, Ras^{VAL12}-induced senescence is drastically impaired. Recently, the NAD-dependent deacetylase SIRT1, a negative regulator of p53, has also been found in NBs. SIRT1 binds to p53 and deacetylases specifically lysine 382, resulting in decreased p53-dependent transcription and PML-induced senescence (Langley et al., 2002).

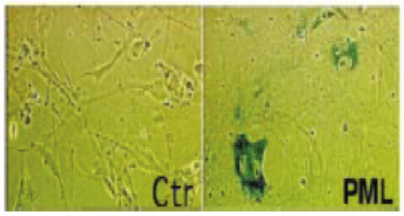

Figure 22. PML induces Premature Senescence. β-Galactosidase staining of Mefs infected with the control vector (Ctr) or PML. A cytological marker of senescence cells is the expression of a senescennce-associated b-galactosidase activity (SA-b-gal).Murine fibroblasts overexpressing PML, morphologically, become flat and enlarged and are strongly positive for SA-b-gal.

2.5.4. PML and telomeres

As previously mentioned a specialized type of PML NBs, called APBs, has been found exclusively in telomerase-negative tumours in which telomeres are maintained by recombination-based alternative (ALT) mechanisms (see above paragraphs). A list of recent works suggest the existence of a functional link between the presence of APBs and the ALT mechanism, and also a role for PML in telomere maintenance.

First of all, different laboratories focused their attention on the dynamic interaction between telomeres and PML NBs. Telomeres are firmly attached to the nuclear matrix throughout the interphase, and are considered important for the intranuclear positioning of chromosomes. However, the use of a fluorescently labelled PNA (peptide nucleic acid) probe that specifically target the telomeric DNA repeats introduced in living human osteosarcoma U2OS cells (ALT positive), allowed to observe the telomeres dynamic in vivo (Molenaar et al., 2003). This study revealed that, while the majority of telomeres are relatively immobile and show

constrained diffusion in a small submicron-sized nuclear volume, a subset of telomeres has a much higher degree of mobility. This work also showed that telomeres have the ability to associate with each other in a dynamic manner, forming telomere clusters. Telomere association might provide a possibility for spontaneous recombination of subtelomeric DNA, which happens at high frequency in somatic cells (Flint et al., 1995), and for recombination-based interchromosomal telomeric DNA exchanges observed in ALT cells. In addition they observed a dynamic movement of telomeric DNA to relatively immobile PML NBs.

Another recent paper focuses on telomeres mobility during interphase and proposes a model to explain the formation of complexes between telomeres and PML NBs (Jegou et al., 2009). The model used in this work is a U2OS cell line that have lac operator repeats stably integrated adjacent to the telomeres of three chromosomes. An autofluorescent LacI repressor bounds to the lacO and allows observing by fluorescence microscopy the telomeres movements. This study confirmed the existence of a majority of telomeres which move within a confined radius, and of a small fraction of telomeres with more extended translocations. In addition they inversely correlated the increased mobility with the length of telomere repeat sequence. The hypothesis is that upon shortening of telomeres, the telosome protein complex, which promotes interactions with other chromatin domains and mediates its anchoring within the nucleus, is disrupted. This event favours the loss of telomeres association with heterochromatin regions, and allows a higher mobility. The mobility of short telomeres could favour their association with a pre-existing PML NB. In the second step of the process, additional PML protein from the nucleoplasm is accumulated at this locus to form the typical APB structure. The accumulation in the PML NBs of many proteins associated with DNA recombination and repair activity, suggests that these structures could function as sites for telomere lengthening (Fig.23). When the number of repeats becomes sufficiently high the shelterin proteins can bind again to re-establish the telomere

complex and promote interactions with other chromatin domains to induce a state of low mobility, as observed for the majority of telomeres (Jegou et al., 2009).

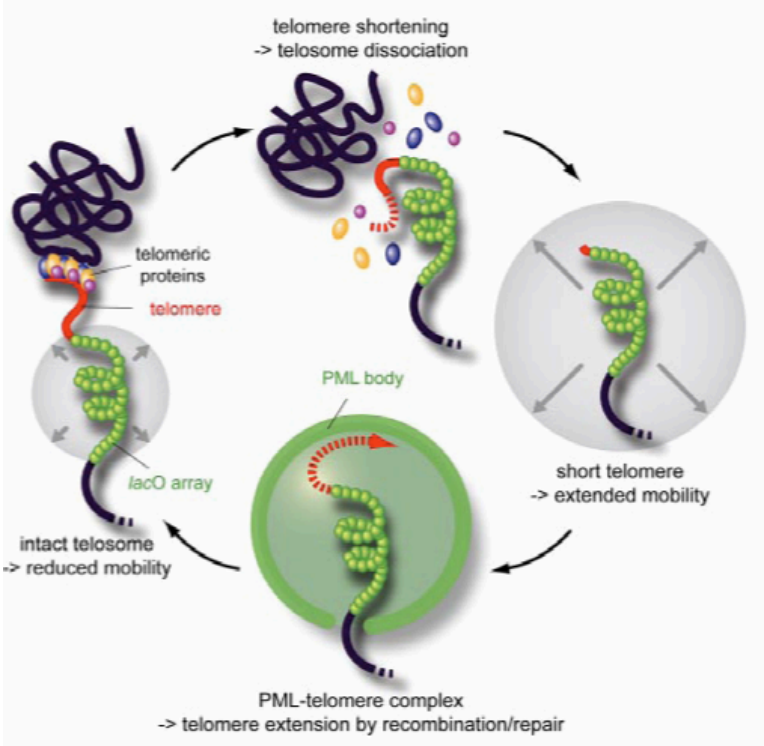

Figure 23. Model for APBs formation. In telomeres with sufficient repeat length the telosome protein complex mediates the anchoring to another chromatin domain. This results in a confined mobility of the telomere that is similar to that of other chromatin loci. When the telomere repeat length is reduced below a critical threshold, the telosome is disrupted, and the anchoring is lost. In this state the telomere displays a significantly extended mobility. The telomere associates with a PML body to form an APB complex, in which the telomere length is increased in a process that involves DNA recombination and repair. The telosome can reform on the extended telomere repeat sequence to reestablish the telomere anchoring associated with the reduced mobility state. (Jegou et al. 2009)

In another paper of Draskovic et al., evidence that PML NBs are not only a marker for ALT cells, but play a direct role in telomere recombination was provided. This work uses an innovative approach to gently enlarge PML bodies in living cells without perturbing their organization. This technique permits to reveal that

PML NBs in ALT cells associate with chromosome ends forming clusters of 2- 5 telomeres. In this way PML NBs bring together chromosome ends and promote telomere-telomere interactions between heterologous chromosomes, providing the required physical proximity for recombination (Draskovic et al., 2009).

All these works, based on innovative and sophisticated microscopy analysis, gave us deep information about the dynamic interactions of telomeres and PML NBs. In addition to these, other recent studies based on biochemical approaches, show PML interactions with factors important for telomere stability. PML IV in fact interact with TERT, the catalytic component of telomerase (Oh et al., 2009). The specificity of TERT interaction with PML IV isoform is due to its C-terminal region. PML IV functions as a negative regulator of telomerase, because cell lines stably expressing PML IV display decreased telomerase activity and shortened telomeres. It is possible that TERT recruitment to PML NBs, through its interaction with PML IV, could favour post-translational modification of the protein with consequent effects on its activity, as in the case of p53.

PML IV also interacts directly with TRF1 and recruits TRF1, and consequently telomeres, to PML NBs in ALT cells. Then, sumoylation of shelterin proteins by MMS21 could occur in PML NBs, favouring the localization of telomeres at PML bodies (Yu et al., 2010). Although details of the molecular mechanism of ALT are largely still unknown, the data suggest an essential role for PML IV in APBs formation.

However, a general link between PML NBs and telomere could be also hypothesized for every cell types, and not only for ALT cells.

PML-telomere associations remained unnoticed except for ALT cells containing ALT associated PML bodies. However, an up-to-date work shows that PML bodies associate temporary with telomeres during their formation, in several cell types, ALT and non-ALT. De novo formation of PML NBs in cells in which they were dispersed by chemical agents, occurs mainly at telomeric

DNA sequences (Brouwer et al., 2009). It is probably in ALT cells only that newly formed PML NBs remain associated with telomeric DNA, while they dissociate rapidly from telomeric sites in other cell types. A potential explanation could be that PML is recruited to SUMOylated telomere binding proteins by the SUMO binding domain present in PML. SUMOylation of PML may than lead to the recruitment of more PML protein and other components of PML NBs.

This study establishes a role for telomeric foci in the recruitment of PML protein, without still giving an explanation of the possible functional meaning of this event.

It would be interesting to explore the role of PML interaction with telomeres, in ALT and non-ALT cells, which could give much insights both into the function of PML protein and in the regulatory pathways of telomere maintenance.

RESULTS AND DISCUSSION

1. PML is recruited to damaged telomeres

1.1. PML NBs localize to telomere in all cell types.

It is well established that PML NBs are localized at telomeres in ALT cell lines but little is known about PML interaction with telomeres of normal cells, or telomerase-positive tumour cell lines.

We investigated the localization of PML at telomeres in different cell types:

- three normal fibroblasts (WI38, MRC-5 and MEFs);
- three telomerase-positive cell lines (HeLa and A549 and U937);
- three ALT cell lines (U2-OS, SK-LU-1 and SA-OS-2), as positive control of the presence of PML at telomeres.

We used ImmunoFISH experiments, in which cells were stained with a fluorochrome-conjugated Peptide Nucleic Acid (PNA) probe that recognizes telomere repeat sequences in interphase nuclei, together with an antibody for the PML protein.

Normal human fibroblast cells, and telomerase-positive cells showed some co-localizations between PML NBs and telomere ends (Fig.1a). The frequency of this co-localizations this cell types ranges from 0,36 to 5,86 co-localizations per cell. This frequency is less than that observed in ALT cell lines, ranging from 3,6 to 9,3 co-localizations per cell (Fig. 1b).

A statistical analysis in each cell type, carried out on 50 cells, shows that the percentage of PML NBs localized to telomeres in normal and telomerase-positive cells is little but consistent, spanning from 4% to 34% of PML NBs total number. The result between all the different non-ALT cell types are comparable, and seem independent of the number of PML spots per cells, rejecting the idea of random associations (Fig.2).

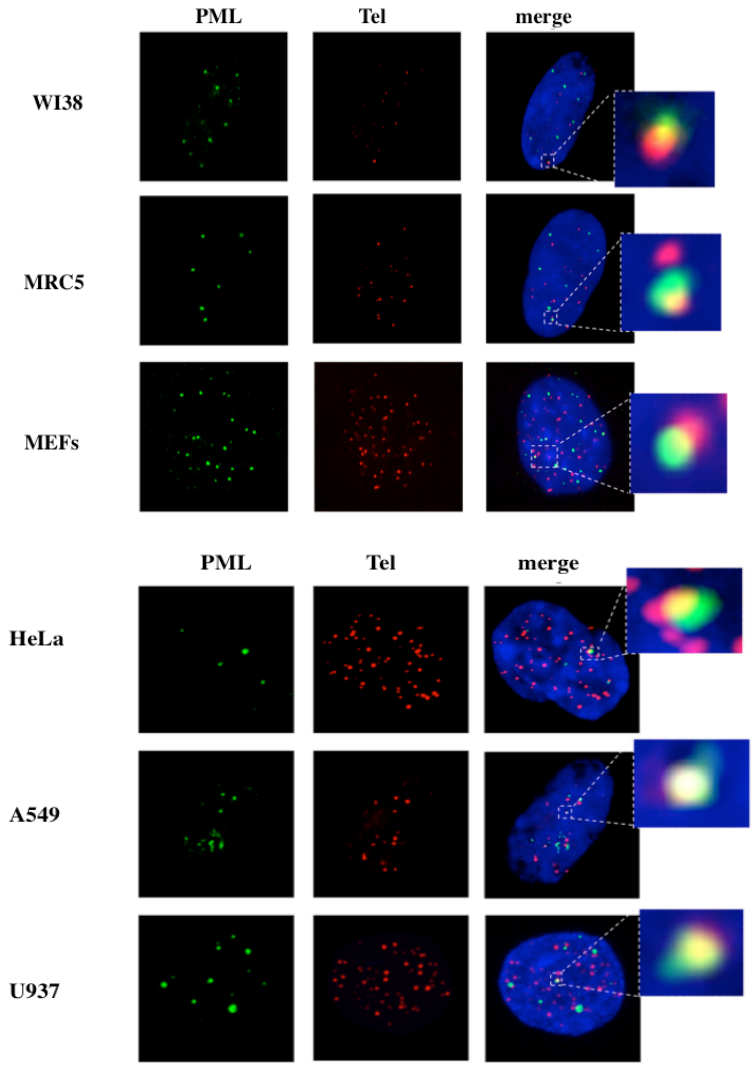

Figure 1a. Localization of PML NBs at telomeres. PML localization at telomeres is evaluated through an ImmunoFISH approach. Cells were stained with a probe recognizing telomeric DNA (red) and with anti-PML antibody (green). DNA was stained with DAPI (blue). Enlargements show PML NBs associated with telomeric repeats. Representative microscopy images of three different normal fibroblasts cell lines (at the top) and three telomerase-positive cell lines (at the bottom) are shown.

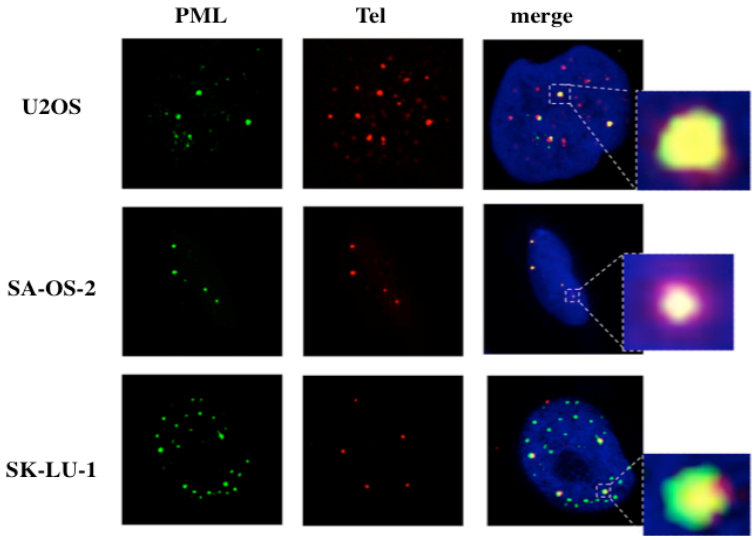

Figure 1b. Localization of PML NBs to telomeres. PML localization to telomeres is evaluated through an ImmunoFISH approach, as in figure 1a. Representative microscopy images of three different types of ALT- positive cell lines.

	Cell Type	n° cells scored	n° PML spots	n° PML/Tel coloc	PML bodies/cell (mean)	PML/Tel coloc/cell (mean)	PML/Tel coloc/cell (%)
Normal cells	WI38	50	372	52	7,44	**1,04**	**14**
	MRC-5	50	406	25	8,12	**0,5**	**6**
	MEFs	50	451	18	9,02	**0,36**	**4**
Tel + cancer cells	U937	50	856	293	17,12	**5,86**	**34,2**
	HeLa	50	200	44	4	**0,88**	**22**
	A549	50	77	20	1,54	**0,4**	**26**
ALT cancer cells	U2OS	50	276	220	5,52	**4,4**	**80**
	SK-LU-1	50	1578	436	31,43	**9,3**	**29,6**
	SA-OS-2	50	312	181	6,26	**3,62**	**58**

Figure 2. Statistic analysis of PML/Telomeres co-localizations. ImmunoFISH staining for telomeric DNA and PML was performed. Fifty cells per type were scored and the number of PML spots or PML/telomeres associations (bold) was counted. The last column shows the percentage of PML NBs co-localizing with telomeres, respect to the total number of PML spots in the cell.

Thus, here we demonstrate that PML NBs can be found to telomeres not only in ALT cells, as showed in several previous works, but also in normal and telomerase positive cells.

The small amount of PML NBs localizing to telomeres in cells in normal growth conditions may reflect a small subset of telomeres wich are in a particular physiological situation that needs the association with this structures. This rare phenomenon can be explained, for example, with the small amount of functional telomeres that can be recognized from the DDR machinery in cells at the steady state (Verdun et al., 2005). Alternatively, the recruitment of PML to telomeres, can be part of DNA-repair mechanism. In fact, it is well established that PML NBs are sites that act as sensors of DNA damage and stress and many DNA repair and checkpoint proteins dynamically localize to PML-NBs (Dellaire and Bazett-Jones, 2004)

1.2. PML NBs are recruited to damaged telomeres after telomere-specific damage.

To investingate the link between PML and the mechanisms involved in telomere damage response, we performed experiments in telomere-specific damage conditions. We took advantage of different systems to induce a specific damage to telomeres, allowing analysis of PML NBs response in different cell types:

- expression of a dominant negative isoform of TRF2 (telomere associated capping protein);
- treatment with RHPS4, a G-Quadruplex that destabilize shelterin structure;
- RNA interference targeting TRF2 protein;

The first model system used to test this hypothesis is provided by HTC75, a telomerase-positive human fibrosarcoma cell line that expresses an inducible dominant negative allele of TRF2 ($TRF2^{\Delta B \Delta M}$), able to bind the endogenous protein and prevent its association with telomeres.

The truncated dominant-negative form of TRF2 is expressed in HTC75 in a doxycyclin-controlled inducible manner. The HTC75 cells, cultured in a medium without doxycyclin, express the dominant negative TRF2 protein (doxycyclin acts as a repressor of gene expression in this system. On the eighth-ninth day after the induction, HTC75 cells underwent senescence due to telomere ends uncapping and shortening, and activation of the DNA damage checkpoint response (van Steensel et al., 1998).

Control HTC75 cells presented a population of cells (25%) with 10 to 30% of PML NBs co-localizing with telomeres. Remarkably, co-localization of PML-NBs with telomeres was found in a higher percentage (75%) of induced senescent cells than in control cells and a subset of these cells reach even 70-80% of PML co-localizing with telomeres (data not shown). In addition, it appears that PML is recruited to Telomere-dysfunction Induced Foci (TIFs), identified as γ-H2AX/Telomere co-localizations (Fig.3a). Statistical analysis revealed a significant increase of PML to TIFs (Fig.3b). These data are the first suggestions that purpose a role for PML in telomere-associated DNA-damage cellular response, in telomerase positive tumour cells.

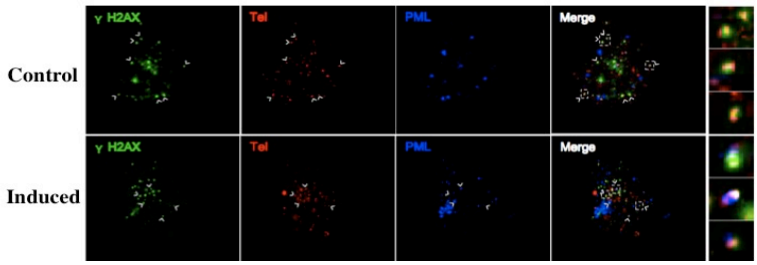

Figure 3a. Recruitment of PML NBs to telomeres in induced senescent cells. HTC75 cells uninduced (control) or induced for TRF2$^{\Delta B\Delta M}$ expression were stained for γH2AX (green), telomeric DNA (red) and PML (blue), following the ImmunoFISH protocol. A representative image reveals the localization of PML to damaged telomeres (telomeres occupied by γH2AX) in induced cells. White arrows indicate co-localizations, some of them shown in enlargements.

Similar results were obtained in experiments using the second model that we choose to induce TIFs: RNA interference of the telomere-capping protein TRF2. Down-regulation of TRF2 protein

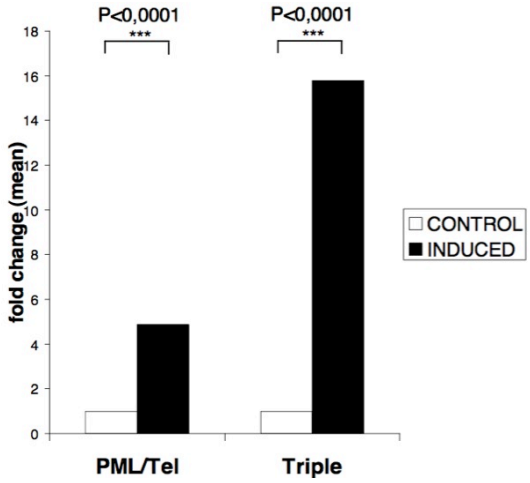

Figure 3b. Recruitment of PML NBs to telomeres in induced senescent cells. HTC75 cells uninduced (control) or induced for TRF2$^{\Delta B \Delta M}$ expression were stained for γH2AX , telomeric DNA and PML, as showed in figure 3a. A statistical analysis of the number of PML/ Telomeres co-localizations and of PML/ γH2AX/ Telomeres triple co-localizations was carried out on 50 cells per type. The graph represents the mean fold change. The P value is based on a two-tailed Student's t-test.

has been abundantly showed to result in telomere uncapping and senescence or apoptosis, depending on p53 status of the cells (Karlseder et al., 1999; Stagno D'Alcontres et al., 2007; Takai et al., 2003).

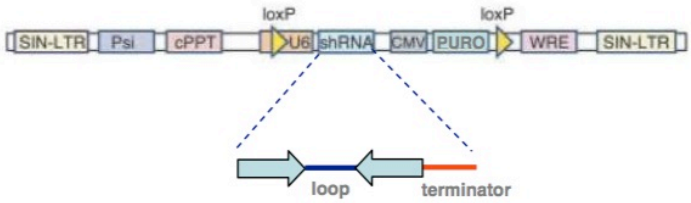

Figure 4. Schematic representation of the shRNA-expressing lentivirus vector pSicoR. U6: RNA polymerase III promoter; shRNA: a sequence able to silence the target gene, as a siRNA, has adapted with a loop sequence to create a stem loop (in red the Pol III terminator stretch); CMV: cytomegalovirus promoter; PURO: resistence for puromycin expressing cassette (Ventura et al., 2004).

We used a short hairpin RNA (shRNA)-expressing lentivirus vector, pSicoR (Ventura et al., 2004). This system allows silencing gene expression as efficiently as synthetic siRNA does, and the shortness of shRNA avoids the antiviral response that would lead to nonspecific inhibition of translation and therefore cytotoxic effects. Moreover, lentiviral vector can be integrated into the genome of the cell, independently by the proliferation of the cell, resulting in its stable expression even in cell with low growth rating.

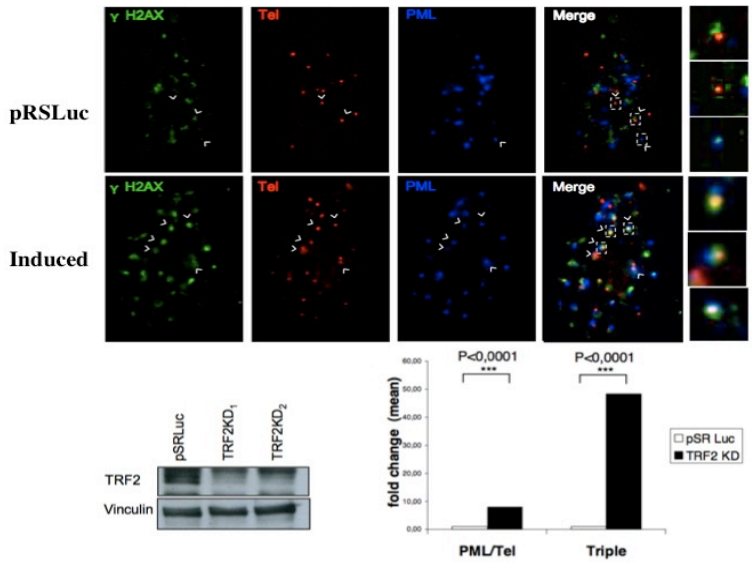

Figure 5. PML NBs association with damaged telomeres in TRF2 knock down cells. WI38 cells were infected with shRNA to inhibit TRF2 protein (TRF2 KD), or with a control vector (pSR Luc). Cells were stained for γH2AX (green), telomeric DNA (red) and PML (blue). The representative image reveals the increase of localization of PML to damaged telomeres (telomeres co-localizing with γH2AX) in cells infected with TRF2 KD1 respect to the control. White arrows indicate co-localizations, some of them shown in enlargements. Lower, on the left, western blotting analysis showing the efficiency of RNAi –mediated knock down in WI38 cells. Lower, on the right, the graph illustrating the statistical analysis of PML/telomeres co-localizations and triple (γH2AX /telomeres/PML) co-localizations, represented as fold change and carried out on 50 cells per type. P value is based on a two-tailed Student's t-test.

An RNA polymerase III promoter, the U6 promoter, drives the expression of shRNA cloned downstream. The shRNA is a short

stretch of inverted DNA sequence that is processed by Dicer to generate siRNA. Downstream of the U6 promoter and the gene specific targeting sequence, there is a CMV-Puro cassette in order to simultaneously produce shRNA and the resistence gene for puromycin, allowing to easily select infected cells (Fig.4).

Different oligos were tested and two of them were chosen for their silencing efficiency in downregulating TRF2 protein expression (TRF2 KD1, TRF2 KD2), evaluated both in western blotting analysis and in indirect immunofluorescence assays (see Western Blotting in Fig.5). As a control we generated a plasmid containing a shRNA that targets luciferase, a gene not present in mammals (pSicoR Luc).

Different normal human fibroblasts, infected with TRF2 KD1 and TRF2 KD2, underwent senescence in few days, as expected, and the number of PML NBs respect to the control (pSicoR Luc) increased. As for HTC75 expressing the dominant negative form of TRF2, also fibroblasts infected with TRF2 KD1 show an increased number of co-localizations of PML to telomeres, and in particular with TIFs (Fig.5). The same result was obtained with the other oligo TRF2 shRNA, TRF KD2, ruling out off-target shRNA effects (data not shown), but not with control shRNAs (pSicoR Luc).

The third model of telomere damage is based on the G-quadruplex ligand, RHPS4. This compound, at high doses, delocalizes POT1 from telomeres and induces TIFs formation in few hours (see introduction, paragraph 2.1.3). Normal fibroblasts (WI38) or ALT cells (U2OS) were treated with 1μM RHPS4 for 24 h. After this treatment, cells show only a small increase of TIFs number. Even if the effects of the treatment are not as strong as for TRF2 protein inhibition, RHPS4 induced recruitment of PML to telomeres in both normal and ALT cells (Fig. 6). All together these data strongly suggest that PML takes part in DNA-damage response to dysfunctional telomeres. In conditions of generalized telomere damage, independently by the system used and by the target cellular system (normal or tumour, ALT or telomerase-

positive), the association of PML NBs with telomeres is greatly enhanced. PML NBs increase in number but are also prevalently recruited to telomeres, because the percentage of PML NBs associated to damage telomeres increases respect to the total number of PML NBs.

Nevertheless, microscopy analysis in living cells is necessary to study the dynamic association between telomeres and PML NBs and to elucidate if there is a redistribution of PML NBs to damage telomeres rather than a de novo formation of PML NBs at these telomeres.

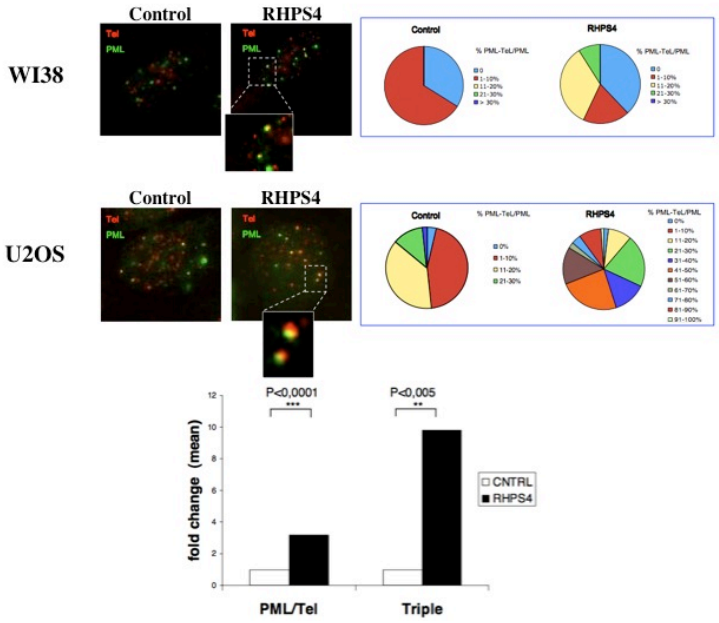

Figure 5. PML NBs recruitment to dysfunctional telomeres induced by treatment with RHPS4. WI38 normal fibroblasts and U2OS ALT cells were treated with RHPS4 1μM for 24h. Co-localizations PML/Telomeres were evaluated by ImmunoFISH experiments and expressed in the graphs as classes of frequency of the percentage of PML found associated with telomeric DNA, respect to the total number of PML spots in the cell, counted in 50 cells. The lower panel consists of the graph illustrating the statistical analysis of PML/telomeres co-localizations and triple (γH2AX /telomeres/PML) co-localizations, represented as fold change and carried out in WI38 cells, on 50 cells per type. P value is based on a two- tailed Student's t-test.

This last hypothesis could be supported by a recent work that shows a role for telomeric DNA in the formation of PML NBs (see introduction) (Brouwer et al., 2009).

1.3. PML NBs are recruited to telomeres of senescent fibroblasts and stem cells.

Each time a cell divides telomereic DNA shorten until it reaches a critical length and it becomes unable to maintain the proper shelterin macromolecular structure, thus unveiling single-strand DNA ends that are recognized by DNA damage surveillance machinery. These events activate the pathways that lead the cell to die or enter senescence (see introduction). For this reason, normal cells in vitro can undergo a limited number of population doublings before entering replicative senescence.

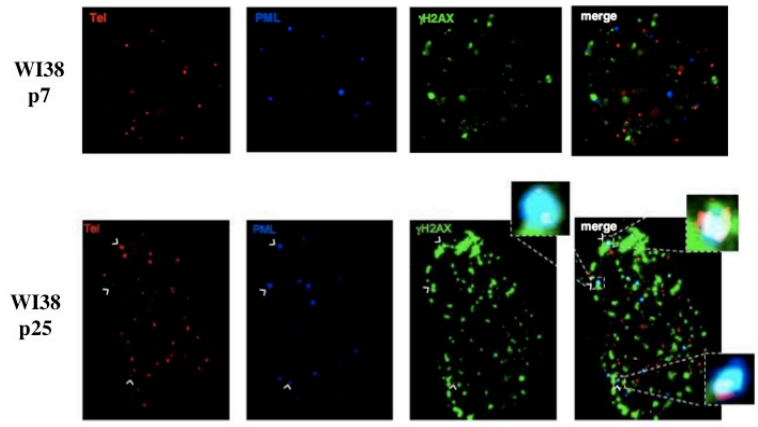

Figure 7a. PML NBs recruitment to dysfunctional telomeres in late passages of human fibroblasts. WI38 were stained for telomeric DNA (red), PML (blue) and γH2AX (green), following the ImmunoFISH protocol. A representative image reveals the localization of PML to damaged telomeres (telomeres occupied by γH2AX) in a late passage (WI38 p25) respect to an early passage (WI38 p7) of culture of human fibroblasts. Some co-localizations are shown in enlargements.

The normal telomere attrition due to serial passages in vitro has been used as a physiological model of telomeric damage. WI38

normal fibroblasts have a finite lifetime of 50 (plus or minus 10) population doublings with a doubling time of 24 hours. We performed ImmunoFISH staining to analyze PML localization to telomeres in WI38 at early passage (p7) respect to WI38 at passage 25, corresponding to about 32 population doublings. At this late passage, WI38 cells start to show senescent phenotype. The number of TIFs per cell increases from 1,78 of the control early passage cells to 4,86 of the late passage cells (Fig. 7a and 7b).

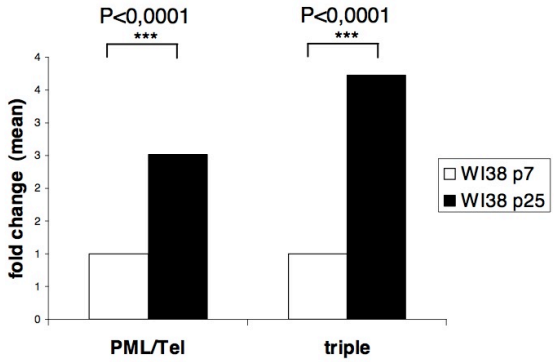

Figure 7b. PML NBs recruitment to dysfunctional telomeres in late passages of human fibroblasts. WI38 were stained for telomeric DNA (red), PML (blue) and γH2AX (green), as in figure 6a. The graph illustrates the statistical analysis of PML/telomeres co-localizations and triple (γH2AX /telomeres/PML) co- localizations represented as fold change, carried out on 50 cells per type. P value is based on a two-tailed Student's t-test.

Statistic analysis on fifty cells revealed that PML NBs increase in number (data not shown) and are associated with telomeres at higher percentage in late passages respect to early passages. PML NBs are mainly recruited to telomeres associated with DNA-damage foci.

Similar results were obtained performing experiments on another normal diploid fibroblasts cell line, MRC-5 (data not shown).

These results demonstrate that PML is involved in the response of the cell to telomere attrition caused by prolonged culture of cells in vitro.

Aging is associated with reduction of stem cells function. The molecular mechanisms of stem cells aging seem to involve telomere dysfunction that leads to induction of cell cycle checkpoints. Although most stem cell compartments express telomerase, the level of telomerase activity is not sufficient to maintain telomere length of stem cells during aging. Stem cells appear to have tighter DNA damage checkpoint control in comparison to somatic cells, which may reflect the need to protect this long lasting cell compartment against malignant transformation (Ju and Rudolph, 2006).

PML is also involved in telomere-associated DNA damage response in aged stem cells? To address the issue we performed experiments on early and late passages of amniotic fluid-derived stem cells. These cells were isolated from amniotic fluid as previously described (Orciani et al., 2008), characterized for stem cells markers in the laboratory of Prof. Donti, and kindly provided. An early (p5) and a late (p18) passage of amniotic stem cells were compared for the number of co-localizations between PML NBs and telomeres, by ImmunoFISH performed with a PNA probe for telomeric DNA and an antibody directed against PML protein, as previously done in WI38 experiments. The number of PML spots associated with telomeres increases significantly in the late passage of amniotic stem cells, which show SA-β-galactosidase (SA-β-gal) activity, a classical marker of cellular senescence (data not shown).

Telomeres co-localizing with PML NBs augment in response to a telomere attrition generated by prolonged culture of primary cells or stem cells in vitro, evidencing that the presence of PML is required not only by a generalized condition of telomere damage, but also by a physiological type of damage. The association of PML with damaged telomeres, generated by telomere shortening that occurs after several rounds of replication, underlines the involvement of the protein in mechanisms of cellular aging.

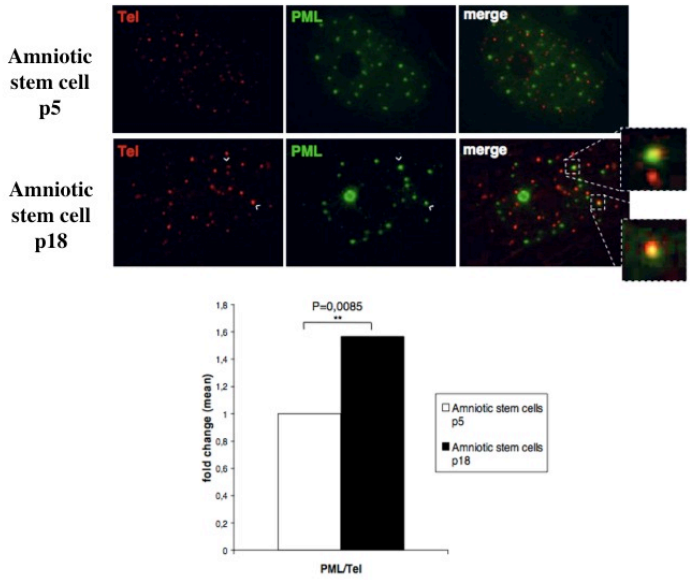

Figure 7. PML NBs recruitment to telomeres in late passages of amniotic fluid-derived stem cells. Amniotic stem cells were stained for telomeric DNA (red), PML (blue) and γH2AX (green), following the ImmunoFISH protocol. A representative image reveals the increase of localizations of PML to telomeres in a late passage (p18) respect to an early passage (p5) of culture of amniotic stem cells. Some co-localizations are shown in enlargements. The lower graph illustrates the statistical analysis of PML/telomeres co- localizations. The statistic was carried out on 50 cells per type (amniotic stem cells at passage 5 versus the same cells at passage 18). P value is based on a two-tailed Student's t-test.

PML could take part in the repair of dysfunctional telomeres or in the signaling towards checkpoints that lead the aged cell to undergo cell cycle arrest or senescence. This role is essential because allows to avoid that cells continue to divide beyond their normal replicative limit and enter a stage of "crisis". Crisis is marked by massive genomic instability, which is the major step towards oncogenic transformation.

The data presented in this chapter demonstrate that PML is present at telomeres to mediate a DNA damage response. These observations prompted us to elucidate whether PML is actively involved in telomeres repair, maintaining telomere stability and preventing aging, or alternatively if it is involved in signaling of unrepaired telomeres towards cell cycle arrest, preventing cancer.

2. PML depletion induces growth inhibition

2.1. PML knockdown leads to premature senescence in normal fibroblast cell lines.

In order to study the effects of PML on cellular growth and telomeres stability, we used the same lentiviral system discussed in paragraph 1.2 to stably deplete TRF2 expression, but in these case the shRNA were designed to target PML mRNA.

Different oligos coding for shRNAs have been designed, and two of them have been chosen for their efficiency and used to perform all experiments. These two oligos target a region of PML within the exon 3, common to all PML isoforms. Hereafetr we will refer to them as PML Knock Down 1 and 2 (PML KD1 and PML KD2). As control we used a lentiviral vector coding a shRNA directed against a region of the gene luciferase, which is not present in mammals (pSicoR Luc). Downregulation of PML was checked by indirect immunofluorescence and western blotting analysis (Fig.9). The number of PML NBs and the protein levels of PML were strongly down-regulated by both PML KD1 and PML KD2, with the best efficiency for PML KD1. To test the stability of lentivirus-induced RNAi, we monitored the expression of PML in cells infected with PML KD1 and KD2 for two weeks after the end of the selection with puromycin. Over this period, cells showed no rise of the protein level (Fig.9).

We used this suitable system to study the effects of PML on the cellular growth of normal and tumor cell lines, performing each experiment at least with the two oligos tested (PML KD1 and PML KD2) to reject the hypothesis of off-target shRNA effects. We knocked down PML in two normal fibroblast cell lines, WI38 and MRC-5, and we observed that PML depleted cells have a remarkable reduced growth rate, as revealed by the growth curves performed with the two independent PML shRNAs (PML KD1 and PML KD2) (Fig.10a).

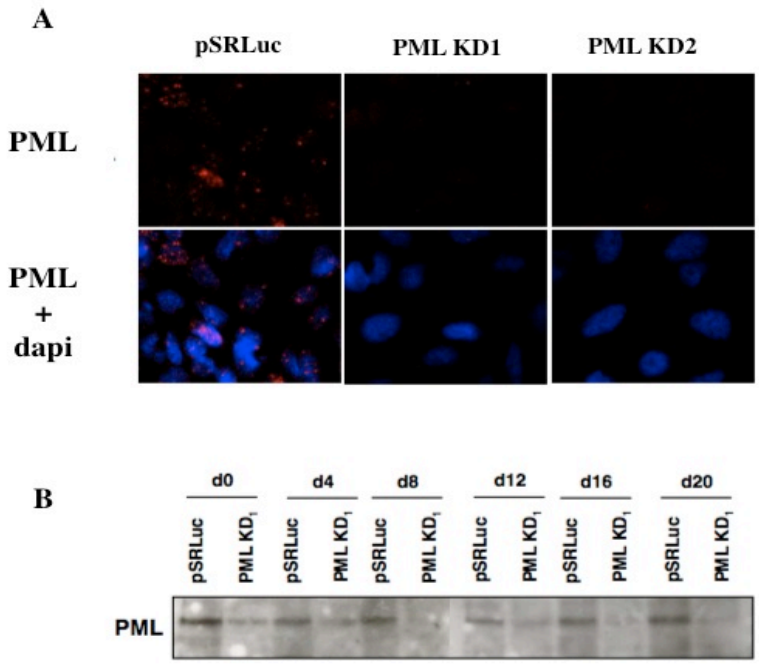

Figure 9. Efficiency of PML RNAi-mediated knock down in U2OS cells. a) Indirect immunofluorescence with an anti-PML antibody shows PML NBs disappearance in cells infected with two independent shRNA for PML (PML KD1 and PML KD2) respect to control cells (infected with pSR Luc). b) Western Blot analysis shows that the PML protein levels are stably reduced by RNAi with PML KD1 for 20 days.

WI38 and MRC-5 PML knock down (PML KD) cells undergo premature senescence before control cells. Senescence is showed by the morphological changes in cells shape that become flattened, vacuolated and enlarged. In addition PML knocked down cells show increased levels of SA-β-galactosidase staining. This assay is a good tool to detect senescent cells between an eterogeneous population as showed for the first time in 1995 (Dimri et al., 1995).

Another feature of senescent cells are the Senescence Associated Heterochromatin Foci (SAHF), heterochromatin structures responsible for E2F target genes repression, usually enriched for histone H3 methylated on lysine 9 (meH3K9) (Narita

et al., 2003). Fibroblasts deleted for PML present a more distinctive localization of meH3K9 than the control cells, consistent with its concentration in the DNA foci of senescent cells (Fig.10c).

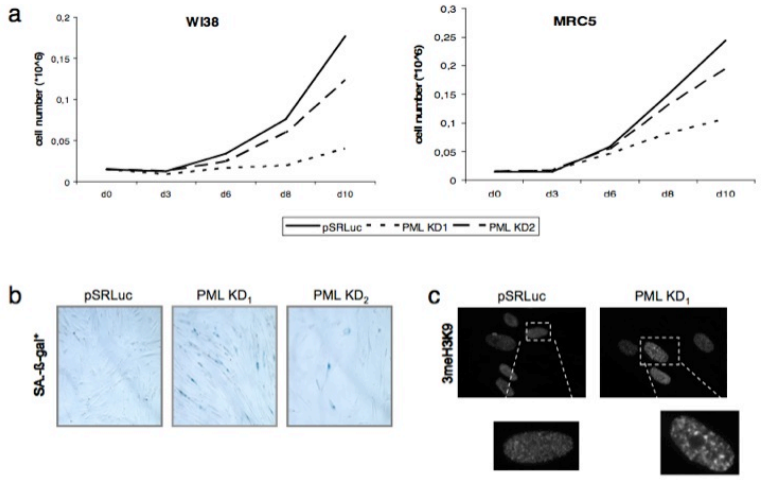

Figure 10. PML knock down in human fibroblasts leads to premature senescence. a) Growth curves of human fibroblasts infected with two different PML shRNAs or a control shRNA (pSR Luc). Cells were counted in the indicated days after selection. Curves are representative of three independent experiments. b) SA-β-gal-staining performed on WI38 cells, 6 days post selection. c) Indirect immunoflorescence with an antibody specific for 3meH3K9 modification shows accumulation of the modified histone in foci, typical of senescent cells.

The pathways activated during senescence in fibroblasts, involve the up- regulation of p53 and its target p21, and/or induction of p16 and ipo- phosphorilation of Rb (see introduction, paragraph 2.1.3). To investigate the pathways involved in premature senescence induced by PML depletion, western blot analysis has been performed on cellular lysates at different days after the end of selection with puromycin of infected cells. Up-regolation of the cell cycle regulators p53, p21 and p16 was detected (Fig.11) and it was maintained for up than ten days, both in WI38 and MRC-5 fibroblasts.

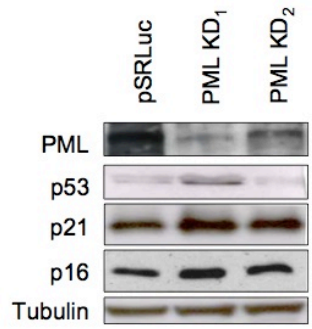

Figure 11. PML knock down in human fibroblasts activates p53/p21 and p16/Rb pathways to induce premature senescence. Western Blotting showing up-regulation of p53, p21 and p16 protein levels in WI38 cells after 6 days of PML RNAi with two constructs for silencing (PML KD1 and PML KD2).

In conclusion, PML knock down causes in human fibroblasts a growth inhibition that has the appearance of premature senescence, as demonstrated by positive SA-β-gal staining at pH6, presence of heterochromatin markers, like tri-metyl-H3K9, and activation of the typical pathways.

2.2. PML knockdown induces growth inhibition in ALT-positive tumour cells.

We extended the analysis of the effects of PML loss on cellular growth to ALT- positive tumour cells.

Two ALT-positive cell lines, U2OS and SK-LU-1, that do not express telomerase, have been infected with lentiviral particles, expressing shRNAs targeting PML (PML KD1 and PML KD2), or the control vector (pSR Luc), as described above. PML depletion was checked by immunofluorescence and western blotting. At the end of selection, $3x10^3$ cells were seeded in a 6 well plate and counted at the indicated days. Growth curves show a significant reduction of growth rate for PML knock down cells, respect to control cells (Fig.12).

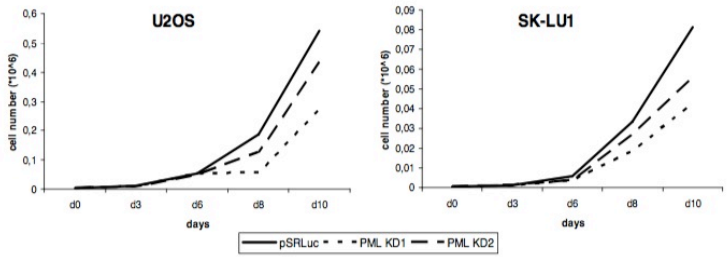

Figure 12. PML knock down causes reduction of growth rate in ALT-positive tumour cells. Growth curves of U2OS and SK-LU1 ALT cells, infected with two different PML shRNAs or a control shRNA (pSR Luc). Cells were counted in the indicated days after selection. The curve reported for each cell types is representative of three independent experiments.

To obtain an independent confirmation of the decreased growth potential of cell lacking PML, we performed a colony formation assay, which measures the clonogenic ability of a cell to divide and produce a colony when plated at low density.

Cells infected with the three different viruses, were plated, after the end of selection with puromycin, at a density of 500 cells in a 6 cm tissue culture dish, in triplicate. After two weeks in culture, colonies were stained and counted to evaluate the colonies number. Alternatively, colonies were counted at the microscopy, than trypsinized to count the total number of cells on the plate. The number of cells divided for the number of colonies gives us the mean value of cells for colony, which represents a measure of colonies size.

ALT cells in which PML is silenced have a reduced capacity to form colonies, evidenced by a lower number and a smaller size of formed colonies, respect to control cells infected with an irrelevant shRNA (Fig.13). Interestingly, the biological effect on cellular growth seems to be dose-dependent, because it is more pronounced for the PML KD1 construct that have stronger effect on PML protein levels than PML KD2. This observation allows us rejecting the objection of an off-target effect.

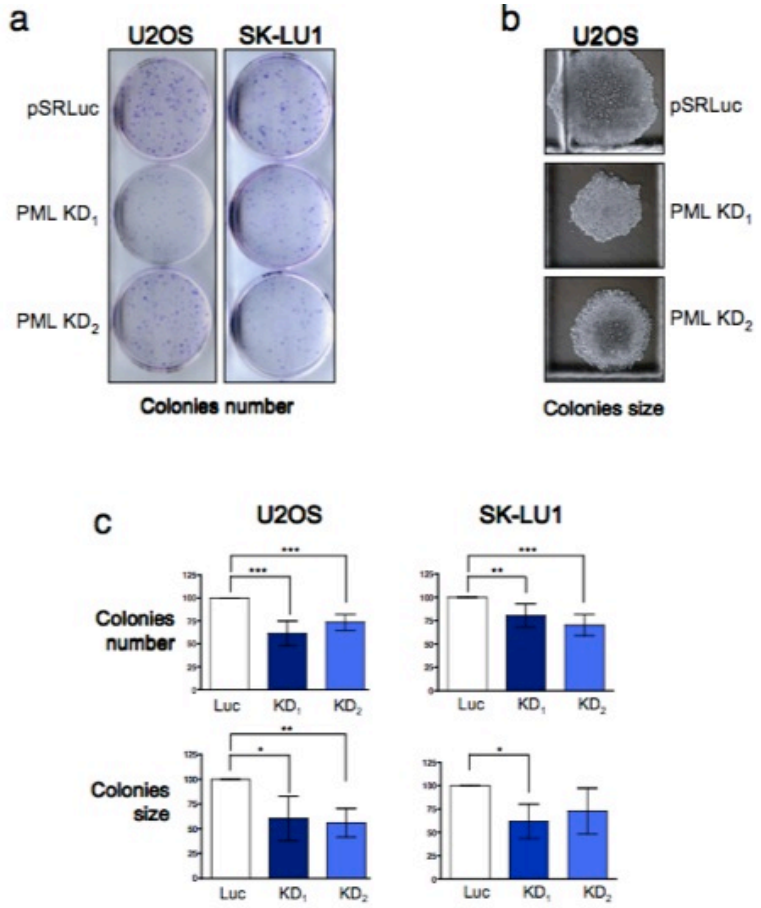

Figure 13. Decreased colonies formation in PML knock down ALT-positive tumour cells. a) Representative clonogenic assay showing decreased colony formation in PML KD1 and KD2 ALT cells compared with cells infected with the control vector (pSRLuc). b) Photos showing the different sizes of single colonies of U2OS treated with the indicated vectors. c) Quantification of the colony formation assay. Graphs of colonies number represent the percentage of colonies respect to the control assumed as 100%. Histograms represent the mean and error bars the s.d. of three independent experiments; in each of them samples were plated in triplicate. Graphs of colonies size represent the number of cells/colony expresses as percentage respect to the control assumed as 100%. Histograms represent the mean and error bars the s.d. of three independent experiments; in each of them samples were plated in triplicate. The differences are statistically significant, as shown by the asterisks.
(*** = P<0,001, ** = P<0,01, * = P<0,05, according to a two-tailed Student t-test).

To elucidate the pathways activated by down-regulation of PML, cellular lysates obtained at different days after stable interference were analyzed by western blotting to detect the levels of the major cell cycle regulators. We observed increase of p53 and p21 protein levels in U2OS cells (Fig.14), just after the end of the selection and for more than ten days (data not shown). Osteosarcoma U2OS cells do not express p16 protein due to hypermethylation of the INK4/ARF locus, so we analyzed the levels of another cyclin-dependent kinase inhibitor involved in the control of cell cycle G1 progression, p15. p15 is up-regulated in U2OS cells lacking PML (Fig.14).

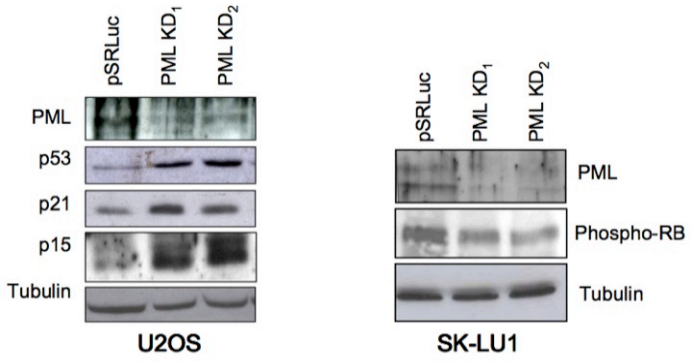

Figure 14. Pathways involved in cell cycle arrest induced by PML knock down in ALT tumour cells. Western Blotting showing up-regulation of p53, p21 and p15 protein levels in U2OS cells, and hypophosphorylation of pRb in SK-LU1 cells, after 6 days of PML RNAi with two constructs for silencing (PML KD1 and PML KD2).

SK-LU-1 is a lung adenocarcinoma cell line with a mutation of p53 that is expressed but not functional and not regulated. They do not express p16 and p15 due to homozygous deletion of the $p16^{INK4}$ and $p15^{INK4B}$ genoomic locus. On the other hand, SK-LU-1 cells express the Retinoblastoma protein (pRb), a cell cycle inhibitor. In the hypophosphorylated state, pRb is active and carries out its role as tumor suppressor by inhibiting cell cycle progression. Phosphorylation by cyclin- dependent kinases (CDK) and cyclins inactivates pRb. We noticed a slight reduction of

phosphorilated Rb levels, according to the possible role of pRb in inhibition of proliferation induced by PML depletion.

Therefore, PML knock down reduces proliferation of ALT tumour cells, as evidenced by growth curves and colony formation assay. This event could involve p53/p21 and/or pRb pathways. Differently from normal fibroblasts, ALT cells do not undergo senescence, even after long time of PML depletion, as evidenced by negativity to SA-β-gal staining performed until two months of interference. The magnitude of the effect seems correlated with the efficiency of PML knockdown, and this could be dependent from the reduction of the association of PML NBs from telomeres.

2.3. PML knockdown induces only a slight growth inhibition in telomerase-positive tumour cells.

Analogously to ALT cells, we performed assays to reveal the effect of PML depletion on tumour cells expressing telomerase.

For this study we used HeLa cells, a cervical cancer cell line which do not express p53 protein, and A549 cells, a lung carcinoma cell lines, positive for p53 but negative for p16 and p14 expression.

Growth curves reveal no effect of PML RNA interference in HeLa cells, while show a slight growth inhibition in A549 (Fig.15).

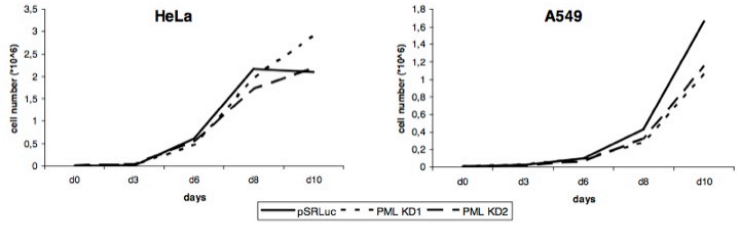

Figure 15. PML knock down in telomerase-positive tumour cells induces only a slight reduction of growth rate. Growth curves of HeLa and A549 cells, infected with two different PML shRNAs or a control shRNA (pSR Luc). Cells were counted in the indicated days after selection. The curve reported for each cell types is representative of three independent experiments.

Colony formation assay displays that no difference in the number of colonies arises after PML silencing. However, a little decrease of size appears especially in A549 cells depleted of PML protein (Fig.16).

Key cell cycle regulators such as p53, p21, p16 and p15 show no substantial protein levels modification (data not shown).

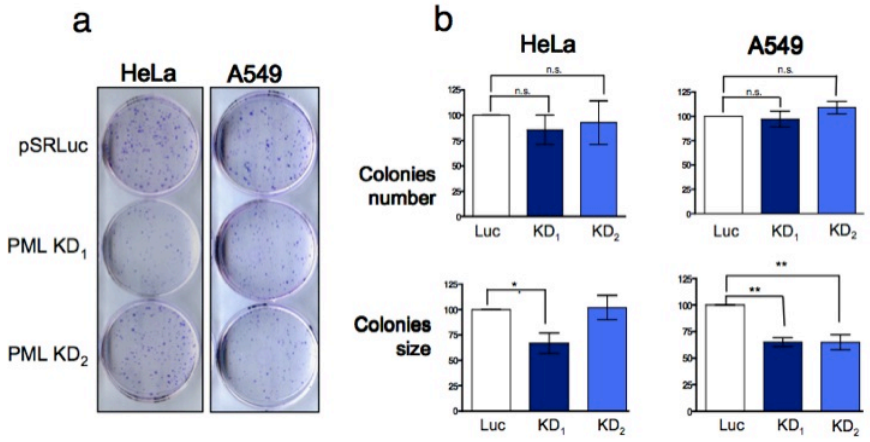

Figure 16. Irrelevant effect of PML knock down in telomerase-positive tumour cells on colonies formation capacity. a) Representative clonogenic assay showing only a small reduction of the size of colonies, but no differences in the number of formed colonies, in PML KD1 and KD2 cells compared with cells infected with the control vector (pSR Luc). b) Quantification of the colony formation assay. Graphs of colonies number represent the percentage of colonies respect to the control assumed as 100%. Histograms represent the mean and error bars the s.d. of three independent experiments, in each of them samples ware plated in triplicate. Graphs of colonies size represent the number of cells/colony expresses as percentage respect to the control assumed as 100%. Histograms represent the mean and error bars the s.d. of three independent experiments; in each experiment samples were plated in triplicate. The differences in the colonies number are not statistically significant (n.s.), while small significant differences are present in colonies size, as shown by the asterisks. (** means P<0,01, * means P<0,05, according to a two-tailed Student t-test).

In conclusion, HeLa and A549 are less affectd by PML loss-dependent growth inhibition than the other types of cells previously analyzed. These data on telomerase-positive tumor cell lines are not completely understood. We could hypothesize that PML effects on proliferation are less evident in telomerase-positive cells. A telomerase-inhibited effect of PML knock down could support the idea that PML loss causes a cell cycle arrest due to its

failed role on telomere maintenance. PML effect could be hidden by telomerase, because this protein could compete with PML for telomeres maintenance. Moreover, it has been recently showed that PML is a negative regulator of Telomerase activity, and the two proteins directly interact (Oh et al., 2009). PML role in telomere maintaince could be masked by Telomerase activity, altought this is in contrast with the work by Oh and colleagues. On the other hand, p53 role could be foundamental for PML suppression growth inhinition. This could be an explanation for irrelevant effects observed in HeLa cells, negative for p53, compared with the moderate but not undetectable growth inhibition in A549 cell line. Definitively, supplementary experiments are in progress and they will shed light on this unsolved issues.

3. PML deficiency induces telomere dysfunctions.

Replicative cellular senescence can be initiated from dysfunctional telomeres, which are recognized by DNA damage response factors and are detected as Telomere dysfunction-Induced Foci (TIFs) (see introduction, paragraph 2.1.3).

We just showed that PML localize to telomeres in all cell types and PML depletion in normal and ALT cells results in severe growth inhibition. This two events can be linked creating the hypotesis that PML depletion cause telomere dysfunctions.

To demonstrate this assertion we analyzed PML knock down cells for the presence of TIFs. First of all we performed this analysis on human normal fibroblasts that enter senescence when PML is loss, as showed above. WI38 and MRC-5 cells were infected with PML KD1 vector or with the control vector (pSR Luc), selected for four days with puromycin and plated on

coverslips. After fixing them, cells were stained following the ImmunoFISH protocol, with a fluorescent labeled PNA probe specific for telomeric repeats, and an antibody directed to γ-H2AX. TIFs, revealed as foci of γ-H2AX associated with telomeres, were analyzed with confocal microscopy (Fig.19) and counted in fifty cells per sample.

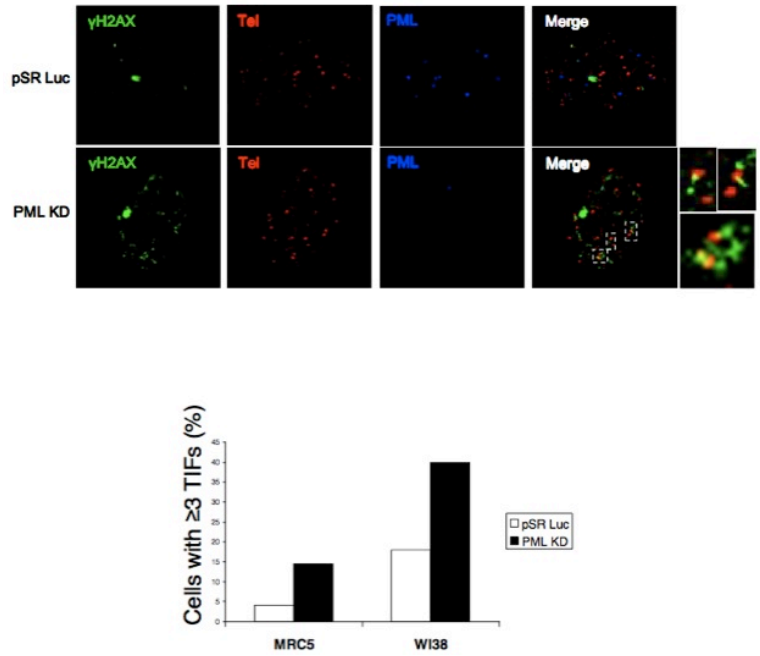

Figure 17. Increased telomere dysfunction in PML knock down MRC-5 cells. Top: MRC-5 cells were efficiently treated for PML interfering, as shown by the disappearance of PML NBs. TIFs, detected by co- localization of γ-H2AX and telomeres, increase in PML knock down cells. Representative images acquired at confocal microscope are shown. Bottom: Graph representing the mean percentage of cells with more or equal than three TIFs per cell. Analysis was performed counting TIFs in fifty cells per sample, in MRC-5 and WI38 normal fibroblasts.

PML knock down cells data shows that the number of telomeres co-localizing with DNA-damage response factors foci substantially increases (Fig.18), and there is an enlargement of population of cells with dysfunctional telomeres (Fig.17). We extended the analysis to tumour cell lines used in the previous experiments, to

determine if the effect of telomere damage is generally caused by PML loss, independently by the cell system.

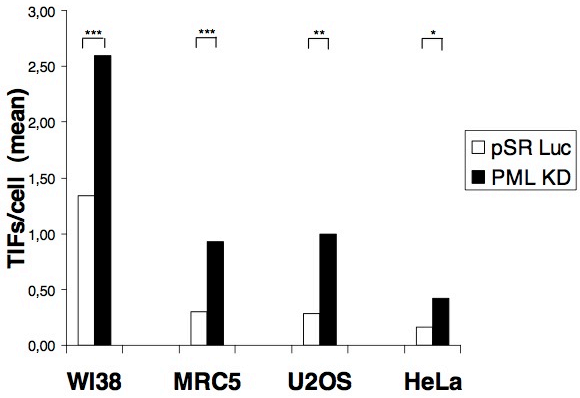

Figure 18. Increased telomere dysfunction in all types of PML knock down cells. WI38, MRC-5, U2OS and Hela cells were efficiently treated for PML interfering. ImmunoFISH using a florescent PNA probe for telomeric repeats and an antibody specific for γ-H2AX was performed. TIFs were detected by co- localization of γ-H2AX and telomeres. Histograms represent the mean of TIFs/cell. Analysis was performed counting TIFs in fifty cells per sample. Significant differences are evidenced by asterisks (*** means P<0,0001, ** means P<0,01, * means P<0,05, according to a two-tailed Student t-test).

The results indicate that PML contributes to telomere stability in U2OS ALT-positive cells, because its depletion results in significant increase of TIFs. Therefore PML knock down can cause telomere dysfunction also in tumour cells.

Telomerase-positive HeLa cells seem to undergo only a modest telomere damage as result of PML depletion, and they present only a limited increment of TIFs. These data are in agreement with the previous experiment in which HeLa cells react to PML loss with a barely perceptible inhibition of growth, evidenced only in a modest reduction of colonies size in cells infected with the construct PML KD1, more efficient for PML silencing. This result could support our hypothesis that PML and telomerase both participate to telomeres maintenance. However, telomerase may be sufficient to stabilize telomeres also when PML is absent, thus hiding the role of PML.

Summarizing, the experiments have identified a crucial role for PML in telomere stability. PML knock down leads in fact to accumulation of telomere dysfunctions, determined by the increase of TIFs. The mechanism at the basis of this event it is not completely understood, but maybe PML loss could destabilize telomere structure or more probably could impair the normal repair of damaged telomeres. The final effect is a transduction of damage signals towards the activation of checkpoints. Both p53/p21 and/or pRb/p16 pathways seem activated in cells lacking PML, with differences depending on the cell type. Increased levels of cell cycle inhibitors lead cells to growth rate reduction that even appears as premature senescence in normal primary cells.

PML role has always been associated with tumor suppression mechanisms and its overexpression causes reduction of proliferation by induction of apoptosis and senescence (see introduction, paragraph 3.3). The results we obtained in this work seem to be in contrast with this knowledge, because we show that also PML depletion leads to growth inhibition, reducing even the proliferation of cancer cells. These data rather underline a role for PML as an oncogene.

PML could play a double role, as tumour suppressor or oncogene, depending on the contest, as two face of the same medal. On one hand, PML act as a "guardian" of telomeres stabilizing and allowing normal growth and preventig the arise of genomic instability, but on the other hand, this should be balanced with its role in p53-mediated senescence.

In support to our findings of a potential role of PML as oncogene, a recent work of Ito and colleagues defines the critical function of PML in haematopoietic stem cell maintenance, in particular of quiescent leukaemia-initiating cells and suggest a new therapeutic approach for targeting possibly cancer-initiating cells by pharmacological inhibition of PML (Ito et al., 2008).

In conclusion, in cancer condition, it could be useful to inhibit the oncogenic function of PML to reduce tumour progression, but

in normal condition PML, stabilizing chromosome end, could have a role in preventing aging and cancer transformation.

4. PML association with phospho-DNA-PKcs in telomere damage response

The group in which I developed the PhD project, was one of the first to clone the translocation t(15;17) and successively four of the seven known PML isoforms (Jensen et al., 2001). An antibody able to visualize the characteristic nuclear domains in which PML accumulates was also generated. These structures, which may recruit a host of proteins, have been associated with many functions such as transcription regulation, genome stability (DNA repair, senescence and telomere maintenance), viral infection, apotosis and tumor suppression. However, the function of PML and PML-NBs is still unclear. To understand the molecular basis of the PML function, the proteins that physically interact with PML have been isolated in vivo by immunoprecipitation of the endogenous PML complex in 293T cells. The PMLIV isoform, tagged to Flag epitope, was over-expressed to a very low level to obtain a size and number of nuclear bodies comparable to endogenous pattern. The co-precipitated proteins were analyzed by mass spectrometry originating a list of the PML interacting proteins: it has been found structural proteins (α e β-actin,…), proteins of the metabolism [Ribosomal Protein L4 (RPL4), RPL5,...] and a wide range of proteins involved in the transcription and modification of RNA [heterogeneous nuclear RiboNuclearProtein G (hn RNP G), hn RNP M, ADAR1...], casein

kinase 2 (CK2), and proteins involved in the DNA-damage response (Mre11, RAD50 and DNA-PKcs) (Fig.19).

Because of its known function in telomere maintenance, we focused our attention on DNA-PKcs and decided to validate its interaction with PML. This was a likely candidate to explain the mechanisms at the basis of PML function in telomere metabolism.

DNA-PKcs is a member of phosphoinositide-3-kinase-related (PIKK) family, which associates with the Ku70/80 heterodimer to form the catalytically active DNA-PK holoenzyme, one of the main components of Non Homologous End-Joining (NHEJ) pathway (see introduction, paragraph 2.1.1), the predominant mechanism of DSB repair. DNA-PKcs associates with telomeric DNA in human cells, and several works reported its role in telomere protection. Inhibition of DNA-PKcs catalytic activity results in telomere shortening, fusions and genomic instability (Bailey et al., 2004; d'Adda di Fagagna et al., 2001; Ruis et al., 2008).

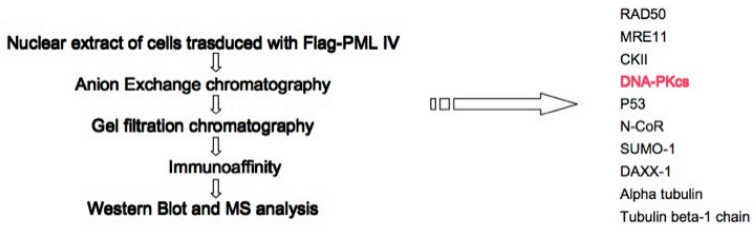

Figure 19. PML complex isolation. On the left, scheme of the passages of the procedure performed to isolate the high molecular complex of PML. On the right, short list of proteins found associated with PML.

4.1. Phospho-DNA-PKcs localizes with PML at telomeres of ALT cells.

ALT cells present a novel class of PML NBs, known as APBs, which contain telomeric DNA and protein involved in DNA recombination, replication and repair.

The results of PML macromolecular complex suggest the interaction of PML with DNA-PKcs, an important factor involved in DNA repair and telomere stability. For this reason we decided to start imaging experiments to investigate an hypothetical interaction in the APB of ALT cells. Interestingly, we noticed by immunofluorescence that PML co-localizes with DNA- PKcs phosphorylated at Thr2609 in U2OS (Fig.20).

Thr2609 is a key regulation site of DNA–PK activity. In response to DNA damage as, for example, ionizing radiation, DNA-PK complex is activated and phosphorylates its targets, among them the subunit DNA-PKcs itself (see introduction, paragraph 2.1.1) (Douglas et al., 2002). Autophosphorylation of Thr 2609 is a Ku-dependent event, important for NHEJ, because mutation of this site impairs DSB rejoining (Ding et al., 2003). Mutant DNA-PKcs protein for residue Thr2609 maintains the protein kinase activity but it is defected for its ability to support ligation of DNA ends (Block et al., 2004b). DNA-PKcs phosphorylated on Thr 2609 co-localizes with γ-H2AX and 53BP1 at the sites of DNA DSBs (Chan et al., 2002). Performing immunoFISH analysis, using a fluorescent-labeled PNA probe for telomeric repeats concomitantly with an antibody directed towards PML and an antibody that specifically recognizes the phosphorylated form of DNA-PKcs at Thr2609, we could observe that PML and phospho-DNA-PKcs co-localize at telomeres of two analyzed ALT cell lines, U2OS and SK-LU-1.

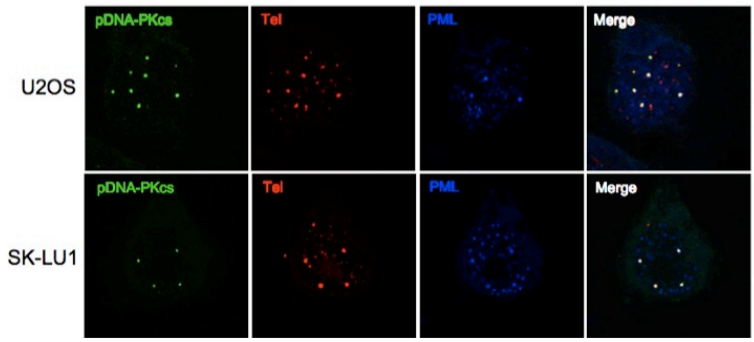

Figure 20. PML co-localizes with phospho-DNA-PKcs at telomeres of ALT cells. U2OS and SK-LU1 ALT cells were stained for DNA-PKcs phosphorylated at Thr2609 (green), telomeric DNA (red) and PML (blue), following the ImmunoFISH protocol. Confocal microscopy revealed the perfect co-localization of the two proteins at telomeres, as large white spots, in both the two cell lines.

The co-localization between the two proteins takes place in particular in large PML NBs, which correspond in ALT cells to APBs, and that associate with foci of telomeric DNA larger than signals from individual telomeres (Yeager et al., 1999) (see introduction, paragraph 1.4.2) (Fig.20). Statistic analysis of visualized co-localizations was performed on fifty cells per sample. The interesting data emerging from this experiment is that PML and phospho-DNA-PKcs associate specifically at telomeres. The graphs in figure 21 show that, even if some spots of PML could localize at telomeres without phospho-DNA-PKcs, all spots of phospho-DNA-PKcs co-localizing with PML are localized at telomeres. In fact, the histogram representing the mean number of PML/phospho-DNA- PKcs co-localizations and the histogram of the triple co-localizations with telomeres are comparable, while the histogram of PML/telomeres co-localizations is higher than the triple's one (Fig.21, top) in both cell lines.

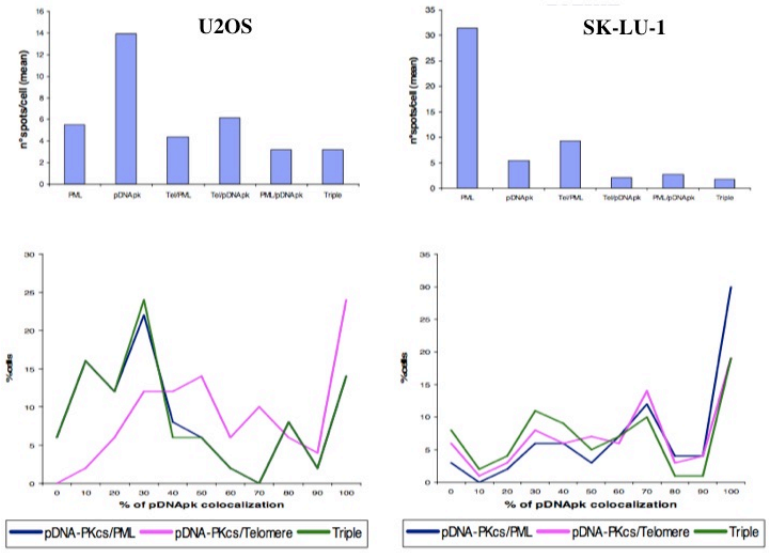

Figure 21. PML association with DNA-PKcs takes part at telomeres. Top: Graphs representing the mean values of spots for cell. Analysis was performed on 50 cells per sample. Bottom: Distributions of population show the percentage of cells that presents a certain percentage of phospho-DNA-PKcs co- localizing with PML (blue line), Telomeres (dark pink line) or both (green line).

In agreement, observing the graphs (Fig.21, bottom) representing the subpopulations of cells which have a certain percentage of phospho-DNA-PKcs co-localizing with PML (blue line), with telomeres (dark pink) or both (green), we can appreciate that the blue and the green curves are quite overlapping, indicating that all phospho-DNA-Pkcs associated with PML is also associated with telomeres. Vice versa, almost all phospho-DNA-PKcs spots present at telomeres are co-localizating with PML NBs, even if in U2OS there are some signlas of the phospho-DNA-PKcs present at telomeres independently from the presence of PML (Fig.21, bottom). In other words, the association between PML and DNA-PK arise at telomeres.

These immunofluorescence experiments not only support the interaction between PML and phospho-DNA-PKcs, noticed by the isolation of the PML complex, but also identified phospho-DNA-PKcs as a novel component of APBs.

Moreover, considering these data it is likely that PML could be necessary to the association of phospho-DNA-PKcs with telomere.

However, some spots of phospho-DNA-PKcs seem to be localized at telomeres in absence of PML in U2OS cell line. This could reflect a simple tecnical problem in detecting PML at those telomeres, or that PML is necessary for the initial association of phospho-DNA-PK or for its autophosphorylation at telomeres and it could then leave the DNA-PK/telomeres/PML tri-complex.

Nevertheless, additional experiments are necessary to evaluate the possibility that a quota of phospho-DNA-PKcs can associate to telomere in a PML- independent manner.

4.2. PML knock down destabilizes phospho-DNA-PKcs from telomeres.

In view of these results, we investigated if PML is responsible for the localization of DNA-PKcs phosphorylated at Thr2609 at telomeres.

To elucidate this purpose, we performed RNA interference for PML in two different ALT cell lines, U2OS and SK-LU-1, and analyzed the co-localization between the phosphorylated DNA-PKcs and telomeric DNA.

RNAi interference was performed as described above, using the PML KD1, that has the strongest effect on PML down-regulation, and the control vector pSR Luc. Cells were stained following the usual ImmunoFISH protocol (Fig.22). Counting fifty cells per sample, it was evident a reduction of phospho-DNA-PKcs association at telomeres (Fig.23). SK-LU-1 cells, expressing very high levels of PML, were silenced following the adapted protocol that consists of two sequential series of infections. The low significance of the result obtained in SK- LU-1 could however reflect the incomplete knock down of PML.

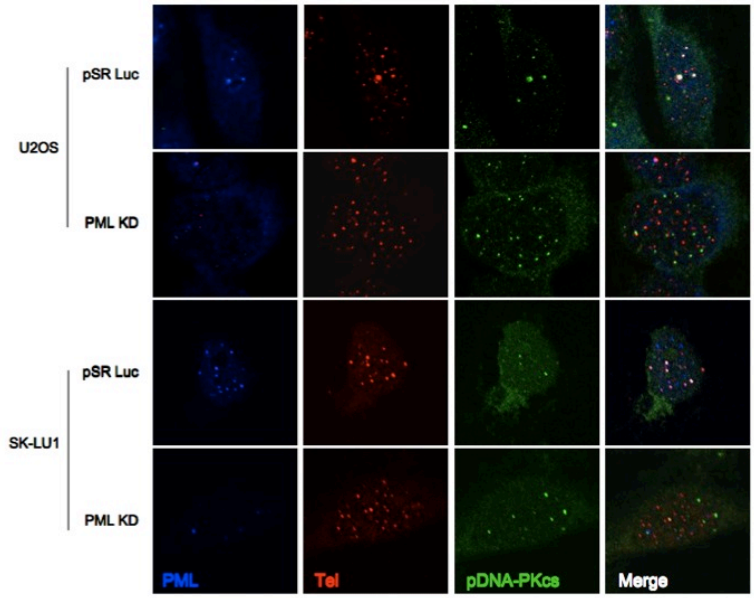

Figure 22. PML knock down destabilizes DNA-PKcs from telomeres of ALT cells. U2OS and SK-LU1 ALT cells were infected with control vector (pSR Luc) or with a vector for PML interference (PML KD). Six days after the end of selection in puromycin, cells were stained for DNA-PKcs phosphorylated at Thr2609 (green), telomeric DNA (red) and PML (blue), following the ImmunoFISH protocol. Confocal microscopy revealed the loss of co-localization of phospho-DNA-PKcs and telomeres, after PML depletion.

The graph in figure 23b underlines a shift of U2OS cells lacking PML towards populations presenting a low percentage of phospho-DNA-PKcs localized at telomeres. However, it seems that not all the singnals of phospho-DNA-PKcs are delocalized from the telomeres after PML interference. This could happens for two reasons: an incomplete PML depletion or a quota of phospho-DNA-PKcs is associated at chromosome ends independently from PML.

In conclusion, PML is necessary to maintain at least a portion of the interactions between phospho-DNA-PKcs and telomeres.

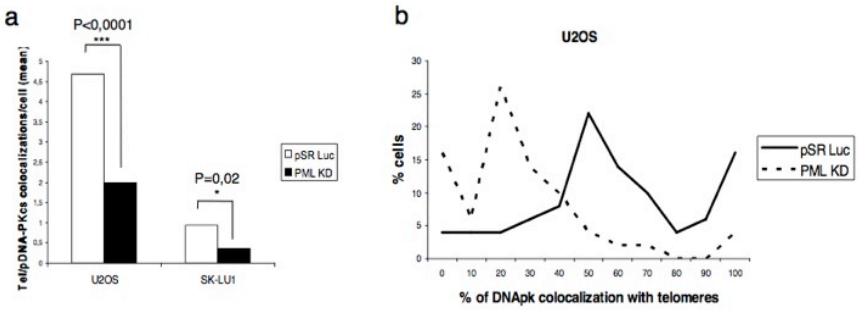

Figure 23. Interaction of phospho-DNA-PKcs with telomeres is impaired by PML loss. a) Graphs representing the mean values of spots per cell. Analysis was performed on 50 cells per sample. P value was calculated according to Student t-test. b) Distribution of cellular population show the percentage of cells that presents a certain percentage of phospho-DNA-PKcs co-localizing with telomeres, in PML KD cells (broken line) versus control cells (unbroken line) .

4.3. PML physically interacts with DNA-Pkcs and with its phosporylated form

The isolation of molecular complex of PML gave the preliminary results of the interaction beetwen PML amd DNA-PK. These were confirmed by immunofluorescence, but a robust biochemical data was still missing.

Thus we performed co-Immunoprecipitation experiments in U2OS cells.

Nuclear extracts of cells immunoprecipitated with an anti-DNA-PKcs antibody, revealed a complex with endogenous PML. Western blot analysis with an antibody against PML that recognizes all isoforms, shows two bands that migrate at the molecular weight corresponding to PML I and PML IV (Fig. 24a). In agreement, nuclear extracts of U2OS immunoprecipitated with the PML antibody, contained DNA-PKcs (Fig. 24b). We also investigated whether PML interacts with DNA-PKcs phosphorylated at Thr 2609 using an antibody targeting this specific post-translational modification. As shown in Fig.24b, there

is a weak but detectable interaction between endogenous PML and the phosphorylated form of DNA-PKcs.

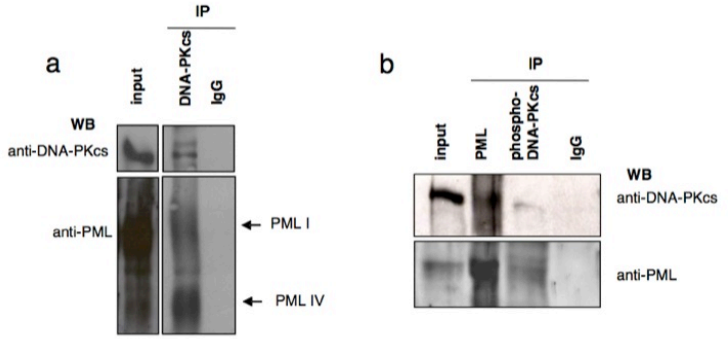

Figure 24. Endogenous PML interacts with DNA-PKcs and its phosphorylated form. a) Nuclear extracts from U2OS cells were immunoprecipitated with an anti-DNA-PKcs antibody. Wester blotting was performed with anti-PML and anti-DNA-PKcs antibodies, as indicated. b) The same nuclear extrats as in a) were immunoprecipitated with anti-PML or anti-DNA-PKcs phosphorylated (Thr2609). Wester blotting was performed with anti-DNA-PKcs and anti-PML antibodies to show the reciprocal interaction. Nuclear lysates were loaded as a control (input).

Therefore, we demonstrated that PML forms a stable complex with DNA-PKcs, an important component of DSB repair. We also show that PML interacts with DNA- PKcs phosphorylated at Thr2609, an activated form of the protein required for NHEJ, in ALT cells.

4.4. Specific telomere damage increases the amount of PML – DNA- PKcs complex.

The localization of PML–phospho-DNA-PKcs complex at telomeres in ALT cells suggests a possible role of this association in telomere stability.

In the experiments presented before we demonstrated that PML is recruited to dysfunctional telomeres. To study whether telomere damage has an effect on the association between PML and phosphorylated DNA-PKcs, we analyzed the complex formed by

the two proteins biochemically, after treatment with RHPS4. This drug leads to TIFs formation and increases PML localization to dysfunctional telomeres, if used at a concentration of 1μM for 24h (see paragraph 2.1.3 of the introduction and paragraph 1.2 of this section).

We used the same treatment to induce telomere damage in U2OS cells, and we performed immunoprecipitation assay with an anti-PML antibody and with the antibody recognizing the phosphorylated form of DNA-PKcs at Thr2609 (Fig.25).

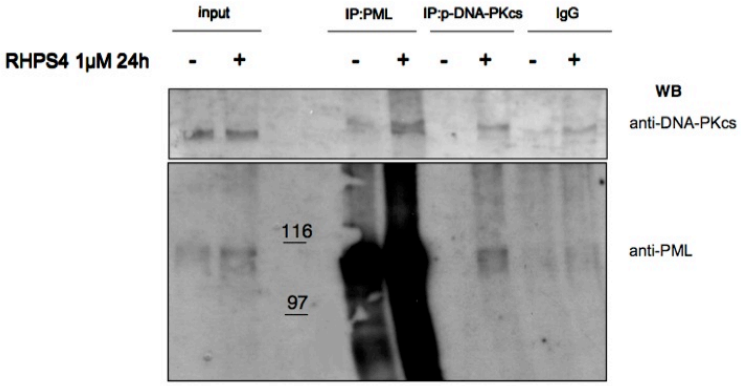

Figure 25. Interaction of DNA-PKcs with PML is increased by telomere damage. Nuclear extracts from U2OS cells, treated (+) or untreated (-) withRHPS4, were immunoprecipitated with anti-PML antibody or with anti-DNA-PKcs-phosphorylated at Thr2609 antibody. Wester blotting was performed with anti-PML and anti-DNA-PKcs antibodies, as indicated. An immunoprecitation with IgG was performed as negative control. Input represents nuclear lysates.

Inputs reveal no differences in total levels of DNA-PKcs. However the antibody directed towards the phosphorylated form, immunoprecipitated a higher quantity of the protein after telomere damage, revealing that under this condition DNA-PKcs can undergo autophosphorylation. As shown before by immunofluorescence, PML protein increases moderately after treatment with RHPS4. PML immunoprecipitated contained more DNA-PKcs after telomere damage, demonstrating that the stechiometry of the complex was increased. Western blotting anti-

PML on immunoprecipitated by anti-phospho-DNA-PKcs (undetectable at basal levels, but significantly increased after treatment) demonstrated that the treatment enhances the association of this phopshorylated form with PML (Fig.25). In conclusion, a specific damage directed against telomeres, not only increases the localization of PML to the chromosome ends, but also leads DNA-PKcs, particularly in its phosphorylated form, to associate with PML. It is possible that PML localized at damage telomeres could recruits proteins involved in recombination and repair, as DNA-PKcs, and regulates their activity.

APBs could represent site of repair for damaged telomeres, present at steady state in ALT cells, where phospho-DNA-PKcs is recruited by PML.

4.5. Phospho-DNA-PKcs localization at PML NBs increases in normal cells after telomere-damage

To investigate if PML co-localization with DNA-PKcs phosphorylated at Thr2609 is present also in non-ALT cells, and if the association of the two proteins in response to a telomeric damage could be a general mechanism, we extended experiments on telomerase-positive tumour cells and on normal cells. An extensively analysis by immunofluorescence revealed that at basal level, telomerase-positive HeLa and A549 cells, have very low abundance of phospho- DNA-PKcs (about 1-2 small spots per cell) and practically none of the spots associate with PML or telomeres (data not shown).

Among an asyncronous population of interfase normal fibroblast cells, only some rare cells present spots corresponding to phospho- DNA-PKcs, and interestingly a certain number of them co-localizes with PML (Fig.26). To understand if this association could take part in response to telomere damage, we induced in two

types of fibroblasts, WI38 and MRC-5, the deprotection of telomeres by TRF2 knock down.

After infections with lentiviruses particles and four days of selection in puromycin, we seeded the cells which were entering senescence on coverslips for immunofluorescence (Fig.27, Top). Indirect immunofluorescence with anti-PML and anti- phospho-DNA-PKcs antibodies showed a high increase of cells positive for phospho-DNA-PKcs spots, which reach 100%, with a raised number of spots per cell (Fig.27, Bottom). TRF2 depletion also leads this protein to localize in PML NBs, as proved by the high number of PML/phospho-DNA-PKcs co-localizations. In summary, PML and DNA-PKcs physically interact. PML associates with the phosphorylated form of DNA-PKcs in APBs of ALT cells at basal conditions. This association increases after telomere damage in both ALT and normal primary cells. These findings suggest that PML-DNA-PKcs interaction has a role in response to telomere damage.

Our data propose that the mechanism of association of damaged telomere to PML NBs and the consequent recruitment of proteins is not limited to ALT cells, but it could be a process common to diverse cellular types. ALT cells, maintaining their telomeres by recombination that lead to a constant presence of cells with telomeres reaching a short length, permit to observe, in a subset of cellular population, phenomena that occur in other cells only under conditions of telomere damage. Generating massive telomere damage allow to notice that also normal and telomerase-positive cells present PML NBs associated with telomeres, and activate autophosphorylation of DNA-PKcs.

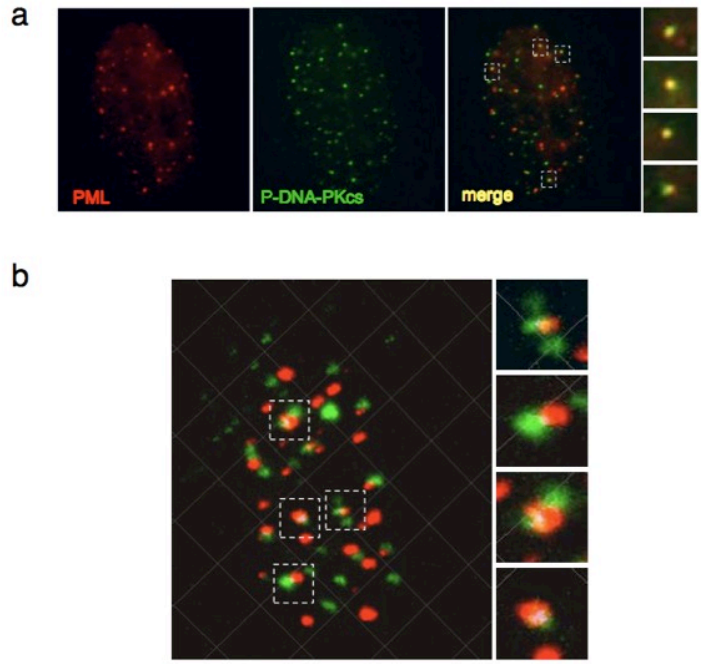

Figure 26. PML co-localizes with phospho-DNA-PKcs in few normal fibroblasts. a) Immunofluorescence for DNA-PKcs phosphorylated at Thr2609 (green) and PML (red) shows a WI38 cell presenting co-localizations. b) An image obtained with confocal microscopy confirming the interaction.

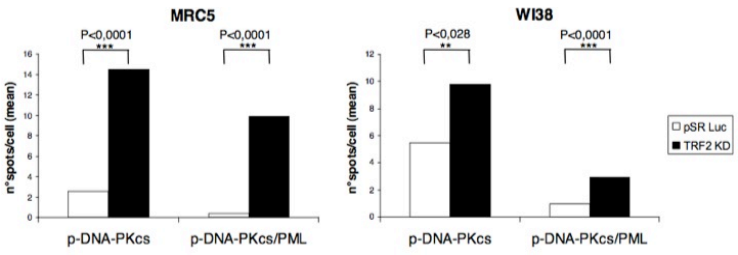

Figure 27. PML co-localization with phospho-DNA-PKcs in normal fibroblasts after telomeric damage. Top) Immunofluorescence for DNA-PKcs phosphorylated at Thr2609 (green) and PML (red) shows the increment of co-localizations between the two proteins in normal fibroblasts depleted forTRF2. Bottom) Graphs representing the mean values of spots per cell. Analysis was performed on 50 cells per sample, treated as in figure 30a. P value was calculated according to Student t-test.

Considering all the results, it is likely that PML could mediate the phosphorylation and recruitment of DNA-PKcs to telomeres in response to damage. This event could be important in telomere maintenance, and we could hypothesize that the accumulation of telomere damage in PML depleted cells is an effect of the destabilization of phospho-DNA-PKcs from telomeres. Ongoing experiments are ongoing to demonstrate this hypothesis.

4.6. PML depletion leads to genomic instability.

These interesting observations let a question arise: which are the molecular mechanisms that could link the observed PML-dependent growth inhibition with DNA-PK destabilization from telomeres?

It is well established that DNA-PK deficiency results in anaphase bridges and genomic instability deriving from abnormal telomeric fusions (Goytisolo et al., 2001). Binucleated Cells (BNs) and Abnormal Nucleous Morphogenesis (AMNs) as Nucleoplasmic Bridges (NPBs), Micronuclei (MNs) and Nuclear Buds (Bs) are biomarker of genomic instability linked to telomeric defects (Pampalona et al., 2010).

Given these considerations we addressed the issue of genomic instability in PML depleted human fibroblast MRC-5 cells.

At first, we simply detected an important increase in binucleated cells stained with DAPI in our asyncronous interphase fibroblasts after PML RNAi using phase contrast microscopy (Fig.28).

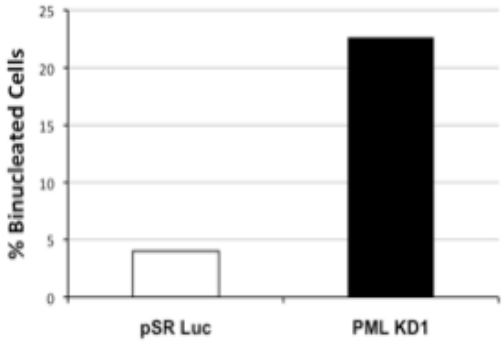

Figure 28. Percentage of binucleated cells in PML knock down MRC-5 cells. The graph shows percentages of binucleated cells in control RNAi and PML Knock Down RNAi (PML KD1) on total asyncronous population, evaluated by DAPI coloration. Number of binucleated cells were determined with phase contrast microscopy.

Following these preliminary observations we performed an experiment using blebbistatin, a selective cell-permeable inhibitor of non-muscle myosin II ATPases, that induces binucleation through citokinesis inhibition (Straight et al., 2003). Using this drug we were able to block our cells after mitosis but before cytokinesis, in order to increase the probability of ANMs . We analyzed only binucleated cells in search of ANMs as markers of genomic instability, evaluating Nucleoplasmic Bridges (NPBs), Micronuclei (MNs) and Nuclear Buds (Bs) by DAPI coloration (Fig.29a). A consistent increase in overall ANMs was detected in PML deficient cells, and, in particular, nucleoplasmic bridges strongly increased respect to control cells (Fig.29b).

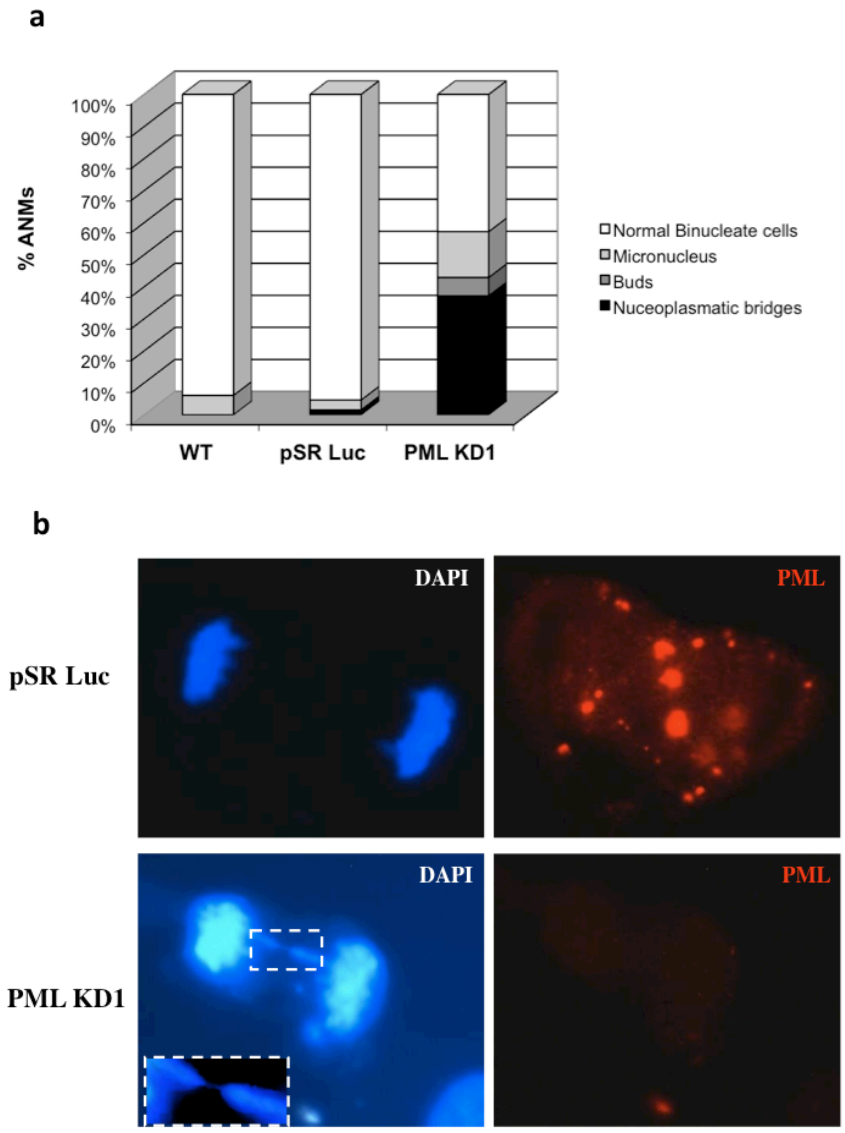

Figure 29. Percentage of ANMs in binucleated MRC-5 cells after PML knock down. a) The graph shows overall ANMs in PML depleted cells. Nucleoplasmic Bridges (NPBs), Micronuclei (MNs) and Nuclear Buds (Bs) were counted and percentages are calculated on total cells. b) The lower panel shows a dividing PML interfered cell with typical nucleoplasmic bridge. Dashed box shows an enlargement of original image.

These defective phenotypes are of great interest in our work, because it is known that they could be directly linked to telomere dysfunction through mechanisms dependent on DNA-PK catalytic activity, as mentioned above. These could confirm our hypothesis that PML ablation leads to DNA-PK phosphorylation defects, resulting in genomic instability and impaired cell growth.

MATERIAL AND METHODS

Cell culture

Primary cells and cell lines
Normal human WI38 fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% North American Fetal Bovine Serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml). Human fibroblasts BJ and their TERT-transfected clone (BJ-TERT)239, MRC-5 fibroblasts, and lung adenocarcinoma cell line SK-LU-1 were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% North American FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml), non-essential amino acids (0,1 mM) and sodium pyruvate (1 mM). 293T cells, cervix adenocarcinoma cell line HeLa, lung carcinoma cell line A549 and osteosarcoma cell line U2OS were maintained in DMEM supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml). Osteosarcoma cell line Saos-2 was cultured in McCoy's Medium supplemented with 15% FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml). HTC75 cell line, kindly provided by T. De Lange19, was maintained in high glucose DMEM supplemented with 10% FBS, penicillin, streptomycin, and 100 ng/ml doxycyclin. Amniotic fluid-derived stem cells were kindly provided by Donti E. All cells were cultured in 5% CO2 at 37°C.

Mouse Embryonic Fibroblasts (MEFs) preparation
MEFs were prepared from day 13.5 embryos derived from WT mice. The whole embryo was minced and dispersed in 10 cm plate in 1 ml trypsin then incubated at 37 °C for 10 minutes. 9 ml of DMEM plus 10% North American FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml) were directly added to the minced embryo and incubated in 9% CO2 at 37 °C until confluence and splitted once. After that cells were used to perform experiments.

Calcium-phosphate transfection
DNA was diluted in 439 µl of ddH2O and mixed with 61 µl of 2M CaCl2. The mixture was added, drop-wise, to 500 µl of 2x HBS (HEPES-Buffered Saline: 50 mM HEPES pH7.1, 280 mM NaCl, 1.5 mM Na2HPO4, final pH7.1). After 15 minutes the precipitate was added to cells plated on a 10 cm dish and removed after 16 hours.

Virus generation and infection for RNA interference
5 µg of lentiviral vector and 2,5 µg of each packaging vector (Ampho and Δ8.2) were cotransfected in 293T cells by calcium-phosphate transfection. Supenatant was collected 36-48 hours after infection, filtered through a 0,45 µm filter and used directly to infect target cells. After 3 hours the supernatant was replaced with a fresh one. Two/three cycles of infections were repeated for two days. After 24 hours from the last cycle of infection, infected cells were selected with puromycin. An adapted protocol was followed to knock down PML in SK-LU-1 cells: at the end of selection (4 days long) cells were submitted again to three cycles of infections, repeated for two days, and puromycin was added newly until the end of the experiment.

Induction of dominant negative of TRF2 in HTC75 cells
To induce the dominant negative of TRF2 in HTC75 cell line, doxycyclin was removed completely from the medium.

RHPS4 treatment
RHPS4 was synthesized as described previously. The drug, used at a concentration of 1 µM for 24 hours, was added to the cells 24 hours after plating.

Growth curves
After selection of PML knock down cells with puromycin, 3x103 tumour cells, or 15x103 primary fibroblasts were seeded in a 6 well plate, in triplicate. Cells were counted at 3, 6, 8 and 10 days after plating. Every experiment was repeated at least three times.

Colony formation assay
Five hundreds cells were seeded in triplicate on a 6 cm dish. Fresh medium was replaced after a week. Two weeks after the plating, colonies number and size were evaluated.
For colonies number, cells were washed twice with PBS, fixed in 5% formaldehyde, rinsed with water and stained for 5 minutes with 0,05% crystal violet. Visible colonies were counted.
Alternatively the number of colonies was counted at the microscope, than cells were collected and counted. The mean number of cells per colony was calculated dividing the number of cells on the plate for the total number of colonies, and represents a measure of the colonies size.
The percentage of colonies or cells/colony was calculated respect the control considered as 100%. All experiments were repeated at least three times. Mean and s.d. were calculated among the experiments.

β-Gal-staining
Cells plated in 6 well dishes were washed in PBS, fixed 10 minutes at room temperature with 4% paraformaldehyde, washed and incubated over night at 37°C with a staining solution containing 1 mg/ml of 5-bromo-4-chloro-3-indolyl β-D- galactoside (X-Gal), 5 mM potassium ferrocyanide, 5mM potassium ferricyanide in a saline solution composed of 40 mM citric acid, 150 mM NaCl2, 2 mM MgCl2 and sodium phosphate pH 6,0. Solution was removed from dishes that were washed 4- 6 times with PBS and cells were analyzed at the microscope.

Protein analysis

Immunofluorescence

Cells were cultured on coverslips, fixed in 4% paraformaldehyde for 10 minutes at room temperature. The fixed cells were washed twice in PBS, permeabilized in 0,1% Triton X-100 for 10 minutes and blocked for 1 hour in blocking buffer (PBS containing 2% BSA). After blocking, cells were incubated in primary antibody diluted in blocking buffer for 1 h at room temperature. Cells were then washed with PBS and incubated with fluorescent secondary antibody in blocking buffer for 45 min at room temperature. After incubation, cells were washed with PBS and their nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Slide were mounted and viewed with a x60 objective on a Olympus BX61 wide-field microscope or a Leika TCSSP2 AOBS confocal microscope. Images were acquired with a CCD camera (Hamamatsu B/W CCD Camera CJ895). For quantification, multiple random fields were captured with the wide-field microscope and 50-100 cells were counted.

The commercial primary antibodies used in this study were as follows: anti-PML (H238) (Santa Cruz, sc-5621), anti-γH2AX (Ser139) (Upstate 05-636), anti-TRF2 (clone 4A794, Millipore, 05-521), anti-Histone H3 (tri methyl K9) (Abcam, ab8898), anti-DNA-PKcs phosphorylated (Thr2609) (Biologend, 612901).

The secondary antibodies were: CY5 donkey anti-Rabbit (Jackson Antibodies), 488 donkey anti-mouse (Alexa), 488 donkey anti-rabbit (Alexa).

Telomere Fluorescence in situ hybridization (T-FISH)

Interphasic cells were grown on coverslips, fixed in 4% paraformaldehyde for 30 minutes at room temperature. The fixed cells were washed twice in PBS, treated with 100 mM glycine for 20 min at room temperature to inactivate paraformaldehyde, and permeabilized in 0,5% Triton X-100/0,5% saponin for 1 h. After two washes in PBS, cells were blocked for 1 hour in blocking

buffer (PBS containing 2% BSA). After blocking, cells were incubated in primary and secondary antibodies as described above for immunofluorescence. Cells were then washed with PBS and treated newly with 4% paraformaldehyde for 30 minutes and 100 mM glycine for 30 min. Then, Telomere-FISH was carried out using the Telomere PNA FISH Kit/Cy3 (DAKO, Glostrup, Denmark), accordingly to the protocol indicated. Briefly, coverslips were immersed in Tris-buffered Saline (TBS) two times for 5 min, and permeabilized with the pretreatment solution containing proteinase K. After washes with TBS, DNA was denatured by heat for 5 min at 80°C in the presence of ten micro liters of Cy3-conjugated telomere specific peptide nucleic acid (PNA) probe in hybridization solution containing 70% formamide. After hybridizing in the dark for 1 hour, slides were briefly immersed in Rinse Solution (supplied with the kit) and then washed twice at 46°C for 5 min in Wash Solution (supplied with the kit). After rinsing with TBS, slides were counter- stained with DAPI, and mounted with antifade solution (VectaShield, Vector laboratories Inc., Burlingame, CA, USA).

Western blotting

For immunoblotting, cells were lysated in SDS Laemmli Buffer, sonicated, boiled, separated by SDS-PAGE and transferred to nitrocellulose membranes, which were then blocked with 5% milk/TBS-T and incubated with primary specific antibodies. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti- mouse IgG (Amersham Bioscience) were used as secondary antibodies, and immunoblots were developed using the ECL reagent (Amersham Bioscience). The commercial antibodies used were: anti-PML (H238) (Santa Cruz, sc-5621), anti-TRF2 (clone 4A794, Millipore, 05-521), anti-p53 (DO-1) (Santa Cruz, sc-126), anti-p21 (C-19) (Santa Cruz, sc-397), anti-p16 (H-156) (Santa Cruz, sc-759), anti- p15 (C-20) (Santa Cruz, sc-612), anti-p14 ARF (C-15) (Santa Cruz, sc-30547), anti-Phospho-Rb (Ser780) (Cell Signaling, 9307), anti-DNA-PKcs (Serotec, AHP318).

Immunoprecipitation

For nuclear extracts preparation, cells were resuspended in E1A buffer (250 mM NaCl, 50 mM Hepes pH7.0, 0,1% NP40, 5 mM EDTA, 1 mM DTT, 50 µg/ml PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin A, 0,2 mM sodium orthovanadate, 50 mM sodium fluorure, 25 mM β-glycerophosphate, 10 mM niacinamide) and kept 15 minutes on ice. The lysates were centrifuged at 3000 rpm for 6 minutes. The supernatant, corresponding to the cytosol was discarded. The nuclear pellets were resuspended in E1A buffer, sonicated three times then centrifuged at 13200 rpm for 15 minutes at 4°C. Nuclear extacts were then pre- cleared with 30 µl Protein-A conjugated beads. Anti-DNA-PKcs (Serotec, AHP318), anti-PML (H238) (Santa Cruz, sc-5621) or anti-DNA-PKcs phosphorylated (Thr2609) (Biologend, 612901) were added to supernatants for 16 h at 4°C. Samples were incubated with Protein-A conjugated beads for 1 h at 4°C and the beads were then washed six times with the E1A buffer. The immunoprecipitated proteins bounds to the beads were dissolved in SDS Laemmli Buffer, boiled, separated by SDS-PAGE and blotted with the indicated antibodies. 50 µg of nuclear extracts were loaded as control (inputs).

Plasmids

For RNA interference, pSicoR lentiviral vector was used232. Oligos coding for the various shRNAs were annealed and cloned into HpaI-XhoI digested pSicoR. Oligo design was as described241. The following oligonucleotides were chosen:

PML KD1
for:
T-(GACCTCAGCTCTTGCATCA)-(TTCAAGAGA)-
(TGATGCAAGAGCTGAGGTC)-TTTTTTC

rev: TCGAGAAAAAA-(GACCTCAGCTCTTGCATCA)-
(TCTCTTGAA)-(TGATGCAAGAGCTGAGGTC)- A

PML KD2
for:
T-(GGGACCCTATTGACGTTGA)-(TTCAAGAGA)-
(TCAACGTCAATAGGGTCCC)-TTTTTTC

rev:
TCGAGAAAAAA-(GGGACCCTATTGACGTTGA)-
(TCTCTTGAA)-(TCAACGTCAATAGGGTCCC)- A

pSicoR Luc
for:
T-(CTTACGCTGAGTACTTCGA)-(TTCAAGAGA)-
(TCGAAGTACTCAGCGTAAG)-TTTTTTC

rev: TCGAGAAAAAA-(CTTACGCTGAGTACTTCGA)-
(TCTCTTGAA)-(TCGAAGTACTCAGCGTAAG)- A

TRF2 KD1 for:
T-(GAGGATGAACTGTTTCAAG)-(TTCAAGAGA)-
(CTTGAAACAGTTCATCCTC)-TTTTTTC

rev:
TCGAGAAAAAA-(GAGGATGAACTGTTTCAAG)-
(TCTCTTGAA)-(CTTGAAACAGTTCATCCTC)-A

TRF2 KD2
for:
T-(GAACAGCTGTGATGATTAA)-TTCAAGAGA-
(TTAATCATCACAGCTGTTC)-TTTTTTC

Rev
TCGAGAAAAAA-(GAACAGCTGTGATGATTAA)-
(TCTCTTGAA)-(TTAATCATCACAGCTGTTC)-A

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