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Characterization of AKTIP, a new protein involved in human DNA replication and telomere metabolism

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GLOSSARY

Telomere: a region of repetitive DNA at the end of linear chromosomes, which protects the end from degradation or loss.
Telomerase: "ribonucleoprotein complex" composed of a protein component, a reverse transcriptase, TERT, and an RNA primer sequence, TERC. It adds new telomeric sequences at chromosomes terminus counterbalancing telomere shortening.
ALT: alternative recombination-based mechanism of telomere lengthening. It could be activated in telomerase negative cells.
TIFs (Telomere Dysfunction-Induced Foci): Dysfunctional, uncapped telomeres, that activate a strong DNA damage response and become associated with DNA damage response factors, such as 53BP1, γ-H2AX, Rad17, ATM, and Mre11.
DNA damage response: A cascade of processes induced by the cell cycle regulator phosphoprotein p53, or an equivalent protein, in response to the detection of DNA damage.
DNA strand break: it involves one or more disruptions of the covalent linkages among phosphodeoxyribose moieties within the sugar-phosphate backbone in one (single stranded (ss) breaks) or in both (double stranded (ds) breaks strands of a DNA molecule). It can be induced both by exogenous factors and endogenous processes.
Shelterin: six-protein complex that enables mammalian cells to distinguish their natural chromosome ends from DNA breaks, represses DNA repair reactions, and regulates telomerase-based telomere maintenance. The components of shelterin specifically localize to telomeres; they are abundant at telomeres throughout the cell cycle; and they do not function elsewhere in the nucleus. Is formed by TRF2, TRF1, POT1, TIN2, TPP1 and Rap1.
Replication fork: the branch-point structure that forms during DNA replication between the two template DNA strands where nascent DNA synthesis is ongoing. Fork progression is mediated by the action of DNA helicases that unwind the DNA and facilitate the movement of the DNA synthesis machinery.

Fragile site: a specific heritable site on a chromosome that is prone to form gap and breaks when the cell is exposed to partial replication stresses.
Nuclear periphery: term that generally refers to the nuclear-membrane bilayer, its associated proteins and the embedded nuclear-pore complexes.
Nuclear lamina: The proteinacious meshwork that underlies the inner nuclear membrane.
Lamins: are rod-shaped proteins of the intermediate filament class. They consist of a head and tail domain that flanks a conserved alpha-helical rod domain. Lamins form parallel homo- and probably heterodimers which, in turn, can polymerize in a head-to-tail fashion. These linear polymers are thought to associate laterally into 10-nm lamin fibres, which form the fibrous lamina meshwork in the nuclear periphery. Mutations in their encoding genes are associated to different diseases collectively named laminopathies.
Laminopathies: A group of diseases that include premature ageing syndromes and certain types of muscular dystrophies and are associated with mutations in genes encoding lamins.
Cell senescence: the phenomenon in which replicatively dividing cells enter a non-dividing or quiescent phase that is accompanied by changes in gene transcription and metabolism.
Progeroid disease: A genetic disorder in which various tissues, organs or systems of the human body appear to age prematurely. These diseases are often called segmental progeroid diseases because they do not fully recapitulate normal ageing. A common feature of such diseases is genomic instability.
E2: An enzyme that forms a thioester bond with ubiquitin (Ub) following transfer from an E1 enzyme. E2 enzymes are referred to as Ub or Ubl-conjugating enzymes because they can conjugate the Ub protein directly to a substrate. However, E2 enzymes often require E3 ligases for proper function.
PCNA (Proliferating cell nuclear antigen): ring-shaped molecule encircling DNA. It slides bidirectionally along DNA to

constitutively monitor genomic integrity. Following DNA damage, ubiquitylation of PCNA is essential for the recruitment of damage-tolerant DNA polymerases, allowing translesion synthesis.
Fluorescence in situ hybridization (FISH): A technique whereby a fluorescently labelled DNA probe is used to detect a particular chromosomal region by fluorescence microscopy.
Chromatin immunoprecipitation (ChIP): A technique that involves crosslinking methods and is used to identify pieces of DNA or chromatin that contact a protein of interest *in vivo*.

SUMMARY

Telomeres are nucleoprotein structures that protect the ends of linear chromosomes. They are composed of long tracts of TTAGGG repeats, telomere specific proteins that form the shelterin complex and several telomere accessory proteins that co-operate to telomere metabolism. Proper telomere maintenance is a crucial process to protect the genome against instability and telomere dysfunction has been linked to tumorigenesis and premature aging. AKTIP gene is the human homologue of *Drosophila* peo, a gene that was recently linked to telomere metabolism.
The aim of this study was to understand if AKTIP could have a role in human telomere metabolism, in analogy with the telomeric function of its homologous in fly. For this purpose we have analyzed the phenotype of human cells in which AKTIP expression was downregulated by RNA interference. In human primary cells AKTIP downregulation triggered the reduction of the mitotic index, proliferation impairment and premature senescence. AKTIP reduction induced a strong DNA damage response proved by the accumulation of the phosphorylated form of proteins involved in DNA damage sensing and signaling such as ATM, p53 and Chk1, by the accumulation of p21 mRNA and by the formation of foci containing DNA damage response proteins. About half of these foci were located at telomeres (TIFs) indicating the presence of dysfunctional telomeres in AKTIP knocked down cells. These data were consistent with the accumulation of aberrant telomeres in MEFs $p53^{-/-}$ observed following the downregulation of murine homologue of AKTIP (named Ft1). AKTIP involvement in telomere metabolism was further suggested by its interaction with telomeric repeats observed by ChIP analysis. Altogether, these findings indicate that AKTIP takes part in telomere maintenance. Interestingly, immunostaining assays showed that AKTIP is not a stable component of telomeres but was found located in the nucleus, mainly at nuclear rim. This particular localization, in

addition with the telomeric role outlined for AKTIP, suggest that AKTIP is a telomeric nonshelterin protein. Consistent with this hypothesis, we observed that Ft1 downregulation caused the formation of chromosomal aberrations in addition to telomeric abnormalities, indicating that AKTIP/Ft1 plays a role not only in telomere maintenance but also in the overall genomic stability, possibly contributing to DNA replication. Indeed, the most prominent telomeric aberration observed in Ft1 downregulated MEFs was the formation of multiple telomeric signals at the ends of chromosomes, also known as fragile telomeres, indicative of replication impairment. In addition, AKTIP downregulation was found to induce an S-phase block of cell cycle progression and a strong reduction of PCNA positive cells in primary fibroblasts, along with an increased sensitivity to drugs that impair DNA replication, as aphidicolin.

Collectively, these data demonstrate that AKTIP is a protein needed for proper DNA maintenance in mammalian cells. In the telomeric context AKTIP likely is a telomeric accessory protein, rather than a shelterin-like protein, because it's conserved in fly, differently from shelterin proteins, has a role in telomere maintenance but is not stably located at telomeres. AKTIP, in addition to its telomeric function, seems to have a more general role in cellular metabolism, as all the other telomeric nonshelterin proteins. In particular our data indicate that AKTIP could be involved in DNA replication.

Considering all the collected data together, our current hypothesis is that AKTIP plays a role in replication of complex DNA structures, including telomeric repeats. Its downregulation could impair the replication fork progression through these DNA regions leading to chromosomal aberrations, DNA damage response and cell cycle alterations, the most prominent phenotypic traits of AKTIP knocked down cells.

INTRODUCTION

TELOMERES: structure and function

The genetic information of eukaryotes is organized on linear chromosomes located in the nucleus. Chromosomes linear nature poses two main challenges for the cell: the so-called end protection problem, that deals with the need to distinguish the natural chromosomal ends from double strand (ds) break to avoid deleterious nucleolytic attack and their recruitment in harmful DNA repair reactions, and the so-called end replication problem concerning the inability of DNA polymerase machinery to complete the replication of lagging strand, leading to progressive erosion of chromosomes ends. Telomeres are the evolutionary answer to both this problems: they are nucleoprotein structures located at the ends of linear chromosomes and either facilitate replication of the chromosomes ends and protect them against erosion and recognition by DNA damage machinery (Chan, 2010; de Lange, 2009).
Telomeric DNA of most eukaryotes is composed of ds short tandem repeats in which the strand running 5'-3' from the centromere towards the chromosome end is generally guanine-rich and cytosine-devoid and for this G/C composition, the two strands of telomeric DNA are called G- and C-strand. The length of this duplex telomeric DNA per chromosomes varies enormously from organism to organism ranging from 300 bp of *S. Cerevisiae* and *S. Pombe* to 10-15 kb in human (Samassekou, 2010) and 20Kb or more for laboratory mice and rats (Paeschke, 2010; Palm, 2008). In mammals, like in most eukaryotes, the telomeric DNA is constituted by tandem repeats of TTTAGG examer. The actual ends of telomeres is not blunt, but is characterized by the presence of a single stranded 3'-protrusion's of the G-strand, called G-tails or G- or 3'-overhang; this is a conserved feature of eukaryotic telomere structure and is essential for telomere function. Also G-strand protrusion length is highly different among eukaryotes and

in mammalian telomeres its length varies between 50-150 nt, which is considerably longer protrusion of most other eukaryotes (McElligott, 1997; Wellinger, 1997). It has not yet been fully elucidated how this overhang can be generated but its formation is likely linked to an active and highly regulated post-replicative 5'-3' exonucleolytic resection of the C-strand. Consistent with this scenario, the 5' end of human telomeres, located on C-strand, is accurately defined and predominantly ends with the sequence ATC-5', while the last base of the 3' end, located in the G-overhang, is variable in telomerase negative cells (Sfeir, 2005; Palm, 2008). This overhang is important for the creation of the t-loop, a lariat structure that contributes to ends protection (Figure 1) (de Lange, 2004). Moreover it provides the substrate for telomerase, a specialized enzyme able to add telomeric repeats to chromosomes ends after cell division (Chan, 2002).
Moving from chromosomes end to centromere in most eukaryotes subtelomeric sequences are present in the subterminal region adjacent to telomere. In higher eukaryotes these regions are enriched of repetitive DNA and typically comprise subdomanis forming a gradient progressing from telomeric repeats at the distal end of subtelomeres to variant repeats and finally degenerated repeats in proximal domain (Louis, 2005). The subtelomeres are enriched in segmentally duplicated DNA compared to genome as whole and in short direct and inverted repeats and AT-rich regions. These DNA sequences are frequently found at more than one telomere in an organism, but are not conserved between different species (Louis, 2005). Moreover, in mammals, whereas telomeres don't contain genes at all, subtelomeres are gene poor (Blasco, 2007). Growing evidences suggest that subtelomeric regions could have a role in telomere length regulation (McCord, 2008).
In yeast only subtelomeric repeats contain nucleosomes, whereas both mammalian telomeres and subtelomeres contain nucleosomes (Figure 1) (Blasco, 2007). These show a slightly altered spacing compared with non-telomeric chromatin: telomeric nucleosomes are characterized by an unusual repeat length that is about 20-40 bp

shorter than bulk nucleosomes spacing and by a low content of linker histone H1 (Pisano, 2008).
A highly conserved feature of telomeric DNA is its heterochromatic status. The first heterochromatic attribute described for telomeric regions is their ability to silence nearby genes, a phenomenon known as "telomere position effect" (TPE). It was described first in *D. Melanogaster* and later reported also in yeast and mammalian telomeres (Blasco, 2007). Consistent with the observation of TPE in mammalian cells, many marks that are usually found in heterochromatin can be found in mammalian telomeres. In particular trimethylation of H3K9 and H4K20 have been identified at mammalian telomeric and subtelomeric domains; for H3K9 this modification is carried out by SUV39H1-H2 (Garcia-Cao, 2004) whereas H4K20 trimethylation is carried out by SUV4-20H1-H2 (Benetti, Gonzalo, 2007). Moreover human and mouse telomeres and subtelomeres can also be found enriched in HP1, that is recruited at telomeres through its affinity for trimethylated H3K9 and it's important for chromatin compaction (Blasco, 2007). Mammalian telomeres and subtelomeres are also characterized by low levels of acetylated H3 and H4 (Benetti, Garcia-Cao, 2007). In contrast to budding yeast that lacks DNA methylation, mammalian subtelomeric sequences are heavily methylated, whereas telomeric repeats cannot be methylated because they lack CpG sequences that are the substrates for mammalian methyltransferase (Gonzalo, 2006). Increasing evidences suggest that there is an important role for histones and DNA methylation in regulating mammalian telomere length and integrity, and an important role of telomere length in regulating the assembly of heterochromatin domains at telomeres (Benetti, Garcia-Cao, 2007; Garcia-Cao, 2004; Blasco, 2007; Schoeftner, 2009). Epigenetic marks at telomeric and subtelomeric chromatin are essentials to repress and regulate recombination events (Gonzalo, 2006; Blasco, 2007; Schoeftner, 2009). However, mammalian telomeres don't exhibit all the features previously described for constitutive heterochromatin, for example human

telomeric DNA is not late replicating in contrast to yeast telomeres or mammalian pericentric heterochromatin (Wright, 1999; Arnoult, 2010).
Considering their compact heterochromatic structure, telomeres were not regarded to be permissive for transcription; nevertheless recently reports showed that telomeric C-strand is frequently transcribed by RNA polymerase II, giving rise to UUAGGG-repeats containing non-coding RNAs named TERRA or TelRNA (Azzalin, 2007; Schoeftner, 2008). Transcripts containing telomeric repeats have been described in mice, humans, and yeast (Schoeftner, 2009) and also in *Drosophila*, telomeres lengthen is dependent on transcription of telomeric regions, suggesting that transcription could be a universal process occurring at the ends of linear eukaryotic chromosomes (Schoeftner, 2009). Currently it's not clear which is the function of TERRA molecules but they are proposed as negative regulators of telomeres length and in this context it has been proposed that they could act inhibiting telomerase or influencing semi-conservative telomeres replication or their chromatin structures (Schoeftner, 2009; Feuerhahn, 2010).
The structure of telomeric DNA, although is generally conserved from yeast to human, is not universal and some exceptions exist. One of these is represented by *Drosophila Melanogaster* in which telomeres consist of tandem array of sequences produced by successive transposition of specialized non LTR-retrotrasposons (see below for detailed description) (Louis, 2005; Blasco, 2007).
Mammalian telomeres are also constituted by a complex of six proteins called shelterin complex (described in details in the next sections) that has a major role in their metabolism and maintenance.

Telomeres and the end protection problem

Concerning the ends protection problem, unprotected linear chromosomes ends can activate two main DNA damage sensing and signaling pathways: one dependent on ATM (Atassia Telangiectasia Mutated) activation, usually induced by the presence of ds-breaks, and the other triggered by ATR (Atassia Telangiectasia and Rad3 related) recruitment, predominantly activated by the presence of single-strand (ss)-breaks (Harper, 2007). Briefly ATM is normally activated following its recruitment at damaged DNA by MRN (Mre11-Rad50-Nbs1) complex that associates with the ds-break, and its auto-phosphorylation, while ATR activation requires the binding of RPA (Replication Protein A) to ssDNA following by ATRIP (ATR interacting protein) recruitment that in turn brings ATR at damaged site. Once activated ATM and ATR activate a phosphorylation-mediated signaling cascade that involves the phosphorylation and the consequent activation of different factors, such as Chk1 and Chk2, and, as finale effect, triggers cell cycle arrest and DNA repair pathway activation (Harper, 2007). Potentially telomeres have the features to activate both these pathway because they are ends and they showed a constitutive region of ssDNA, so mechanisms have to be evolved to avoid their activation at natural ends. Moreover two DNA repair pathway can be activated in mammalian cells to repair ds-breaks and are reported to be activated at unprotected chromosomes ends: Non Homologous Ends Joining (NHEJ) and Homologous Recombination (HR). NHEJ is an error prone repair pathway that joins together ds-breaks in a sequence-independent way; NHEJ is activated essentially when the heterodimer Ku70/80 binds a free end and a key factor of this pathway is DNA ligase IV that promotes the ligation of the ends (Lieber, 2010). When NHEJ is activated at unprotected telomeres it can lead to formation of end-to-end fusions that can block the cell cycle progression or, if checkpoint mechanisms are bypassed and the cells with fused chromosomes enter in mitosis, they can create problems during

chromosomes segregation and can become the starting point of a breakage-fusion-bridge cycle, source of genomic instability (O'Sullivan, 2010; Palm, 2008). HR, in principle, is an error free repair mechanism mainly when it's activated following S-phase and sister chromatids can be exploited as template. It requires Rad51 to promote strand invasion and following processing of derived structures that can involve different nucleases (Heyer, 2010). Unprotected telomeres could be subjected to different types of inappropriate HR that could have all detrimental effects because they could cause unequal exchanges between two chromosomal ends or they could bring to loss of chromosomal regions and to chromosomal rearrangements that can threaten genome stability (Palm, 2008). In recent years it has become clear that telomere dysfunction could have a major role in the establishment of genome instability that could lead to cancer development, underlying the importance of a complete understanding of telomere metabolism and biology (O'Sullivan, 2010).

How telomeres solve the end protection problem: t-loop and higher order structure

Telomeres solve the ends protection problem following two different but interlinked strategies: the formation of higher-order DNA structures that sequester the linear ends, and the constitutive binding of a specialized protein complex, the shelterin, that either has an active role in avoiding the recognition of chromosomes ends as ds-breaks and facilitates the formation and/or stabilizes these protective secondary structures (de Lange, 2010; Paeschke, 2010; Chan, 2010). A well documented example of telomeric higher order structure with protective function is the t-loop (Figure 1), a large duplex lariat structure identified first in human and mouse cells by electron microscopy (Griffith, 1999) and later observed in a large variety of different organisms such as trypanosomes (Munoz-Jordan, 2001), ciliates (Murti, 1999), plants (Cesare, 2003) and *C. elegans* (Raices, 2008). T-loops are likely formed by

3'-overhang looping back and by its strand invasion of duplex telomeric repeats, displacing the G-strand at the base of the loop, forming a so-called displacement loop (D-loop) (Palm, 2008; de Lange, 2004). The size of the loop is highly variable and it seems to not influence its function; it ranges from 0.3 Kb in trypanosomes (Munoz-Jordan, 2001) to 30 Kb in mice and 50 Kb in peas (Cesare, 2003); moreover it varies also between telomeres of a single cell (Palm, 2008). It has been proposed that T-loop formation, hiding telomere ends, could prevent the access of DNA–binding ends factors involved in DNA repair, such as MRN complex or Ku70/80, inhibiting in this way, respectively, the activation of ATM-mediated DNA response and of NHEJ pathway (de Lange, 2009). It has been suggested that shelterin components and in particular TRF2, described in detail below, could have a role in promoting t-loop formation and stabilization due to its ability to facilitate t-loop formation (Griffith 1999; Stansel, 2001) and to promote strand invasion *in vitro* (Amiard, 2007). However it has not yet been determined if TRF2 is necessary for t-loops maintenance *in vivo*. Moreover some other aspects of t-loop biology has not yet been fully elucidated, for example is not clear if t-loops are present at each telomere *in vivo*, or if their formation is cell cycle regulated and if and how they are resolved to allow telomeres replication (Paeschke, 2010; Palm, 2008). An alternative higher order structure proposed for telomeres is the G-quadruplex; its formation involves the association of four different guanines into a cyclic Hoogsteen hydrogen bonding arrangement (Maizels, 2006). G-overhang can form intra- or inter- molecular G-quadruplex structures *in vitro* but their *in vivo* occurrence has been demonstrated only in ciliates (Paeschke, 2008; Paeschke, 2010), so further investigations are needed to clarify if these structures are present at telomeres of higher eukaryotes cells *in vivo*, to understand how their formation could be regulated and how they can contribute to telomere maintenance (Paeschke, 2010).

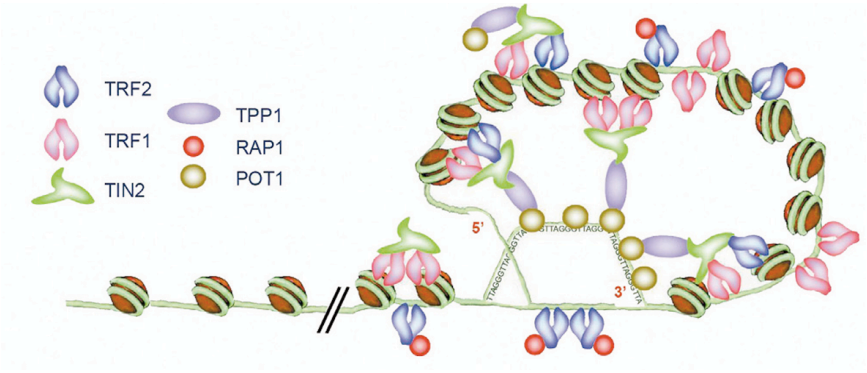


Figure 1: Model of mammalian telomeres structure.
G-overhang folds back and invades upstream dsDNA, forming the t-loop. Shelterin components and nucleosomes, in brown, are represented. Adapted from Pisano, 2008.

How telomeres solve the end protection problem: shelterin

The second solution to the end protection problems in mammalian cells is represented by the above mentioned six-subunits protein complex, that is part of telomeres, called shelterin (Figure 1). Shelterin enables cell to distinguish their natural chromosomes ends from ds-breaks, represses DNA repair reactions and regulates telomerase mediated telomere-lengthening, as described in the next sections (Palm, 2008). The components of shelterin complex specifically localize to telomeres; they are abundant at telomeres throughout the cell cycle; they work exclusively at telomeres and don't have function elsewhere in the nucleus (Palm, 2008). The specificity of shelterin for telomeric DNA is due to the recognition of TTAGGG repeats by two of its components: TRF1 and TRF2 (Telomeric Repeat binding Factor 1 and 2) that bind the ds part of telomeres with their Myb/SANT-type DNA binding domain (Broccoli, 1997; Palm, 2008; de Lange 2010). The complex contains also a third DNA binding protein, POT1 (Protection Of Telomeres 1) that binds ss TTAGGG repeats with its two OB (oligosaccharide/oligonucleotide binding) fold domains; POT1

binding activity is crucial for telomeres protection but doesn't have a role in anchoring the shelterin complex at telomeres (Loayza, 2003; de Lange 2010). POT1 is recruited at telomeres through its interaction with another component of shelterin complex, TPP1 (known also as TINT1, PTOP, PIP1) (Liu, 2004; Ye, Hockemeyer, 2004; de Lange, 2010). TPP1 binds to TIN2 (TRF2- and TRF1-Interacting Nuclear protein 2), the linchpin of shelterin complex, that interacts also with TRF1 and TRF2, providing a bridge between the shelterin components that bind to ds and ss telomeric DNA and connects also TRF1 and TRF2 (Ye, Donigian, 2004; O'Connor, 2006; de Lange 2005; Palm 2008). The last shelterin component is Rap1, the human ortholog of the yeast Repressor/Activator Protein 1, that is recruited at telomeres through its interaction with TRF2 (Li, 2000; Chan 2010). Shelterins form a stable complex also in the absence of telomeric DNA (Palm, 2008) and the TRF1/TRF2/TIN2/Rap1 core of shelterin is very abundant; a recent study suggests that human telomeres could contain hundred of copies of this complex potentially covering all telomeric DNA (Takai, 2010). The same study estimates also that POT1/TPP1 complexes are less abundant, about 50 copies per telomere, but it indicates that POT1 is likely to be present in excess of its ss binding site (Takai, 2010; de Lange, 2010). The shelterin complex is highly conserved in mammals, including mice that are frequently used for gene targeting studies. The only distinctions in rodents is the presence of two POT1 genes, Pot1 a and b, both interacting with Tpp1 and approximately equal abundant at telomeres, but with different functions (Hockemeyer, 2006). Shelterin proteins are essential to survival of mammalian cells, as their depletion either drives cells into cellular senescence or results in early embryonic lethality (Chan, 2010).
Each protein of the complex plays a specific role in chromosomes ends protection and in telomeres homeostasis and, for this reason, we are going to analyze them more in detail. TRF1 and TRF2 share a common domain structure consisting of the TR homology domain (TRFH) and a C-terminal SANT/Myb DNA-binding

domain, which are connected through a flexible hinge domain (Broccoli, 1997). The N terminal part of the two proteins is different and confers them the possibility to interact with different telomeric accessory proteins and to recruit specific subset of accessory factors to telomeres: the N terminus of TRF2 contains a Gly/Arg rich domain, named GAR domain or basic domain, whereas TRF1 has acidic aminoacids at its N terminus (Broccoli, 1997; Palm 2008). Both TRF1 and TRF2 recognize telomeric DNA by their SANT/Myb domain and bind TTAGGG repeats as homodimers or oligomers formed through homotypic interaction in the TRFH domain; they don't interact directly (Broccoli, 1997; Fairall, 2001; Court, 2005). Both TRF1 and TRF2 are subjected to regulative modifications, such as parsylation, ubiquitylation, sumoylation or phosphorylation, but the functional consequences of these modifications are not yet been fully understood (Palm, 2008). TRF2 plays a main role in chromosomes ends protection selectively blocking the activation of ATM (Denchi, 2007) and NHEJ pathways at telomeres (van Steensel, 1998; Smogorzewska, Karlseder, 2002; Celli 2005). Indeed when TRF2 is deleted in mouse embryonic fibroblasts (MEFs) or inhibited with a dominant negative allele in human cells, most, if not all, telomeres become sites of a DNA damage activation signal that is mediated by ATM. Cytologically, this event is readily detectable as the formation of DNA damage foci located at telomeres named TIFs (telomere dysfunction-induced foci) containing DNA damage response factors, as γH2AX, 53BP1, MDC1 and activated ATM (Takai, 2003; Celli, 2005; Dimitrova, 2006). Following TRF2 depletion TIFs form in all stages of interphase cells (Konishi, 2008). This DNA damage response activation appears to be ATM dependent and when ATM or components of MRN complex are genetically inactivated in MEFs the deletion of TRF2 fails to induce TIFs formation (Celli, 2005; Denchi, 2007; Attwool, 2009; Deng, 2009; Dimitrova, 2009). Consistent with the activation of ATM pathway, TRF2 deletion triggers phosphorylation of Chk2 and the activation of p53/p21 pathway leading to cell cycle, prevalently G1/S, arrest.

In fibroblast this arrest is accompanied by senescence while in other cell types it can induce apoptosis (van Steensel, 1998; Karlseder, 1999; Smogorzewska 2002). Currently two models are proposed to explain how TRF2 can block ATM activation at telomeres: in the first model TRF2 can directly repress ATM activation given that it can interact with ATM and its overexpression can inhibit ATM activation also at nontelomeric sites (Karlseder, 2004); the second model invokes a key role for TRF2 in maintaining a higher-order structure at telomeres in which the DNA free ends sensed by ATM are hidden, as in t-loop structure, because, as mentioned before, TRF2 seems able to promote t-loop formation *in vitro* (Griffith 1999; Stansel, 2001). According to this second model, t-loop impedes MRN loading on DNA end by simple occlusion, preventing ATM activation (de Lange, 2010). TRF2 has also a crucial role in blocking NHEJ activation at telomeres. Indeed TRF2 inhibition, in p53 KO MEFs, generates numerous chromosome end fusions, resulting in long strings of joined chromosomes (van Steensel, 1998; Smogorzewska, 2002; Celli, 2005). NHEJ takes place primarily in G1 phase, but also postreplicative fusions are observed in TRF2 KO cells (Konishi, 2008). The depletion of other shelterin components don't lead to this massive telomeric fusions phenotype suggesting that TRF2 is the principle responsible for NHEJ inhibition at telomeres. However TRF2-mediated repression of telomere fusions is sufficient to protect chromosome ends in G1, but after DNA replication additional mechanisms are required and these involve POT1 and also nonshelterin proteins, like Apollo (de Lange, 2009). The molecular mechanism by which shelterin prevents NHEJ activation at telomeres is still unclear. The current model is similar to the t-loop model for ATM repression; again TRF2 could act in favouring the formation or stabilization of the t-loops that are expected to block Ku70/80 loading on chromosomes ends thereby preventing NHEJ activation at its first step (de Lange, 2010). TRF2 is important also in repression of HR at telomeres, although the heterodimer Ku70/80, an accessory factors recruited

at telomeres through its interaction with shelterin components, has a key role in telomere protection from this kind of DNA repair and in particular in inhibiting telomere sister chromatid exchanges (TSCEs) (Celli, 2006). However the highest incidence of TSCEs is observed when both TRF2 and Ku70 are depleted (Celli, 2006).
TRF1 has a minor role in telomere ends protection; it stabilizes and enforces POT1 binding to telomeres and, as discuss below, it's principally involved in regulating telomerase dependent telomeres elongation and in semi-conservative telomeric repeats replication. In this last context the absence of TRF1 can activate an ATR mediated DNA damage response at telomeres during S-phase progression (Sfeir, 2009). Nevertheless this DNA damage activation is due to a replication problem that arises at telomeric repeats when TRF1 is deleted and can lead to fragile-site-like phenotype at telomeres (Sfeir, 2009), that is analyzed more in detail in the next section.
Rap1 is a constitutive binding partner of TRF2 and it's dependent on TRF2 for its telomeric localization and stability (Li, 2000; Li, 2003). It is dispensable for telomere protection from ATM and NHEJ activation at telomeres because deletion of Rap1 in TRF2 proficient cells doesn't lead to telomere fusions or TIFs formation (Martinez, 2010; Sfeir, 2010). Rap1 seems to have an important role in repressing HR; indeed in Ku70 KO context, Rap1 depletion induces high frequency of TSCEs (10% of chromosome ends) (Martinez, 2010; Sfeir, 2010). How Rap1 protects telomeres from HR is not clear; it's likely that Rap1 has an interacting partner that block HR (de Lange, 2010). Moreover Rap1 has a role in telomere length regulation.
A crucial role in telomere capping is played by POT1. This shelterin protein in its N terminus has two OB-fold domains with which it can recognize G-overhang (Lei, 2004; Loayza, 2004). POT1 contains also a third OB-fold domain at its C terminus (Theobald, 2004). POT1 has a preference for TAGGGTTAG site at 3' end and its binding is stimulated by TPP1; however it can bind at many positions along G-overhang, suggesting that POT1/TPP1

can bind ss telomeric repeats in a 3' end independent way, indicating that POT1 can bind also D-loops when telomeres are in t-loops configuration (Palm, 2008). POT1 has a crucial role in repressing ATR pathway activation at telomeres. Simultaneous depletion of Pot1 a and b from MEFs results in TIFs formation at most telomeres and elicits a cell cycle arrest; this DNA damage response is unaltered in ATM-deficient cells but it's diminished when ATR is inhibited (Hockmeyer, 2006; Denchi, 2007). POT1 depleted cells show phosphorylation of both Chk1 and Chk2 and this modification is reduced in the absence of functional ATR (Denchi, 2007). In mouse cells, Pot1a is the primary factor in repressing ATR signaling whereas Pot1b has a subsidiary role in this context (Hockmeyer, 2006); however Pot1b is important for G-overhang length regulation (Hockmeyer, 2008). Since ATR signaling is activated following RPA binding to ssDNA, a simple competition model has been proposed to explain how POT1 represses ATR pathway at telomeres. According to this model POT1 and RPA compete for the binding to ssDNA at telomeres ends and the presence of POT1 at ss telomeric repeats would block RPA binding thereby preventing ATR activation (de Lange, 2009). Consistent with this model, normally RPA is not observed at telomeres but when both Pot1 a and b are depleted, RPA is detectable at telomeres; moreover RPA is required for ATR pathway activation at unprotected telomeres (Gong, 2010). However RPA is a very abundant protein, in contrast POT1 is much less abundant; moreover the affinity of POT1 for ss telomeric DNA is in the same range of RPA binding affinity, even when POT1 is bound to TPP1 (de Lange, 2010). Despite these considerations POT1 is able to exclude RPA from ss telomeric DNA; likely POT1 wins the competition with RPA for ss TTAGGG repeats because it's tethered to telomeres by the other shelterin components. Indeed through its TPP1-TIN2 link, POT1 accumulates at telomeres and this connection provides POT1 with two telomeric binding sites, one through POT1-ss telomeric DNA interaction and the other through protein-protein interaction that

connects POT1 to TRF1 and TRF2 and so to ds TTAGGG repeats (de Lange, 2009; de Lange, 2010). POT1 is also important for NHEJ repression after DNA replication. Its involvement in inhibition of this pathway is demonstrated by the mild telomeres fusion phenotype that is observed in human cells following POT1 reduction and in mouse cells depleted of both Pot1 a and b (Veldman, 2004; Hockemeyer, 2005). Interestingly these fusions involve sister chromatids indicating a postreplicative event (Hockemeyer, 2006). The current model proposes that POT1 loading on the ss overhang keeps the repair pathway inactive until higher-order telomeric structure is reformed after semiconservative replication; this last process involves also nonshelterin telomeric proteins, as Apollo (de Lange, 2010). Moreover POT1 is required for the repression of HR at telomeres. Indeed, as happens for Rap1, the depletion of both Pot1 a and b in the context of Ku70 null mice, induces high incidence of TSCEs (Palm, 2009). For HR repression it could be important the ability of POT1 to compete with ssDNA binding proteins, because HR beginning involves the binding of both RPA and Rad51 to ssDNA (de Lange, 2009).
TIN2 has a crucial role in stabilizing shelterin complex and so both in ends protection and in telomeres length regulation. Indeed TIN2 is able to interact simultaneously with TRF1, using its C terminus, and with TRF2, using its N terminus (Ye, Donigian, 2004); in this way TIN2 forms a bridge between TRF1 and TRF2 stabilizing and enforcing the binding of the two proteins at telomeres. Consistent with this, TIN2 reduction in human cells has a profoundly destabilizing effect on shelterin, inducing a reduction of TRF2 telomere-bound levels. Moreover TIN2 recruits TPP1, and therefore POT1, to telomeres, using a third protein interaction site distinct from its TRF2 binding site but located at its N terminus. This kind of interaction has a crucial importance for POT1 recruitment at telomeres and for POT1 dependent telomere ends protection from ATR pathway (O'Connor, 2006).
As TIN2 also TPP1 takes part in ends protection promoting POT1 recruitment at telomeres. Indeed TPP1 can bind both POT1

through a centrally located interacting domain, and TIN2 through its C-terminal domain (Liu, 2004; Ye, Hockemeyer, 2004). At its N terminus is present an OB-fold domain that can interact with telomerase suggesting that this shelterin component can be involved also in telomeres length regulation (Xin, 2007). TPP1 is essential to recruit and stabilize POT1 at telomere ends (Hockemeyer, 2007) and its depletion or the expression of a mutant form of TPP1 unable to bind POT1, leads to removal of all POT1 from telomeres generating a telomere deprotection phenotype consistent with POT1 loss (Hockemeyer, 2007; Liu, 2004; Kibe, 2010). Moreover TPP1 is essential also for POT1 nuclear localization: POT1 mutants lacking TPP1 interacting site, are excluded from the nucleus and also TPP1 downregulation diminishes the amount of nuclear POT1 (Chen, 2007).

How telomeres solve the end protection problem: nonshelterin telomeric proteins

In addition to shelterin complex, mammalian telomeres contain a large number of nonshelterin telomeric proteins, also called accessory proteins, that give an important and essential contribution to chromosomes ends maintenance and protection. The nonshelterin proteins are typically less abundant at telomeres than shelterin and some of them are only transiently associated to telomeres; moreover, unlike shelterin components, these proteins have also nontelomeric function. Most of them are involved in DNA transactions, such as DNA repair, DNA damage signaling, chromatin structure and often are recruited to telomeres by interaction with shelterin components, in particular TRF2 and TRF1 (Palm, 2008). Since nonshelterin proteins normally function in DNA processing, at one hand they facilitate shelterin tasks and take part in chromosomes ends protection, but on the other hand, these factors are potentially harmful for telomeres. This paradox suggests that shelterin must carefully control and regulate nonshelterin proteins actions, although mechanisms used for this

control have not yet be fully elucidated (de Lange, 2005). There are a lot of nonshelterin factors associated to telomeres and acting in their metabolism; for most of them telomeric function has been elucidated but for some of them it is still enigmatic. Among the best characterized nonshelterin telomeric proteins, particularly interesting are DNA-PKcs and the above mentioned heterodimers Ku70/80. These proteins normally promote NHEJ but they associate to telomeres through interaction with shelterin; ironically they are involved in NHEJ and HR repression at telomeres. Indeed mouse cells depleted of DNA-PKcs show a significant increase in end-to-end fusions (Bailey, 1999; Goytisolo, 2001; Bailey, 2001) indicating that its function is important for chromosomes ends protection. Moreover all the three subunits of DNA-PK have found to be associated to telomeres (d'Adda di Fagagna, 2001). Ku70/80 is a very important factor in telomeres protection from HR and it has been observed at telomeric repeats (Hsu, 1999). Depletion of TRF2 or POT1 or Rap1 alone is not sufficient to induce TSCEs; when Ku70/80 is present, even when NHEJ is blocked, exchanges are rare (Celli, 2006). How Ku70/80 acts to prevent TSCEs has not been established but it may be related to its general ability to repress homologous recombination, or it could be important the association with shelterin to repress HR at telomeres (de Lange, 2010). Another DNA damage repair protein that acts paradoxically at telomeres is ERCC1/XPF; this nuclease is implicated in the processing of G-overhang after telomere damage but it's essential to prevent the recombination of telomeric ends with TTTAGG-like interstitial sites (Zhu, 2003). This phenomenon can potentially lead to terminal deletions in which chromosomes lose all sequences distal the interstitial TTTAGG repeat array and produce an extrachromosomal elements, double minute chromosomes (TDMs) that contain the deleted segment with most of the original telomeres (Palm, 2008). These TDMs are observed in cells lacking ERCC1 (Zhu, 2003) and it has been proposed that ERCC1/XPF endonuclease could promote the cleavage of the strand-invaded intermediate (Palm, 2008). Another nonshelterin protein that can

counteract TDMs formation is the RecQ helicase WRN that acts particularly when telomeres are short (Laud, 2005). WRN is important also to repress TSCEs formation; indeed, when it's absent, in a telomerase negative setting, frequent TSCEs are observed (Laud, 2005). It has been proposed that WRN can block recombination at telomeres moving the Holliday junction, deriving by strand-invasion, toward end terminus (Palm, 2008). The engagement of WRN at telomeres is paradoxically because it has been demonstrated that *in vitro* WRN can resolve the t-loop structures and degrade G-overhang (Opresko, 2002; Opresko, 2004). However WRN is recruited at telomeres by TRF2 in S-phase (Crabbe, 2004; Machwe, 2004) and it's important also for telomeres replication because in the absence of WRN, S-phase dependent telomere loss has been observed (Bai, 2003; Crabbe, 2004). It has been proposed that WRN could be important to unwind G-quadruplex structures from telomeric repeats that could impede telomere replication (de Lange, 2005; Paeschke, 2010). Apollo nuclease is another nonshelterin protein that has been implicated telomeres replication and in the generation of G-overhang after replication. Indeed the reduction of Apollo in human cells induces TIFs and DNA damage response activation in S-phase cells, suggesting its involvement in telomere replication (Lenain, 2006; van Overbeek, 2006). Apollo depletion generates fusions that involve the leading ends (Lam, 2010; Wu, 2010) and for this reason it has been proposed that TRF2 recruits Apollo at the leading ends of telomeres to regenerate G-overhang at this strand that presumably is blunt right after replication (de Lange, 2010). It's possible that the regeneration of G-overhang after replication requires also the recruitment at telomeres, maybe operated by TRF2, of other nonshelterin factors as MRN complex or ATM (de Lange, 2010).

Other nonshelterin telomeric identified proteins are: Bloom helicase (BLM), PARP1 and 2, tankyrases, Rad51, 9-1-1 complex, ORC, HP1 proteins, PINX1, PIN1, FEN1, SLX4, Mus81, BRCA1

but it's likely that this list will grow (de Lange, 2005; Palm, 2008; de Lange, 2010; Chan, 2010; Giannone, 2010).
Recently three additional nonshelterin telomeric factors are identified in mammalian cells and, in particular, in human cells, that are the homologues of yeast CST complex components (Miyake, 2009; Surovtseva, 2009). Budding yeast telomeres are not protected by a shelterin-like complex; although the ds telomeric repeats are bound by Rap1 and two associated factors; these proteins are not involved in chromosomes ends protection. Instead this function is fulfilled by a trimeric complex, CST, comprised of Cdc13, Stn1, Ten1, which bind G-overhang. In yeast CST plays a dual role in telomeres protection and modulation of their replication (Price, 2010). This complex is related to RPA complex but performs a different biological function (Gao, 2007; Sun, 2009). The mammalian CST complex is formed by Ctc1-Stn1-Ten1 (Miyake, 2009), localizes at telomeres but several lines of evidences suggest that it can have both telomeric and nontelomeric functions: it binds ssDNA in a sequence independent manner and shows also nontelomeric localization (Price, 2010). Depletion of CST components in human cells leads to an increase in G-overhang length and induces telomere dysfunctions and genome instability (Miyake, 2009; Surovtseva, 2009). It has been proposed that CST complex in mammalian cells has a minor role in chromosomes ends protection but it could play a role in G-overhang generation and maintenance (Dai, 2010). Basing on the analysis of the nature of telomere dysfunctions generated by its loss, it could be important for telomeres replication but it can also take part in replicative processes in presence of replication stress. In this context, specifically, this complex may serve as a DNA polα/primase recruitment factor at telomeres or where replication stress induces the accumulation of ssDNA and the need to re-initiate leading or lagging strand synthesis (Price, 2010; Sampathi, 2011).

How telomeres are replicated and the end replication problem

Telomere maintenance is based on conventional semiconservative replication, which accounts for the bulk of telomeric DNA synthesis, and additional mechanisms could be present to extend G-strand, which as discussed below, shortens each replication round. The replication of eukaryotic genomes initiates bidirectionally at defined origins. At chromosomes ends, the last origin is expected to be responsible for the replication of telomeric sequences (Gilson, 2007). Currently, except in few organisms, there has been little evidence of initiation of DNA replication within the telomeric repeats themselves (Makovets, 2004), so telomeric replication is thought to start from an origin located in subtelomeric region (Liew, 2009; Sampathi, 2011). Replication forks move bidirectionally from subtelomeric origins towards telomeric repeats; replication terminates when the fork reaches the chromosome end. Given the conserved directionality of the telomeric repeats, the new G-strand is always synthesized by leading-strand synthesis, and the new C-strand by lagging-strand synthesis; it means that parental G-strand is replicated by discontinuous lagging synthesis generating new C-strand and vice versa parental C-strand is replicated by leading synthesis generating new G-strand (Liew, 2009). Due to the inability of DNA polymerase to replicate in 3'-5' direction and the consequent necessity of primers to start the replication of parental G-strand, the removal of the last 5' primer from the newly synthesized C-strand leaves it incomplete. In this way the newly replicated C-strand would progressively shorten, eventually compromising telomere function and resulting in loss of genetic information. This has become known as the end replication problem. Most organisms solve this matter by the action of telomerase, a reverse transcriptase that is able to add new telomeric repeats at chromosomes ends and that is active in specific tissues and cell types. Moreover different mechanisms involving retrotransposition or recombination can compensate this erosion (Gilson, 2007), as

discussed more in detail in the next section. The lagging end resulting from removal of the last primer is thought to have already a G-overhang, whereas the leading end resulting from replication is thought to be blunt ended. 5' resection is necessary for 3'-overhang generation and Apollo, a nonshelterin telomeric protein, has been implicated in this process (Wu, 2010). Apollo probably is not the only nuclease that mediates the generation of 3'-overhang at leading strand because when Apollo is absent leading telomere fusions occur but are not frequent (Lam, 2010; Wu, 2010), suggesting that there is another pathway that can generate 3'-overhang when Apollo is missing. One possibility is that the ATM signaling at telomeres lacking Apollo is responsible for 5' resection. MRN mediated ATM activation can induce the resection at telomeres, as happens at ds-breaks, most likely through CtIP nuclease (de Lange, 2010). Indications of ATM and MRN involvement in G-overhang generation come from experiments in which TRF2 is deleted in cells lacking MRN or ATM (Attwool, 2009; Dimitrova, 2009).

Telomeres appear to be one of the most difficult-to-replicate genomic regions: during the passage through telomeric repeats, replication fork faces a number of potential challenges due to their long G-rich repetitive nature and their unique chromatin structure (Sampathi, 2011). Moreover, due their terminal position, stalled fork in the telomere cannot be rescued by a converging fork, and is likely to result in incomplete telomeric DNA replication (Liew, 2009). Replication fork stalling at telomeres can activate a ATM/ATR mediated DNA damage response leading to cell growth arrest or creating a situation that may lead to accelerated telomere attrition (Sampathi, 2011). For these reasons telomeres resemble fragile sites resulting from defects in replication and are prone to deletions and chromosome rearrangements (Sfeir, 2009). Telomeric proteins facilitate replication fork passage through telomeric repeats ensuring their complete replication. One of the challenges that replication fork need to solve during telomeric DNA replication is the presence of secondary structures such as G-

quadruplex or t-loop, described above. The formation of these structures may contribute to telomere function, but these structures have to be tightly regulated as they could impede replication fork passage and telomerase mediated elongation (Paeschke, 2010). Their resolution requires the actions of RecQ helicases BLM and WRN; these proteins can unwind G-quadruplex structures (Mohaghegh, 2001) and they could also resolve telomeric D-loop to allow passage of replication fork and/or telomerase access (Crabbe, 2004). WRN and BLM activity at telomeres is regulated and stimulated by shelterin proteins, in particular TRF2 and POT1 (Opresko, 2002; Lillard-Wetherell, 2004; Opresko, 2004). Moreover WRN deficient cells specifically lose telomeres replicated by lagging-strand synthesis (Arnoult, 2009) and, confirming its importance for telomere replication, WRN localizes at telomeres during S-phase (Crabbe, 2004).
Recently it has been emerged a predominant role for TRF1 in efficient telomere replication. Loss of TRF1 leads to aberrant telomere structural changes resembling common fragile sites, which activate ATR pathway during S-phase; moreover single molecule analysis of replicated DNA reveals that the absence of TRF1 weakens replication efficiency, suggesting that TRF1 promotes efficient replication of telomeric DNA by preventing fork stalling (Sfeir, 2009; Martinez, 2009). It's not completely clear how TRF1 promotes efficient telomeric replication, but it has been proposed that it acts recruiting additional factors as BLM or RTEL1 to stalled replication fork at telomeres promoting its stabilization and re-starting (Sfeir, 2009; Paeschke, 2010).
Mammalian telomeres, although heterochromatic and difficult to replicate, differently from yeast telomeres that are replicated in concert late in S-phase (Friedman, 1995), replicate throughout all S-phase (Wright, 1999).
In mammalian somatic cells, that lack telomere length maintenance pathway, each round of DNA replication is accompanied by a net loss of 100-200 bp, as consequence of incomplete replication of the ends and of the post-replicative processes that are involved in

restoration of overhang and protective structure formation. Telomere shortening at each cell division is more than that predicted from primer removal and it's likely that postreplicative phenomenon of C-strand resection, described above, happens at both replicated ends (Huffman, 2000). As a function of the rate of replication associated shortening and initial telomere length, a somatic cell can only undergo a defined number of doublings before telomeres become critically short, lose their protective properties and send cells into a terminal arrest termed replicative senescence, or cause cell death (O'Sullivan, 2010). This mechanism limits the replicative lifespan of individual cell and probably of some cellular compartments in organisms and therefore is a tumour suppressive pathway that prevents cells from becoming immortal. The fact that most cancer need to activate a telomere length maintenance pathway for survival, emphasizes the protective role of telomere attrition (O'Sullivan, 2010). The bulk of telomeres in human primary fibroblasts that enter in senescence seems to be around 4kb (Karlseder, 2002) and only one telomere that reaches a critically short length is enough to induce cell cycle arrest (Hemann, 2001). Critically short telomeres activate a DNA damage response that is similar to that triggered by the presence of uncapped telomeres and ultimately induce cell cycle arrest or apoptosis (d'Adda di Fagagna, 2003). However germ line cells, and highly proliferative tissues require the activation of telomere length maintenance pathway to counteract the loss of terminal DNA at each cell division.

How telomeres solve the end replication problem: telomerase

Most eukaryotes counteract telomere attrition thanks to telomerase (Greider, 1985; Greider, 1987). This is a ribonucleoprotein enzyme with two components: a highly conserved reverse transcriptase (TERT) (Nakamura, 1997; Lingner, 1997) and an associated template RNA (TERC) (Feng, 1995). TERT is related to the reverse transcriptase of non-LTR retroposons and group II introns

and like these, it extends the 3' end of a DNA, rather than RNA, primer (Nakamura, 1998; Smogorzewska, 2004). The RNA component of telomerase diverged quickly in evolution, but TERCs from different organisms share common structural features, including a pseudoknot and an open loop containing the template for telomeric repeats synthesis (Romero, 1991; Chen, 2000). The template region in mammalian TERCs (AAUCCCAAUC) serves for both the annealing with 3'-overhang and the addition of one telomeric repeat per elongation step (Palm, 2008). The primer for telomerase is the chromosome terminus, which can be positioned on an alignment site in TERC such that the 3' end of the telomere is adjacent to the template sequence. Extension of the telomere terminus results in the addition of one telomeric repeat, and repeated alignment and extension steps can endow chromosome ends with the direct repeat arrays typical of telomeres. After elongation of 3' end, C-strand synthesis is presumably required to create ds telomeric DNA (Smogorzewska, 2004). Telomerase activity is strongly suppressed in human somatic cells; its activity is restricted to ovaries, testes and highly proliferative tissues (Cong, 2002). This regulation takes place primarily regulating transcription levels of hTERT gene, whereas hTERC is virtually ubiquitous. For this reason, forced expression of hTERT in human primary fibroblasts is sufficient to reconstitute telomerase activity and counteract telomere erosion, immortalizing the cells (Ramirez, 2001). Tumour cells require a telomere maintenance system for long-term proliferation, and in the majority of cases, 85%, this is provided upregulating hTERT (Henson, 2002). However telomerase activity per se doesn't induce transformation (Morales, 1999; Smogorzewska, 2004), and, although telomerase is necessary for immortalization, hTERT is not an oncogene (Hahn, 1999; Hahn, 2002; Smogorzewska, 2004); moreover, cancer cells can activate also alternative mechanisms to counteract telomeres attrition.

Despite variation in the length of individual telomeres within a cell or an organism, the average telomere length of telomerase-positive

cells is kept in a narrow species-specific range, indicating a balance between telomere erosion and telomere elongation. This equilibrium is due to regulation of telomerase *in cis* by telomeric protein (Palm, 2008). The key regulatory principle in telomere length homeostasis is the presence of a negative feedback in which the product of telomerase, the telomeric DNA, binds to an inhibitor of telomerase in an amount proportional to telomere length (van Steensel, 1997; Marcand, 1997). In mammalian cells shelterin complex represents telomerase inhibitor that regulate its activity; indeed the level of shelterin bound to telomeres increases with the number of TTAGGG repeats and is used to "count" telomere length (Palm, 2008). TRF1 has a key role in this process; indeed TRF1 binds to ds telomeric repeats and the total number of TRF1 molecules per chromosome end is correlated with the length of telomeric tract (van Steensel, 1997). Moreover, TRF1 is a negative regulator of telomerase since its overexpression causes telomeres shortening, whereas its partial inhibition, through expression of a dominant negative allele, causes telomeres elongation (van Steensel, 1997). Experiments performed using a mutant TRF1 tethered to a subtelomeric site, showed that TRF1 can limit telomerase dependent elongation *in cis* (Ancelin, 2002). Moreover, negative regulators of TRF1 that inhibit its binding to telomeres, as tankyrase 1 and 2, are positive regulators of telomerase activity; indeed their overexpression removes TRF1 from telomeres leading to telomere elongation (van Steensel, 1997) enforcing the idea that TRF1 is a key regulator of telomere lengthening. Also other shelterin components as TIN2, TPP1 and POT1 behave as negative regulators of telomerase (Palm, 2008). TIN2 appears to stabilize TRF1 at telomeres and protects TRF1 from being modified by tankyrase inhibiting TRF1 dislodgement activity (Kim, 1999). POT1 has a crucial function in the direct inhibition of telomerase. Diminished POT1 loading at 3'-overhang or its replacement with a mutant lacking of ssDNA binding domain causes a telomerase dependent telomeres elongation (Loayza, 2003; Ye, Hockemeyer, 2004). Moreover POT1 and telomerase compete *in vitro* for ss

overhang binding (Lei, 2005) suggesting that POT1 could act as a terminal transducer that relays the information about telomere length from TRF1 to chromosomes ends (Palm, 2008). Consistent with this model is the finding that TPP1 depletion causes telomeres elongation; its removal reduces POT1 recruitment at telomeres leaving chromosomes ends accessible to telomerase (Liu, 2004). Data on TRF2 and Rap1 are also consistent with a role as negative regulators of telomeres length but their contribution is less defined (Li, 2003; O'Connor, 2004; Smogorzewska, 2000; Palm, 2008). This regulation system, in which shelterin proteins are used to "count" telomere length and to regulate telomerase activity, ensures that in a cell, telomerase acts preferentially at short telomeres that present low levels of bound shelterin, elongating them and is less active at long telomeres that presents a higher quantity of bound shelterin avoiding to add further repeats. As a consequence, telomeres, in a given telomerase positive cell, tend to converge to a similar median telomere length setting (Smogorzewska, 2004).

How telomeres solve the end replication problem: alternative lengthening mechanisms

Mammalian cells have the ability to activate telomerase-independent telomere maintenance pathways, collectively termed ALT (Alternative Lengthening of Telomeres) (Bryan, 1997; Reddel, 2003; Neumann 2005). All cancer telomerase negative cells, around 15%, maintain their telomeres through ALT mechanisms (Reddel, 2001). ALT cells, differently from telomerase positive cells, are characterized by heterogeneous length phenotype with an average length higher than telomerase positive cells, for human cells is around 20 kb, and a wider distribution of telomeres length of the same cells, in human cells ranging from 3 kb to 50 kb. Telomere length distribution in ALT cells is dynamic, with fluctuation in length occurring on individual

telomere during cellular proliferation and with rapid and drastic changes in individual telomere length (Henson, 2002). Moreover ALT cells show frequent sequence exchanges between sister telomeres (Londono-Vallejo, 2004). ALT mechanism of telomere maintenance is based on intertelomeric HR; different models can explain ALT mechanism: a ssDNA at the end of one telomere can invade dsDNA of another telomere and can use it as a copy template, or telomere lengthening can be achieved by a recombination mediated rolling circle of replication that can also use t-loops as templates (Henson, 2002). ALT cells are also distinguished by the presence of nuclear structures referred to as ALT-associated PML bodies or APBs (Yeager, 1999) that are different from the other PML bodies for their content: it includes telomeric DNA and shelterin TRF1 and TRF2 and a lot of proteins involved in DNA replication and recombination as Rad51, Rad52, RPA, Mre11, Rad50, Nbs1, BLM and WRN. They could represents sites specialized for ALT telomere elongation (Henson, 2002).
Another alternative mechanism of telomere elongation based on retrotransposition is present in *Drosophila* and some related Dipteran species (Biessmann, 1990), as discussed more in detail below.

Drosophila Telomeres

Drosophila telomeres are remarkable because they are maintained by telomere-specific retrotransposons, rather than by the telomerase that maintains telomeres in the other eukaryotic organisms. While *Drosophila* telomeres appear to be physically very different from those of other species, the differences are not so strong as they can appear and moreover the function of telomeres is totally conserved in *Drosophila* despite the different mechanisms used to reach it (Mason, 2008).
In *Drosophila*, telomeres, as those of the other eukaryotes, are composed of tandem repeats, but while in most eukaryotes they are simple and short (6-8 bp), in fly repeats are longer (6-12 kb) and more complex. Differently from the other organisms in which telomeres are devoid of genes, in *Drosophila,* telomeric repeats codify for the proteins involved in retrotrasposons mobilization. Indeed in this organism terminal repeats consist of arrays of telomere-specific non-long terminal repeats (LTR) retrotransposons, HeT-A, TART and TAHRE (collectively HTT elements) (Figure 2), present on multiple copies on normal telomeres and able to "heal" broken chromosome ends (Mason, 1995; Pardue, 1997; Abad, 2004; Pardue, 2005; Mason, 2008). All three elements have very long 3' untranslated regions (UTR); TART and TAHRE each have two open reading frames (ORFs) whereas Het-A has only one ORF (Frydrychova, 2008). ORF1, which is found in all three elements, encodes a GAG-like protein that binds to RNA retroelement and transports it back to the nucleus (Rashkova, 2002). ORF2 encodes a reverse transcriptase (RT) and it lacks in Het-A, so it's likely that the RT required for Het-A transposition derives from TAHRE and acts *in trans* also on Het-A (Figure 2) (Abad, 2004; Shpiz, 2007).

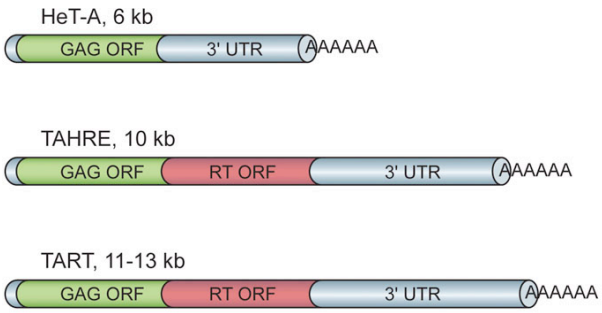


Figure 2: Telomeric non-LTR retrotrasposons.
GAG and RT ORFs are indicated. 3'UTR are long at least 3 kb and 3'oligo(A) tail used to attach at chromosomes ends are indicated by AAAAAA. Adapted from Frydrychova, 2008.

In *Drosophila* these three elements are involved in telomere elongation that, as in all other organisms, is necessary to counteract telomere shortening due to ends incomplete replication. Telomere elongation by addition of HTT elements to the chromosome end can occur by two distinct mechanisms, terminal conversion or targeted transposition (Frydrychova, 2008). HTT elements specifically transpose at chromosome ends and likely this specificity is due to Het-A GAG activity that can associate to chromosome ends (Figure 3) (Rashkova, 2003). Although the details of this process remain unresolved, in the proposed mechanism, RT of HTT elements could use free 3' terminus at chromosome end to prime reverse transcription and new HTT elements are added specifically at chromosome end in an head-to-tail arrangement with their oligo(A) tails always facing towards the centromere (figure 3) (Mason, 2008).
Gene conversion is a recombination based mechanism in which HTTs at the end of a chromosome are used as template for the elongation of another chromosome following a strand invasion step, that either involved sequences on the homologous

chromosome or tandemly arranged sequences on the same chromosome (Mikhailovsky, 1999; Kahn, 2000). Both in *Drosophila* and in the other eukaryotes that present telomerase based telomere elongation, telomeres are a product of a reverse transcription reaction and, as in the other species, although alternative recombination based elongation mechanisms are present, RNA-templated extension predominates also in *Drosophila* (Pardue, 2005; Frydrychova, 2008).

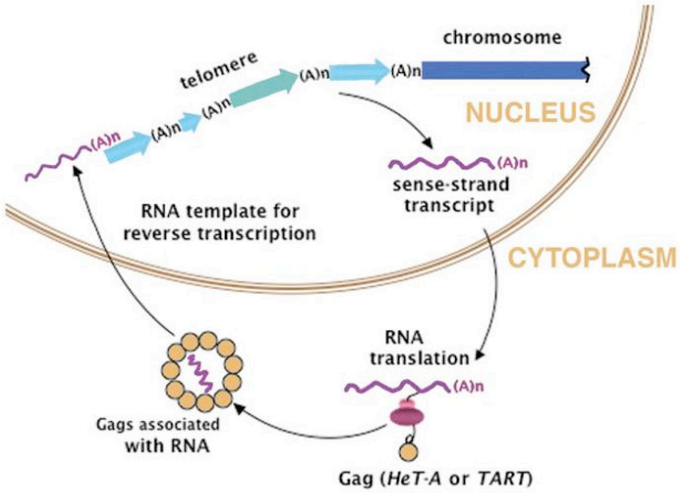


Figure 3: Model for extension of chromosomes end by telomeric retrotransposons. Arrows represent head to tail array of Het-A (blue) and TART (green) that form the telomere. Transcription of an element of the array provides sense RNA (purple) that is translated in cytoplasm to yield GAG protein (yellow). This protein associates with RNA and delivers it to chromosome end where the RNA is reverse transcribed onto the chromosome. Adapted from Pardue, 2011.

As in the other eukaryotes, telomere lengthening is under the control of telomere associated proteins. Among them HP1 encoded by *Su(var)205*, as described more in detail later, is the only identified protein that takes part both in telomere length regulation and in telomere capping. Mutations in *Su(var)205* cause telomere elongation enhancing both the transcription of HeT-A and TART and the frequency of their transposition at chromosome ends. Moreover also gene conversion is increased in *Su(var)205* mutants and involves tandem telomeric arrays of the same chromosome (Savitsky, 2002). These findings indicate that HP1 is a negative regulator of *Drosophila* telomeres length.
Heterodimer ku70/80 is an important negative regulator of telomere lengthening in *Drosophila*, but differently to its role in mammalian cells, it doesn't take part in telomere protection (Cenci, 2005). Mutations in ku70 and ku80 encoding genes increase the terminal transposition of HeT-A and TART without affecting their expression and enhance also terminal gene conversion both with tandem sequences of the same chromosome and of the homologous chromosome (Melnikova, 2005).
Drosophila telomere elongation is also promoted by mutation in *Tel* and *E(tc)* genes; Mutations in *Tel* induce a substantial increase in HeT-A and TART addition at chromosome ends (Siriaco, 2002) whereas mutations in *E(tc)* trigger terminal elongation by gene conversion using tandem arrays of the same chromosome as template (Melnikova, 2002).
Despite the obvious differences, HTTs sequence exhibits a strand bias in nucleotide content that resembles that seen in telomerase-generated telomeres sequences in other organisms (Mason, 2008) and they could form G-quadruplex structures (Abad, 1999; Mason, 2008). Moreover also in *Drosophila*, moving from chromosome end toward the centromere, telomere associated sequences (TAS) are present adjacent to HTT arrays. TAS region consists of several kilobases of complex satellite sequences, which varies among telomeres, although there are sequence similarities (Mason, 2008). It seems evident that HTT has a unique pattern of histones

modifications, including both heterochromatic and euchromatic marks as K3Me3K9, K3Me3K27 and H4AcK12 respectively. Nevertheless in *Drosophila*, as in the other organisms, telomeres are considered heterochromatic for two reasons: they contain repetitive DNA and they are able to repress the expression of genes inserted into telomeric regions. This telomere position effect (TPE) has been described first in *Drosophila* and later in the other eukaryotes; in fly the source of this effect was localized to TAS (Biessmann, 2005; Mason, 2008).
Not all telomeres in *Drosophila* include HTT array and moreover, several indications suggest that a telomeric protective cap can be formed in a sequence independent way (Cenci, 2005). Indeed, although the concept of telomere was conceived by Muller to account for the failure to recover terminally deleted chromosomes after X irradiation (Muller, 1938), subsequent experiments have demonstrated that these terminal deficiencies can be recovered in the presence of *mu2* mutation in females (Mason, 1984). These terminal deficiencies can be transmitted over many generations in a wild type background (Mason, 1995) and their molecular analyses have shown that they don't terminate with HTTs and continuously recede losing 75 bp per fly generation; for this reason, they never terminate with the same DNA sequence suggesting a sequence-independent mechanism for *Drosophila* telomere capping (Biessmann, 1990; Cenci, 2005). Moreover terminal deficiency chromosomes lacking HTT array have been found in natural populations (Mechler, 1985) and also in laboratory stocks (Mason, 2004). In addition, telomere capping proteins are normally recruited at the ends of terminally deleted chromosomes (Fanti, 1998; Cenci, 2003; Cenci 2005) enforcing the idea that *Drosophila* telomeres are capped in a sequence independent way and that virtually any DNA sequence has the ability to form the nucleoprotein complex that protects the ends of chromosomes, a feature that is proper of this organism.
Telomere protection and maintenance also in *Drosophila* requires the end specific binding and the action of different proteins. The

identification of these proteins has mainly relied on the isolation of mutants that show frequent telomere fusions in larval brain cells; molecular analysis of the genes specified by these mutants has allow the identification of loci required to prevent end-to-end fusions (Cenci, 2005). Among the identified proteins four of them seem to localize and work exclusively at telomeres; they form a complex called terminin that has all the features of shelterin and has been proposed to be its functional analog (Raffa, 2011). Terminin components are: HOAP (HP1/ORC-associated protein) (Cenci, 1997, Cenci, 2003), Moi (Raffa, 2009) and Ver (Raffa, 2010); mutations in their encoding genes, *Caravaggio (cav), Modigliani (moi)* and *Verrocchio (ver)* respectively, cause very high frequency of telomeric fusions and produce multicentric linear chromosomes that resemble little "trains" of chromosomes. Moreover they interact directly with each others and they localize exclusively at the ends of polytene chromosomes (Raffa, 2011). HOAP localizes to telomeres also on mitotic chromosomes (Cenci, 2003; Cenci 2005), whereas Moi and Ver were not detected at mitotic chromosomes ends, probably due to their low abundance (Raffa, 2009; Raffa, 2010). An additional terminin protein is HipHop (HP1-HOAP interacting protein), which has been identified among the proteins that co-precipitate with HOAP (Gao, 2010). HipHop directly interacts with HOAP, specifically localizes at both mitotic and polytene chromosomes ends and seems to work only at telomeres (Gao, 2010). Very little is known about the structural features of the complex: it's clear that HipHop and HOAP are mutually dependent for their stability and that Moi and Ver are mutually dependent for their localization at telomeres that requires also HOAP (Raffa, 2011). Moreover HOAP binds ds DNA of different sequences, although with different affinities and Ver contains an OB-fold domain that is similar to that of Rpa2/STN1 and binds ssDNA of different sequences (Raffa, 2010; Raffa 2011). HOAP and HipHop localize to the ends of terminally deleted chromosomes suggesting that these proteins bind chromosome ends independently of terminal DNA sequences

(Raffa, 2011). Currently it's unknown if Moi and HipHop directly bind DNA. Moreover mutations in *cav, moi* or *ver* not cause telomere elongation indicating that terminin, differently from shelterin, is not implicated in telomere length control (Raffa, 2011) enforching the idea that telomere elongation and telomere protection are partially separate processes in *Drosophila* (Cenci, 2005).
In addition to end-to-end fusions, the presence of uncapped telomeres triggers also a DNA damage response in *Drosophila* cells as happens in the other eukaryotes. Among the terminin, HOAP seems to play a key role in preventing not only telomere fusions but also DNA damage checkpoint activation (Ciapponi, 2008). Indeed mutations in *cav* gene lead to extensive end-to-end fusions and affect cell cycle progression. This response is dependent on ATR pathway activation, because mutations in the genes encoding for proteins involved in this pathway can alleviate the cell cycle block (Cenci, 2005; Musarò, 2008). The activation of this DNA damage response seems independent from ATM pathway and it has not yet been elucidated if the cell cycle arrest happens at G1/S phase transition or in G2/M transition (Ciapponi, 2008). Moreover HOAP-depleted telomeres also cause cell cycle arrest during the metaphase-to-anaphase transition due to spindle assembly checkpoint (SAC) activation that is likely mediated by BubR1 recruitment at telomeres that require both ATM and ATR pathway (Musarò, 2008). These findings indicate that in *Drosophila,* similar to mammals and yeast, dysfunctional telomeres, HOAP-depleted, trigger DNA damage response activation that lead to interphase cell cycle arrest. Moreover, in *Drosophila* unprotected telomeres recruit BubR1 that activates SAC, blocking mitotic division of cells with dysfunctional telomeres escaped the DNA damage checkpoint and preventing in this way genomic instability (Ciapponi, 2008). Depletion of Moi or Ver don't activate a DNA damage response or SAC (Cenci, 2009) suggesting that these terminin proteins are essential to hide chromosome ends from the DNA damage machineries that mediate

telomere fusions but are not required to prevent checkpoint activation (Raffa, 2011). It has not yet been fully elucidated if HipHop is necessary to protect telomeres from DNA damage response.
In addition to telomeric specific proteins, Drosophila telomeres require also accessory proteins for their proper protection; these nonterminin proteins, although necessary for telomeric capping have also other localizations and other functions in the cell, as nonshelterin telomeric proteins in mammalian cells. Genetic and molecular studies have identified seven of these proteins required for telomere protection from fusion events: HP1, UbcD1, Mre11, Rad50, Nbs, ATM and WOC (Raffa, 2011). Among them one of the most important for telomere capping is HP1 (Fanti, 1998). This protein is involved in different cellular processes as gene silencing, chromosome structure and transcriptional regulation in addition to telomere protection (Fanti, 2008; Vermaak, 2009). HP1 localizes to the ends of polytene chromosomes in addition to chromocenter and many euchromatic bands (Fanti, 1998; Fanti, 2003). Mutations in its encoding gene cause high level of telomere fusions (Fanti, 1998). Moreover HP1 is the only telomeric protein for which has been described a function both in ends protection and in telomere length regulation (Frydrychova, 2008). HP1 interacts with HOAP (Badugu 2003, Cenci, 2003), Moi but not with Ver (Raffa, 2009; Raffa, 2010) and localizes to the extremities of terminally deleted chromosomes, as terminin proteins. However HP1 should not be considered a terminin component, because it doesn't localize exclusively at telomeres and has multiple telomere-unrelated functions (Raffa, 2011). HP1 doesn't seem necessary for HOAP localization at telomeres because in *Su(var)205* mutant strains, mitotic telomeres accumulate regular amount of HOAP (Cenci, 2003).
UbcD1 is the first gene shown to be required to prevent telomeric fusions in *Drosophila* (Cenci, 1997). It encodes for an highly conserved E2 ubiquitin conjugating enzyme that has role in different cellular processes (Cenci, 1997); it remains to be

determined which are its telomeric targets and which function, regulative or degradative, could have the modification mediated by UbcD1. In *Ubcd1* mutants HOAP and HP1 are correctly localized at telomeres, suggesting that UbcD1 is not necessary for their recruitment to chromosome ends (Cenci, 2003).
Woc is another telomeric nonterminin factor; it encodes for a zinc-finger protein that is located at the ends of polytene chromosomes and co-localizes with euchromatic bands that associate with initiating form of polymerase II (Raffa, 2005). Woc localizes at telomeres independently of the other telomeric proteins and is not required for terminin localization at telomeres (Raffa, 2011).
In *Drosophila*, as in mammalian cells several proteins involved in DNA damage sensing and repair are involved in ends protection. Mutants in *Mre11, Rad50* and *Nbs1* exhibit telomere fusions and chromosome breaks in larval brain cells (Bi, 2004; Ciapponi, 2004; Ciapponi, 2006; Oikemus, 2006), although their incidence is lower than that of terminin mutants. Moreover *Mre11, Rad50* and *Nbs1* depletions reduce HOAP accumulation at mitotic telomeres and both HOAP and HP1 localization at the ends of polytene chromosomes (Bi, 2004; Ciapponi, 2004; Ciapponi, 2006; Oikemus, 2006); *Mre11* mutants show also a reduction of Moi localization at polytene telomeres (Raffa, 2009). These findings suggest that MRN complex components are necessary to recruit terminin at chromosome ends likely because interaction of ends with MRN complex results in conformational changes that facilitate terminin recruitment at telomeres (Cenci, 2005). However, even in the absence of MRN activity, mitotic chromosomes retain the ability to recruit low levels of HOAP that are likely enough to partially protect chromosome ends, as suggested by the finding that MRN components mutants display fewer fusions than terminin mutants (Cenci, 2005; Rong, 2008, Raffa, 2011). As in mammalian cells, also ATM kinase is important for telomere protection in *Drosophila*. Mutations in its encoding gene result both in telomere fusions and chromosome breaks in larval neural cells (Queiroz-Machado, 2001; Bi, 2004;

Oikemus, 2004; Silva, 2004) and don't affect HOAP localization at mitotic telomeres although they reduce HOAP accumulation at the ends of polytene chromosomes (Bi, 2004; Oikemus, 2004). It's not clear how ATM prevents telomere fusions but it seems to act in a different way in comparison to MRN components (Cenci, 2005, Raffa 2011). Mutations in ATR and ATRIP encoding genes don't cause telomere fusions but increase the incidence of them in *tefu/atm* mutants, suggesting that ATM and ATR/ATRIP play partially redundant roles at telomeres (Bi, 2005; Raffa, 2011).

In conclusion, while at a first glance telomeres in *Drosophila* appear totally different in comparison of those of the other eukaryotes, they share several features and are functionally equivalent. It has been hypothesized that *Drosophila* has a telomerase positive ancestor and during the transition from a telomerase-based to transposon-based telomere elongation mechanism, *Drosophila*, rapidly evolved specialized proteins, teminin, to bind chromosome ends independently of DNA sequence. According to this hypothesis, terminin proteins are not conserved in the other eukaryotes and shelterin proteins are not conserved in flies, while telomeric associated nonterminin and nonshelterin proteins are largely conserved from flies to mammals, and many of them play telomere-related functions in both organisms. These findings indicate that the main difference between *Drosophila* and human telomeres is in the telomeric sequence and in the protective cap that specifically binds these different structures (Raffa, 2011). For these reasons and considering the main conservation in humans of nonterminin proteins, cytological screenings of mutant strains that allow the identification of new proteins of this type in *Drosophila*, may lead to the discovery of new human telomeric proteins.

Recently it was identified a new telomeric gene *pendolino (peo)* by a cytological screening of larval neural cells of tardive lethal mutant strains (Cenci, manuscript in preparation). Mutations in *peo* gene cause high incidence of telomeric fusions with a mean of 4 fusions per metaphase. These fusions in anaphase generate

chromosomal bridges that often are resolved without break inducing polyploidy and/or aneuploidy. Moreover, *peo* telomeric function was confirmed by cytological screening of strains obtained crossing *peo* heterozygous mutants with *Su(var)205* or *cav* or *UbcD1* heterozygous mutants: mutations in *peo* increase the frequency of telomeric fusions in these double mutants demonstrating that these genes genetically interact. GST-pull down experiments and two hybrid assays shown that PEO protein interacts physically with HOAP but not with HP1 (Cenci, manuscript in preparation). *Peo* encodes for a protein that shares similarities with E2 ubiquitin conjugating enzyme but it lacks of a cysteine important for catalytic reaction so it is not clear which is its molecular role. *Peo* is conserved in mammals and its human homologue AKTIP is the subject of this study.

AKTIP and Ft1: state of art

AKTIP gene is located on long arm of chromosome 16 (16q12.2), and encodes for a 33 KDa protein. Very little is known about this human protein: it was identified as an interactor of Ser/Thr kinase B (PKB)/AKT1 by a cDNA screening using a GFP-AKT as bait (Remy, 2004) and it takes its name from this identified interaction. In this overexpression-based study, AKTIP seems to act as a protein bridge between AKT1 and its upstream kinase PDK1 increasing the efficiency with which it regulates AKT1 activation and contributing in this way to modulate its activity (Remy, 2004). However in a subsequent study AKTIP was found to interact with all three human Hook proteins (1-2-3) but the authors failed to identify AKT1 among AKTIP interactors. They suggest that this difference is due to different experimental conditions used and that the interaction previously found, could be ascribed to overexpression conditions (Xu, 2008). In this study AKTIP is proposed as a member of a complex that could be involved in vescicle trafficking and/or fusion via the HOPS complex (Xu, 2008).
Recently AKTIP/FTS was identified as a factor involved in cervical carcinogenesis; it is overexpressed in the progressive grade of cervical cancer and negatively regulates tumour suppressor p21 (Cinghu, 2011). It was identified as a putative target to improve radiation therapy of cervical cancer cells. Indeed, silencing of AKTIP/FTS in HeLa cells was found to promote cell cycle arrest and apoptosis following radiation; however it's not clear the molecular mechanism underlying this process (Anandharaj, 2011).
Preliminary bioinformatic and biochemical studies conducted in our laboratory have allowed the identification of AKTIP as a member of Ubiquitin E2 variant (UEV) family. These proteins are characterized by the presence of a domain that has obvious similarity with UBC domain (Figure 4) but lacks the catalytic cysteine that is crucial for ubiquitin transfer reaction and they have

different roles in ubiquitin conjugation from that performed by canonical E2s (Broomfield, 1998; Sancho 1998). UEV proteins play a central role in the assembly of K63-linked polyubiquitin chains (Hofmann, 1999; Ulrich, 2000), a regulative modification involved in different signaling pathways (Hofmann, 2009). The best characterized UEV proteins in mammals are MMS2 (Hofmann, 1999) and UEV1a (Andersen, 2005); they work together with a canonical E2, Ubc13 in the assembly of K63-chains and are involved in DNA damage response. A well known target of the regulative ubiquitilation operated by these two UEVs is PCNA, a replicative factor that has a key role in replication fork progression and regulation (Moldovan, 2007). This particular modification is important to trigger the DNA damage tolerance that allows resumption of DNA synthesis in presence of lesions that block the replication forks progression and that induce prolonged fork stalling potentially leading to fork collapse (Chen, 2011).

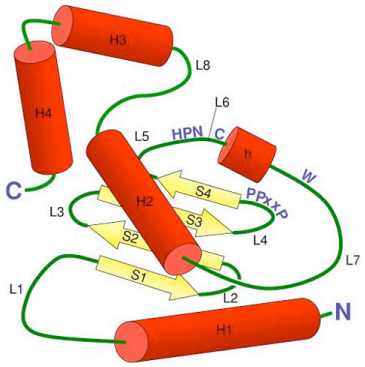


Figure 4: Schematic 3D structure of ubiquitin-conjugating enzyme E2 family.
Alpha helices are represented in red and beta strands by yellow arrows. Important residues and motifs are represented. Adapted from Michelle, 2009.

Murine homologue of AKTIP is Ft1. Ft1 gene is located on chromosome 8, D region and it was identify in 1997 as one of 6 genes deleted in a mouse strain obtained by insertional mutagenesis, Fused Toes (Ft) (Lesche, 1997). Ft/Ft homozygous embryos die at 9-10 day of embryonic development and display several abnormalities in the head development characterized by the absence of telencephalon and mesencephalon; moreover, they show a phenotype described as *situs inversum viscerum* characterized by the absence of right-left symmetry. Ft/+ heterozygous animals are characterized by thymic hyperplasia and fusions of fore limb digits (Figure 5).

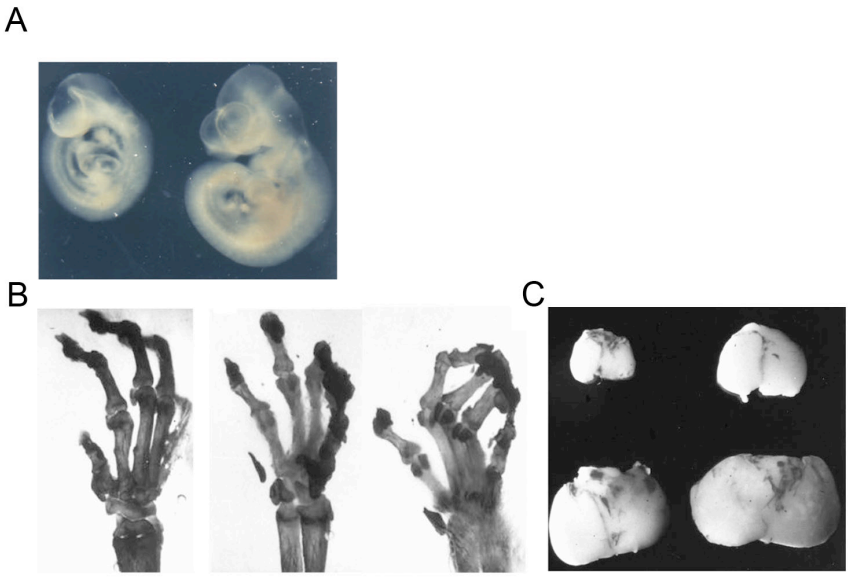


Figure 5: Phenotypic aberrations of Ft mutant strain.
(A): *Ft/Ft* (left) 9.5- day old embryo is represented in comparison with stage matched wild type embryo (right). (B): skeletal stain of a wild type right fore limb (left) is compared to right fore limbs of *Ft/+* mice (right). (C): Thymus from 6 weeks old wild type mouse (top on the left) is compared with ones of an age matched *Ft/+* mouse (top on the right); a thymus from a 4 and 6 months old *Ft/+* mice are presented (bottom from left to right). Adapted from van der Hoeven, 1994.

These phenotypic features are linked to alteration of programmed cell death during morphogenesis (van der Hoeven, 1994). This aberrant phenotype is caused by a deletion of 1.6 Mb including 6 genes: Ft1, Fatso (Fto), Ftm, Iroquois (Irx) 3, 5 and 6 (Peters, 2002). It's not clear which is the specific contribution of each of these genes simultaneously deleted in Ft mutants to the determination of the aberrant observed phenotype. However Irx genes deletion could have a main role in the mutant phenotype because they show a spatio-temporal regulated expression pattern in developing limbs, in craniofacial areas, in the developing central nervous system and in the heart and several of these tissues are affected in mutant strain (Peters, 2002). Ft1 was the first gene to be identified as deleted in Ft mutation because it is the most proximal to the integration site. Its expression is completely absent in Ft/Ft embryos and is reduced in Ft/+ mice. Ft1 gene contains two different transcription's starting sites and two polyadenylation sites leading to the formation of different transcripts that differ in 5' and 3' UTR and then encode for a single protein of 32 KDa. In adult mice, Ft1 is present in all organs analyzed. However, the expression level and the ratio of the Ft1 transcripts seem different between organs. Highest expression is present in kidney, testis, and brain, and lowest in spleen and liver, otherwise its expression is uniform in embryos starting from the day 9 of development (Lesche, 1997). It has not been defined which is Ft1 molecular function and which is its contribute to Ft phenotype.

AIM OF THE PROJECT

Due to the paucity of information available about AKTIP/Ft1 function in mammalian cellular metabolism we were interested in clarifying which is the molecular role of this protein in human cells. In particular the aim of this study was to understand if AKTIP/Ft1 has a role in human telomere metabolism and it's important for telomere maintenance in mammalian cells, in analogy of the telomeric role of its homologue peo in *Drosophila*.
This research could improve our knowledge about telomeres function and physiology, an expanding field that has a crucial importance due to the implication of telomeres in high relevant processes such as replicative ageing and genomic instability that is linked to cancer development; despite the growing number of information about telomere biology some questions, such as some aspects of telomere replication, remain open.
This work could also be crucially important to the field of evolutionary biology of telomeres and it can open unattempted ways to look for new human telomeric proteins studying the mammalian homologues of *Drosophila* telomeric genes, despite the difference in their telomeres structure.

RESULTS

AKTIP downregulation triggers cell cycle block and premature senescence

To analyze the molecular function of AKTIP in mammalian cells we have chosen a reverse genetic approach studying the aberrant phenotype deriving by AKTIP or Ft1 lentivectors-mediated RNA interference, in human and murine cellular cultures. We have tested different short hairpin (sh) interfering sequences, five for AKTIP and three for Ft1 and transduction of human primary fibroblasts (HPF) and $p53^{-/-}$ murine embryonic fibroblasts (MEF) with the corresponding recombinant vectors (LV-shAKTIP or LV-shFt1) has resulted in a specific and significant target mRNA reduction (60%-90% for AKTIP and 80%-95% for Ft1) (figure 6 A-B). We have then tested LV-shAKTIP 09, 11, 13 efficiency also at AKTIP protein level in the three cellular systems that we have used for the subsequent experiments, HPFs, 293T and HeLa cell lines (figure 6 C, where LV-shAKTIP 11 is shown) and we obtained a significant and reproducible reduction of target protein. Therefore we have used these lentivectors for all the experiments described below. For Ft1, among lentivectors tested, we have chosen LV-shFt1 70 that ensured the best combination of target mRNA reduction and low cellular toxicity and we have used it for all the experiments described below. As controls we used untransduced mock cells or cells transduced with a vector encoding a scrambled sequence (LV-ctr).

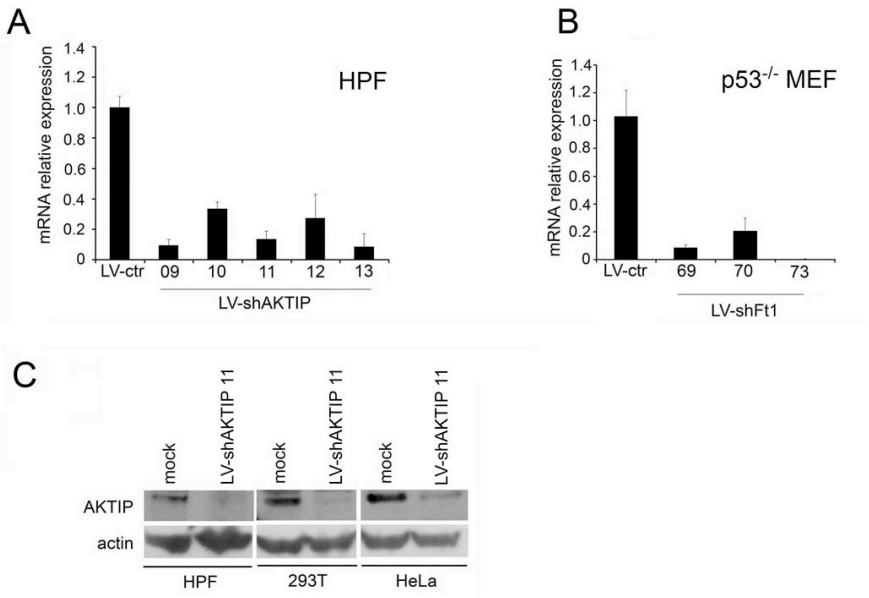


Figure 6: Efficient AKTIP/Ft1 downregulation by RNA interference.
(A-B): cDNAs obtained retrotranscribing total RNA extracted one week post infection (p.i.) from HPFs (A) or p53 $^{-/-}$ MEFs (B) transduced with the indicated recombinant lentivectors, were analyzed by Real Time-PCR performed using AKTIP (A) or Ft1 (B) specific primers. In both cases LV-ctr transduced cells were used as control. The samples were analyzed in duplicate and the mean value is reported; SD is shown. (C): Western blot showing reduced expression of AKTIP in HPFs, HeLa and 293T cells whole protein extracts collected 10 days post LV-shAKTIP 11 transduction compared to untransduced cells (mock). Probing with an anti-βactin antibody was used to normalize samples.

The first phenotypic trait that we have analyzed was the effect of AKTIP downregulation on cell cycle progression because it's known that dysfunctional telomeres, due to replicative attrition of telomeric repeats or shelterin loss, activate a DNA damage response resulting in cell cycle arrest and either apoptosis or cellular senescence (van Steensel, 1998; D'Adda di Fagagna, 2003; Karlseder, 1999; Smogorzewska, de Lange, 2002). Cytological analysis of HPFs showed that AKTIP downregulation induced a strong reduction (75%- 88%) of mitotic index compared to control cells (figure 7 A). Western Blot analysis of protein extracts from AKTIP knocked down (KD) HPFs displayed a high increase in cyclin A level, 12-18 fold those of control cells, and a more modest increase in cyclin B and cyclin E levels (figure 7 B) suggesting the induction of cell cycle slow down or arrest following AKTIP downregulation. Moreover, staining for senescence associated β-galactosidase (SA-β-GAL), an empirical marker of cellular senescence (Dimri, 1995), showed that, starting from eleven days post transduction, the frequency of SA-β-GAL positive cells was significantly higher following AKTIP downregulation in HPFs compared to control cells (figure 7 C-D). AKTIP KD HPFs displayed also morphological signs of senescence including large and flat shape. Collectively, these results indicate that AKTIP downregulation significantly affects cell cycle progression and cellular proliferation.

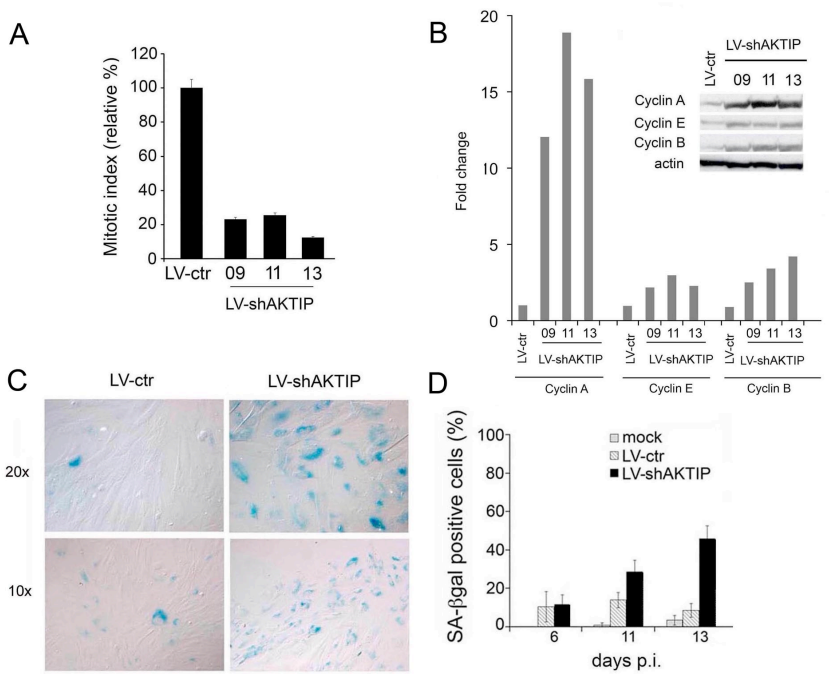


Figure 7: AKTIP downregulation triggers cell cycle arrest and premature senescence.
(A): mitotic index analysis of LV-shAKTIP (09, 10 and 13) and LV-ctr transduced HPFs 10 days p.i. Samples were analyzed in duplicate and mean value is reported; SD is shown. (B): Western Blot analysis of protein extracts from LV-shAKTIP 11 or LV-ctr transduced HPFs 10 days p.i. Probing with an anti-βactin antibody was used to normalize samples. Normalized band intensity quantification analysis is shown. (C-D): untreated (mock), LV-shAKTIP 11 and LV-ctr transduced HPFs, were tested for SA-β-GAL staining at the indicated time points and analyzed by contrast microscopy. (C): exemplificative images of SA-β-GAL stained LV-shAKTIP and LV-ctr transduced HPFs 13 days p.i. are reported. (D): analysis of SA-β-GAL positive cells is presented**.** Samples were analyzed in duplicate and mean value is reported; SD is shown.

AKTIP downregulation triggers DNA damage response activation

In agreement with the data presented above, in HPFs AKTIP downregulation impaired cellular population doublings kinetics compared to control cells (figure 8 A, left panel) confirming the detrimental effect of AKTIP reduction on cellular proliferation. However we couldn't observe a similar response neither in 293T nor in HeLa cell lines: both these cell lines showed normal proliferation rates despite the reduction of AKTIP (figure 8 A, central and right panel). Considering that these two cell lines are characterized by altered p53 and pRb pathways (Louis, 1997; Sullivan, 2002; Scheffner, 1990; Dyson, 1989) that are wild type in HPFs, these results suggest that proliferation impairment caused by AKTIP reduction is induced by the activation of a cellular response that involved these two pathways. Since p53 is the main effector of cellular DNA damage response (Rinn, 2011) we hypothesized that AKTIP downregulation activated a DNA damage checkpoint in HPFs. Indeed Western Blot assays of protein extracts from AKTIP KD HPFs showed an accumulation, compared to control cells, of the active (phosphorylated) form of different proteins involved in DNA damage sensing and signaling (Harper, 2007), such as ATM (ATM PSer 1981), Chk1 (Chk1 PSer 345) and p53 itself (p53 PSer15) (figure 8 B). The activation of p53 following AKTIP downregulation was further confirmed by the increase of p53 protein level observed in protein extracts form AKTIP KD HPFs compared to control cells (figure 8 B, p53 tot) and also by the induction of its direct transcriptional target p21 (Rinn, 2011; Beckerman 2010), as shown by the accumulation of p21 mRNA in AKTIP downregulated HPFs assayed by Real Time PCR (figure 8 C).

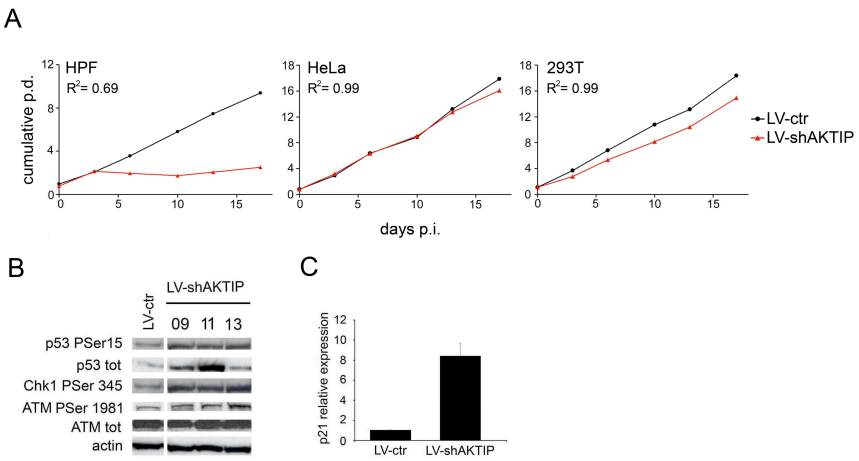


Figure 8: AKTIP downregulation impairs proliferation in HPFs triggering DNA damage response activation.
(A): cumulative population doublings (p.d.) of HPFs, HeLa and 293T cell lines (from left to right) transduced with LV-shAKTIP (red) and with LV-ctr (black) are reported. Curves correlation index (R^2) is shown. (B): protein extracts from LV-shAKTIP (09, 11, 13) and LV-ctr transduced HPFs, were analyzed by immunoblotting for the proteins indicated on the left. Probing with an anti-βactin antibody was used to normalize samples. (C): cDNAs obtained retrotranscribing total RNA extracted one week p.i. from LV-shAKTIP 11 or LV-ctr transduced HPFs were analyzed by Real Time PCR using p21 specific primers. Samples were analyzed in duplicate and mean value is reported. SD is shown

The induction of a strong DNA damage response following AKTIP downregulation was also confirmed by cytological assays. In AKTIP KD HPFs we observed the formation of discrete foci containing proteins involved in DNA damage signaling and repair such as γH2AX, the phosphorylated form of ATM (ATM P) and 53BP1 (Harper, 2007) (figure 9 A-B). Both the percentage of cells containing five or more foci and the mean number of foci per cell were significantly higher in AKTIP KD HPFs compared to control cells (figure 9 C-D). The DNA damage signaling and repair proteins that we have analyzed γH2AX, ATM P and 53BP1 co-localized at most of the foci induced by AKTIP downregulation, suggesting a coordinated response similar to that induced by a genotoxic agents, such as ionizing radiation (IR) that we have used as control (figure 9 E-F). DNA damage signaling and repair foci triggered by downregulation of AKTIP were very similar in the shape to those induced by IR (figure 9 A-B, the first two lines of both panels). These results indicate that AKTIP downregulation in HPFs is sufficient to trigger the activation of a strong DNA damage response without any treatment with genotoxic agents. This response, in turn, likely induces cell cycle progression block, proliferation arrest and premature senescence described above.

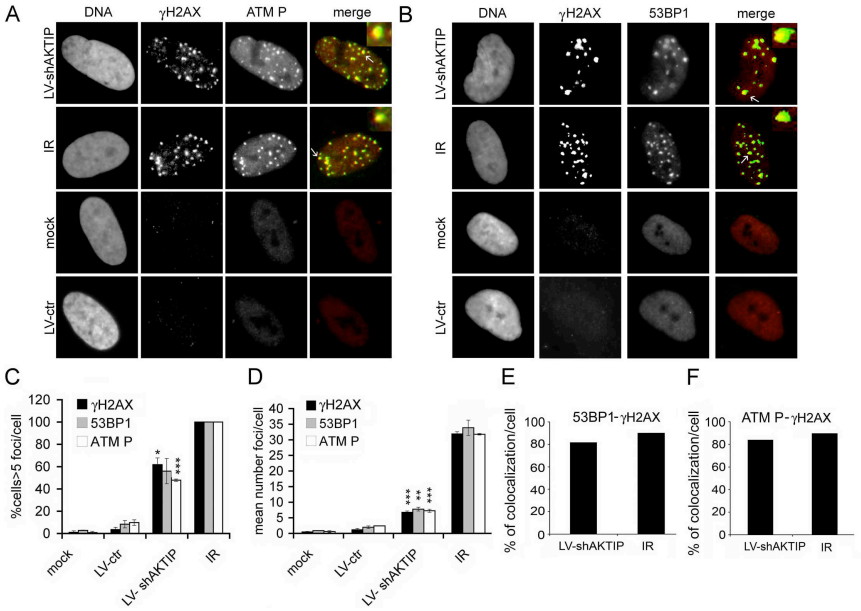
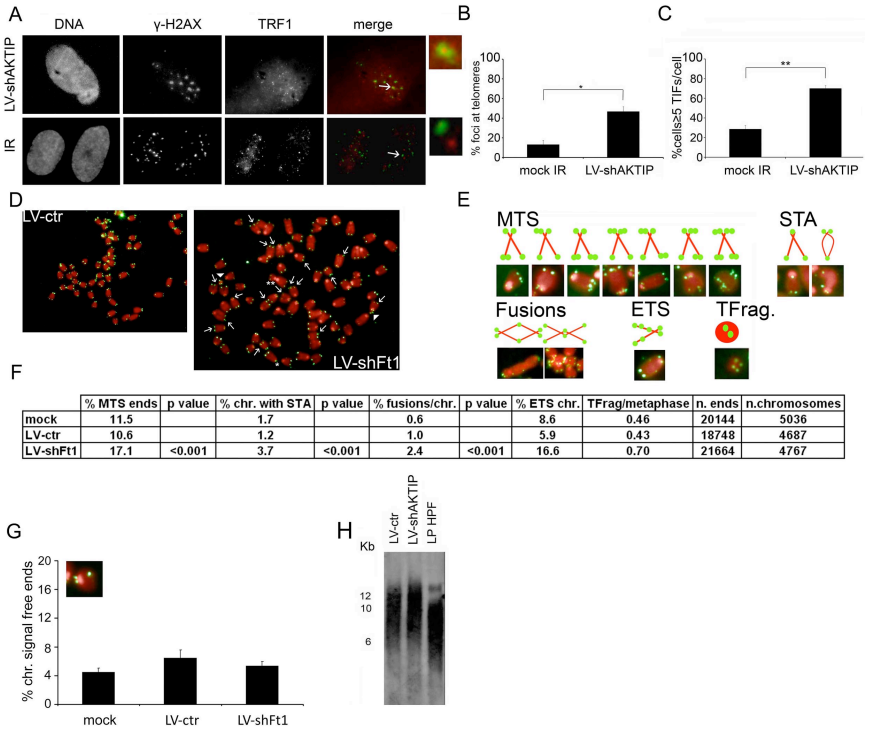


Figure 9: AKTIP downregulation induces DNA damage signaling and repair foci. (A-B): immunofluorescence (IF) showing the formation of nuclear γH2AX foci (A-B, green in merge), Ser1981 phosphorylated ATM foci (A, red in merge) and 53BP1 foci (B, red in merge) in untreated (mock), LV-shAKTIP 11 and LV-ctr transduced HPFs, 5 days p.i. Untransduced cells treated with IR 1 Gy were used as positive control for DNA damage foci formation. A magnification of the focus indicated by the arrow in merge image is reported in the upper right corner. DNA was stained with DAPI. (C-D): percentage of γH2AX (black bar), 53BP1 (grey bar) and Ser1981 phosphorylated ATM (white bar) foci positive cells (C) and mean number of foci per cell (D) are reported. Cells with more than 5 foci were considered positive. Values presented are the mean of two independent experiments; SD is shown. p values were based on Student t-test and statistical analyses were obtained comparing sample values with both controls values ($p< 0.05$:(*), $p<0.01$:(**), $p<0.001$:(***)). (E-F): percentage of foci containing both protein γH2AX and 53BP1 versus foci containing only γH2AX or 53BP1 (E), and percentage of foci containing both protein γH2AX and Ser1981 phosphorylated ATM versus foci containing only γH2AX or Ser1981 phosphorylated ATM (F) are shown.

AKTIP downregulation induces TIFs formation and multiple telomeric aberrations

Dysfunctional telomeres, as those caused, for example, by the loss of shelterin components, like TRF2, result in the formation of TIFs (telomere dysfunction-induced foci) that are DNA damage foci located at chromosome ends (Takai, 2003). TIFs formation can be monitored citologically and is considered a good means to quantify the extent of telomere dysfunction (Palm, 2008). For this reason we have decided to investigate if the DNA damage foci that we observed in HPFs following AKTIP downregulation, described above, were TIFs. For this purpose we immunostained the cells with both anti-TRF1, that can be used as a marker of telomeres in interphase cells (van Steensel, 1997), and anti-γH2AX antibodies (figure 10 A). Untransduced X-rays treated cells were used as control for the formation of DNA damage foci not linked to telomeric sites. We found that in AKTIP KD HPFs about 50% of γH2AX foci co-localized with TRF1, a frequency that is significantly higher than that observed in control irradiated cells in which more than 80% of γH2AX foci didn't co-localize with TRF1 (figure 10 B). In AKTIP KD HPFs more than 60% of cells displayed 5 or more TIFs per cell (figure 10 C), with an average of about 7 TIFs per cell. These results are comparable with those previously reported for shelterin KD (Takai, 2003) suggesting that AKTIP is important for telomere maintenance.



F

	% MTS ends	p value	% chr. with STA	p value	% fusions/chr.	p value	% ETS chr.	TFrag/metaphase	n. ends	n.chromosomes
mock	11.5		1.7		0.6		8.6	0.46	20144	5036
LV-ctr	10.6		1.2		1.0		5.9	0.43	18748	4687
LV-shFt1	17.1	<0.001	3.7	<0.001	2.4	<0.001	16.6	0.70	21664	4767

Figure 10: AKTIP/Ft1 downregulation induces TIFs and multiple telomeric aberrations.

(A): IF showing the co-localization of γH2AX (green in merge) with telomeric sites marked by TRF1 (red in merge) in LV-shAKTIP 11 transduced HPFs, 5 days p.i. A magnification of the focus indicated by the arrow in merge image is reported on the right. DNA was stained with DAPI. (B-C): percentage of γH2AX foci located at telomeres in each cell (B) and percentage of TIFs positive cells (C) is reported. Cells with 5 or more γH2AX foci co-localizing with TRF1 were considered TIFs positive. The results shown are the mean of two independent experiments. SD is shown. p values were based on Student t-test ($p<0.05$: (*) and $p<0.01$:(**)). (D): representative images of telomeric FISH (in green) of metaphase spreads from LV-ctr or LV-shFt1 70 transduced $p53^{-/-}$ MEFs, one week p.i. DNA was stained with DAPI (false coloured in red). Most prominent telomeric aberrations are indicated: arrow: multiple telomeric signals (MTS), arrow heads: fragment with telomeric signals (TFrag.), *: sister telomere association (STA) and **: interchromosomal telomeric fusion. (E): Examples of telomeric aberrations observed following Ft1 KD are reported; a schematic representation with the classification of telomeric aberrations considered, is shown. (F): percentage of MTS per end and the percentage of STA, ETS and fusion per chromosome were determined in untreated (mock), LV-ctr or LV-shFt1 transduced $p53^{-/-}$ MEFs. Total number of ends and

chromosomes considered for each sample is reported. For TFrag. the frequency per metaphase was determined and value shown is the mean of two independent experiments. p values shown were based on χ^2 test and statistical analyses were obtained comparing sample values with both controls values; (G): frequency of chromosome with one or more telomeric signal free ends (an example is reported) is presented. Values shown are the mean of two independent experiments; SD is shown. (H): Hinf I/RsaI digested genomic DNA extracted 13 days p.i. from HPFs transduced with the indicated lentivectors was fractionated on agarose gel and after blotting, telomeric DNA was detected with TTAGGG repeats probe. On the left marker fragments size (in Kb) positions are indicated. Genomic DNA of late passage (LP, passage 30) HPFs was used as control.

Considering that TIFs represent the activation of a DNA damage response at dysfunctional telomeres and the high incidence of TIFs observed in HPFs following AKTIP downregulation, we decided to investigate the nature of the dysfunctions accounting for DNA damage response activation at telomeres in AKTIP KD cells. We have then used $p53^{-/-}$ MEFs that are frequently employed to study telomeric aberrations because the absence of p53 let to overcome the cell cycle block induced by the presence of dysfunctional telomeres allowing cells proliferation despite the presence of altered telomeres and the observation of telomeric aberrations in metaphase cells (Smogorzewska, Karlseder, 2002; Celli, 2005, de Lange 2010). FISH assay using a telomeric specific probe on metaphase spreads from LV-shFt1 70 transduced $p53^{-/-}$ MEFs, revealed the presence of different kinds of telomeric aberrations (figure 10 D). The most prominent aberrant telomeric phenotype induced by Ft1 downregulation was the presence of chromatids with multiple telomeric signals (MTS, figure 10 D-F); their incidence was significantly higher in Ft1 KD MEFs than in control cells (figure 10 F). This kind of telomeric aberrations which are known also as “telomeric doublets” or “fragile telomeres”, was recently linked to replication problems, although the specific mechanism that lead to their formation was not fully elucidated (Sfeir, 2009; Ye, 2010; Martinez, 2009; Martinez, 2010; Pennarun, 2008; Pennarun, 2010). In Ft1 KD MEFs there was also a significant increase in the incidence of sister telomere associations (STAs, figure 10 D-F) that was two fold higher than in control

cells and occurred at the ends of both short and long arm. Also this kind of telomeric aberrations was previously described to be linked to alteration of proper telomere replication (Sfeir, 2009; Ye, 2010; Martinez, 2009; Pennarun, 2008; Pennarun, 2010). Ft1 KD MEFs displayed also an increase, although not statistically significant, in the incidence of chromosomes with extra-telomeric signals (ETSs figure 10 E-F) and of fragments with telomeric signals (TFrag. figure 10 D-F). Ft1 downregulation induced also a significant increase in the frequency, although very low, of chromosomal fusions both with maintenance and loss of telomeric signal at the fusion point (figure 10 D-F). The incidence of fusions per chromosome, up to 2%, is comparable of that observed following Ft1 KO (Sfeir, 2010) but lower than that observed in TRF2 KO cells (Celli, 2005; Konishi 2008).

The telomeric phenotype triggered by AKTIP/Ft1 downregulation was not linked to a massive loss of telomeric repeats as it was demonstrated by the absence of any significant increase in the frequency of chromosomes with one or more telomeric signal free ends in Ft1 KD MEFs compared to control cells (figure 10 G). In addition, Southern Blot analysis displayed that there was no change in the migration of telomeric restriction fragments from genomic DNA of AKTIP KD HPFs compared to control and that these fragments were higher than those obtained from late passage (30 passages) HPFs that had lost part of their telomeric repeats due to replicative erosion (figure 10 H), suggesting that there was no massive telomeric loss following AKTIP downregulation.

Collectively, these data reveal that AKTIP/Ft1 is important for telomeres metabolism and its necessary for their proper maintenance considering that its downregulation causes the formation of multiple telomeric aberrations. Among these, the high incidence of MTSs and STAs suggests that AKTIP is more likely involved in telomere replication rather than in telomere protection.

AKTIP interacts with telomeric repeats but it doesn't reside stably at telomeres

Considering the telomeric phenotype caused by AKTIP downregulation, we have decided to investigate whether AKTIP interacts with telomeric repeats attempting to clarify which is its molecular function in telomeres metabolism. To address this problem we have used a ChIP assay: chromatin from untreated HPFs was immuno-precipitated using a anti-AKTIP commercial antibody or control IgG; hybridization with a telomeric repeat specific probe revealed the presence of telomeric DNA in AKTIP interacting chromatin (figure 11 A on the left). The interaction between AKTIP and TTAGGG repeats was also detected in HeLa cell line (figure 11 A on the right). The specificity of this interaction was supported by the lack of hybridization signal when it was performed using a nontelomeric probe such as that specific for Alu repeats both in HPFs and in Hela cells (Figure 11 A and C). A further confirmation of the specificity of the interaction between AKTIP and telomeric DNA came from the reduction of hybridization signal obtained using a telomeric specific probe on AKTIP immuno-precipitated chromatin derived from AKTIP KD HeLa cells (figure 11 B).

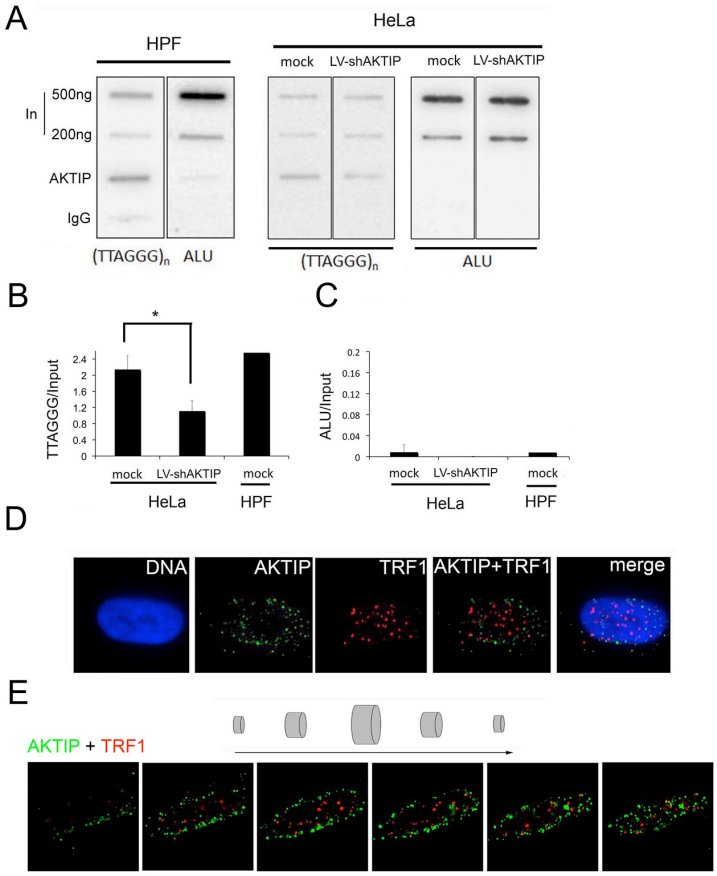


Figure 11: AKTIP interacts with telomeric DNA but doesn't reside stably at telomeres.
(A): telomeric ChIP of untreated HPFs (on the left) and of untreated and LV-shAKTIP 11 transduced HeLa cells (on the right) showing AKTIP interaction with telomeric DNA. Chromatin was immunoprecipitated with antibodies indicated on the left; dot blots were hybridized with TTAGGG repeats probe or with Alu repeats probe, as indicated on the bottom. (B-C): quantification of ChIP values for telomeric repeats (B) and for Alu repeats (C) after normalization to the input (indicated in A) was determined. Mean value of two experiments is reported; SD is shown. (C): IF of wild type (wt) HPFs, showing the absence of massive co-localization between AKTIP (green) and TRF1 (red). Cells were Triton X-100 extracted before IF. DNA was stained with DAPI (blue). (D): six optical sections captured at 1μm Z steps of IF cells are presented.

Given that AKTIP interacts with telomeric DNA we examined by cytological screening its possible co-localization with TRF1, again used as a marker of telomeres in interphase cells. Immunostaining with both anti-AKTIP and anti-TRF1 antibodies in HPFs revealed the lack of an obvious co-localization between the two proteins (figure 11 D). This finding became particular evident looking at optical sections captured at 1μm Z steps above and below the focal plane of immunostained cells (figure 11 E); their analysis underlined that AKTIP and TRF1 have different localization in the nucleus with AKTIP that is highly enriched at nuclear rim (for detailed analysis of AKTIP localization refer to the next section) and TRF1 that is distributed throughout the nucleoplasm.
These data indicate that AKTIP interacts with telomeric DNA but it's not a stable component of telomeres lacking of co-localization with TRF1. This last finding, in addition to the AKTIP emerging telomeric role, indicates that AKTIP has to be considered as an accessory telomeric protein, which are defined as proteins that, differently from shelterin components, don't reside stably at telomeres but are important for proper telomeres maintenance (Palm, 2008).

AKTIP co-localizes with nuclear lamina and it's important for genome stability

To investigate further AKTIP molecular function we have analyzed in detail its cellular localization.
Immunostaining with an anti-AKTIP antibody of untreated wild type HPFs revealed both a cytoplasm and nuclear localization (figure 12 A). Following extraction with a mild detergent that removed the soluble part of the proteins allowing the study of subnuclear localization (Zhu, 2000), AKTIP displayed a nuclear localization, with a peculiar punctate pattern with high enrichment at nuclear rim both in HPFs and in HeLa cells (figure 12 A). AKTIP immunostaining signal was strongly reduced in AKTIP KD HPFs (figure 12 A) confirming the specificity of the anti-AKTIP antibody used. Moreover a highly enriched localization at nuclear periphery was obtained using anti-Flag antibody on extracted 293T cells previously transfected with a Flag tagged version of AKTIP protein (figure 12 A). We have then evaluated AKTIP distribution in optical sections of extracted immunostained cells (figure 12 B). We have quantified AKTIP signal in two focal sections and in the two more distal sections, above and below the focal plan, considering, for each section, AKTIP amount in two regions of the nucleus, a peripheral one (edge), and a central one. Comparison of obtained values confirmed AKTIP enrichment at nuclear periphery (figure 12 C).
This peculiar distribution recalls that of lamins, intermediate filaments that are the principal constituents of nuclear lamina, a filamentous network of proteins associated to the inner nuclear membrane. In mammals two types of lamins compose the nuclear lamina: A-type (A and C) and B-type (B1 and B2) (Dechat, 2010).
Immunostaining of HPFs with both anti-AKTIP and anti-Lamin B1 antibodies displayed high co-localization of the two proteins at nuclear periphery (figure 12 D), suggesting the possibility of an interaction, direct or indirect, between AKTIP and lamin proteins.

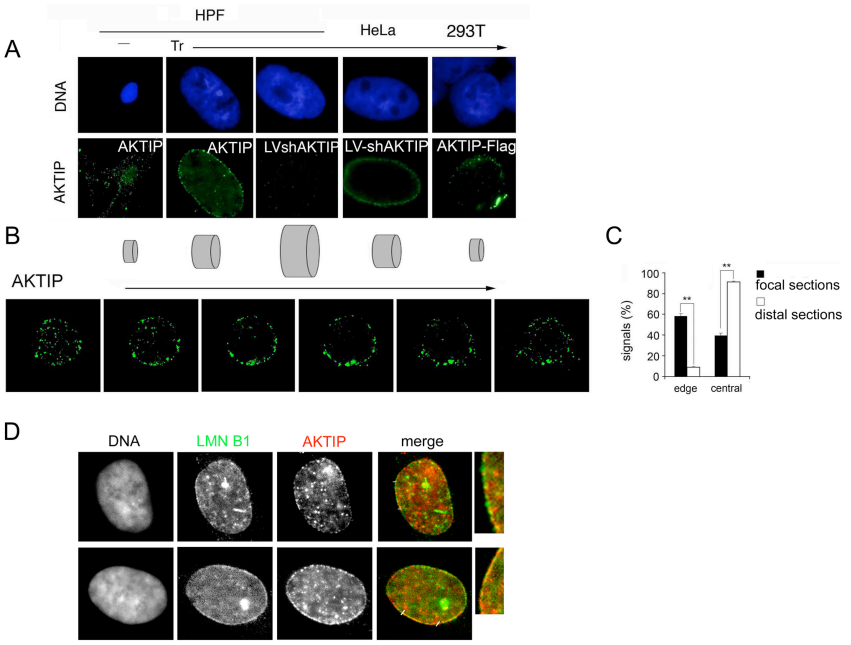
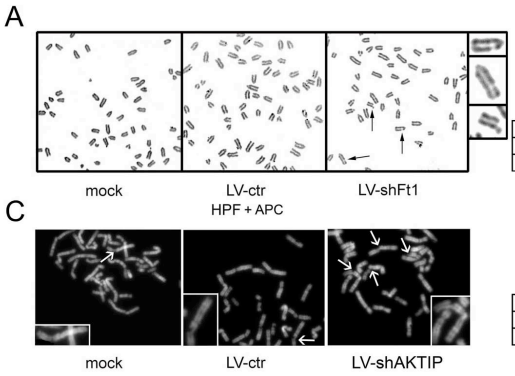


Figure 12: AKTIP in the nucleus localizes mainly at nuclear rim and co-localizes with lamin B.
(A): IF of wt HPFs and HeLa cells showing AKTIP (green) localization. LV-shAKTIP transduction in HPFs resulted in a reduction of the signal (third column from the left). A similar localization was obtained using an anti-Flag antibody (green) on 293T cell line expressing AKTIP-Flag (on the right). Where indicated the cells were Triton X-100 pre-treated. DNA was stained with DAPI (blue). (B): six optical sections captured at 1µm Z steps of immunostained with anti-AKTIP HPFs are shown. (C): analysis of AKTIP signal localization in the nucleus obtained comparing the signal distribution in the two more distal sections (the first and the last from the left in B) and in the two focal sections (the third and fourth from the left in B) in 23 immunostained cells, is reported. The percentage of signals located in the central part of the nucleus and the percentage of those located at the edge of the nucleus were calculated. The values presented are the mean of two experiments. SD is shown. p values were based on a Student t-test ($p < 0.01$:(**)). (D): IF of wt HPFs showing AKTIP (red in merge) and Lamin B1 (green in merge) co-localization at nuclear rim. Two representative images are presented. DNA was stained with DAPI. A magnification of rim indicated in merge is presented on the right.

Given the importance of lamins for the maintenance of genome stability (Gonzalez-Suarez, Redwood, Perkins, 2009; Gonzalez-Suarez, Redwood, Gonzalo, 2009) and the suggestion that AKTIP, as telomeric nonshelterin protein, could have also a nontelomeric function, we decided to assess the putative contribution of AKTIP in genome stability maintenance. Cytological analysis of metaphase spreads from Ft1 KD $p53^{-/-}$ MEFs showed that Ft1 downregulation induced a 4 fold increase compared to control cells of chromosomal aberrations including breaks, gaps, fragments and complex rearrangements (figure 13 A-B). These aberrations could be a direct consequence of AKTIP/Ft1 downregulation and/or a consequence of telomeric fusions-breakage-bridge cycle (O'Sullivan, 2010). Nevertheless the low frequency of telomeric fusions observed in Ft1 KD MEFs, the absence of massive telomere erosion in AKTIP KD HPFs and the lack of any increase in telomere loss in Ft1 KD MEFs, described above, argue against a strong contribution of this second possibility in our context.
Moreover we observed that AKTIP KD HPFs were more sensitive to low doses of aphidicolin (APC) in comparison to control cells. The APC doses used in our experimental conditions are enough to create replication stress and are commonly used to induce fragile sites expression (Durkin, 2007); fragile sites are genomic regions prone to replication fork stalling or collapse (Glover, 2005; Durkin, 2007). Analysis of metaphase spreads from AKTIP downregulated HPFs treated with low doses of APC displayed a significant 1.5-2 fold increase in chromosomal breaks or gaps compared to control cells (figure 13 C-D).



B

	aberrations/ metaphase	p value	n. metaphase
mock	0.3		50
LV-ctr	0.38		50
LV-shFt1	1.3	p<0.001	50

D

	%breaks and gaps/ chrom.	p value	n. chrom.
mock	0.019		942
LV-ctr	0.026		1689
LV-shAKTIP	0.038	p< 0.05	1615

Figure 13: AKTIP is important for genomic stability maintenance.
(A): Giemsa stained metaphase spreads from untreated, LV-ctr and LV-shFt1 transduced $p53^{-/-}$ MEFs, one week p.i., showing the presence of chromosomal aberrations following Ft1 downregulation indicated by the arrows and magnified on the right. (B): percentage of chromosomal aberrations per metaphase was considered and mean values of chromosomal aberrations observed in 50 metaphases are reported. p value reported was based on Student t-test; statistical analysis was obtained comparing sample value with both controls values. (C): DAPI staining of untransduced, LV-ctr and LV-shAKTIP transduced HPFs, following 24h incubation with aphidicolin (APC) 0.4 μM showing the presence of breaks/gaps indicated by arrows and magnified in the lower corner. (D): percentage of breaks and gaps per chromosomes is reported and the number of analyzed chromosomes is indicated. p value shown was based on χ^2 test and statistical analysis was obtained comparing sample value with both controls values.

Collectively, these results indicate that AKTIP in the nucleus is mainly located at nuclear rim where it co-localizes with Lamin B1 enforcing again its classification as accessory telomeric protein. In agreement with this, the data presented above indicate that AKTIP plays a role in genome stability maintenance and strengthen its possible involvement in DNA replication not only of telomeres, as described above, but also of other genomic regions, like fragile sites, that pose a challenge for replication machinery.

AKTIP downregulation impairs proper DNA replication

Some phenotypic features deriving by AKTIP/Ft1 downregulation described so far suggest that AKTIP could contribute to DNA replication. We thus analyzed cell cycle distribution of AKTIP KD HPFs by flow citometry analysis (FACS) of BrdU/PI stained cells. Control asynchronous cells analysis displayed that part of the cellular population, around 25% of analyzed cells, was in S-phase of cell cycle; these cells had a DNA content, measured by PI incorporation, that was intermediate between those of G1 and G2 and a significant BrdU incorporation (figure 14 A, left panel). In contrast ten days after transduction, FACS analysis of AKTIP KD HPFs revealed that a consistent proportion of cells, 45%, was blocked in S-phase, because they had DNA content proper of S-phase cells but they couldn't incorporate BrdU, as indicated by their low BrdU content (figure 14 A, right panel), suggesting a strong reduction or absence of active replication following AKTIP downregulation. These data suggest the activation of an intra-S checkpoint in AKTIP KD HPFs (Branzei, 2009).
The strong reduction of active replication in HPFs following AKTIP downregulation indicated by FACS analysis was further confirmed by cytological analysis of untreated or mild detergent extracted cells, as described above, immunostained with an anti-PCNA (Proliferating Cell Nuclear Antigen) antibody. PCNA is a constituent of replication fork and it's important for the elongation phase of replicative process; recent findings suggest that this protein has a crucial importance for the control and regulation of DNA replication (Moldovan, 2007). In unextracted cells anti-PCNA immunostaining revealed its nuclear localization during all interphase (figure 14 B, on the left). We couldn't find any significant difference in PCNA positive cells frequency between control cells and AKTIP KD HPFs analyzed ten days post transduction (figure 14 C) suggesting that AKTIP downregulation didn't affect nuclear PCNA localization. Instead following mild detergent extraction, that eliminates soluble proteins, anti-PCNA

immunostaining allows to visualize only chromatin bound PCNA molecules that are part of active replication forks (Chagin, 2010). Therefore in this experimental conditions PCNA positive cells are S-phase cells with fully competent replication forks. Immunostaining with anti-PCNA antibody following detergent treatment revealed that, ten days post transduction, AKTIP downregulation strongly reduced the proportion of PCNA positive cells compared to controls (11% in AKTIP KD HPFs in comparison to 25-30% of control cells) (figure 14 D).
Moreover, as mentioned before, following detergent extraction PCNA marks replication foci allowing to visualize their positions that change during replication progression: early S-phase is characterized by a high number of small foci distributed in the internal part of the nucleus; mid S-phase is characterized by a reduction of replication foci number and by a switch in their localization from the interior of the nucleus to the peripheral part; finally late S-phase is characterized by an increase in the dimension of replication foci that assume also a more irregular shape and are distributed both at nuclear periphery and scattered throughout the interior part of the nucleus (examples are reported on the left of figure 14 E) (Dimitrova, 2002; Chagin, 2010). Thus replication foci nuclear distribution reflects the stage of S-phase progression (Chagin, 2010) and we have exploited PCNA immunostaining in extracted cells to identify which proportion of S-phase cells is in each stage of S-phase. In asynchronous control cells most of PCNA positive cells had a pattern compatible with early S-phase (figure 14 E); this finding is in agreement with previous report that indicates this stage as the longest in primary cells (Dimitrova, 2002). AKTIP downregulation triggered an altered distribution of PCNA positive cells in the different S-phase stages, with a significant ($p<0.05$) reduction of early S-phase cells and a corresponding increase of mid and late S-phase cells (figure 14 E).

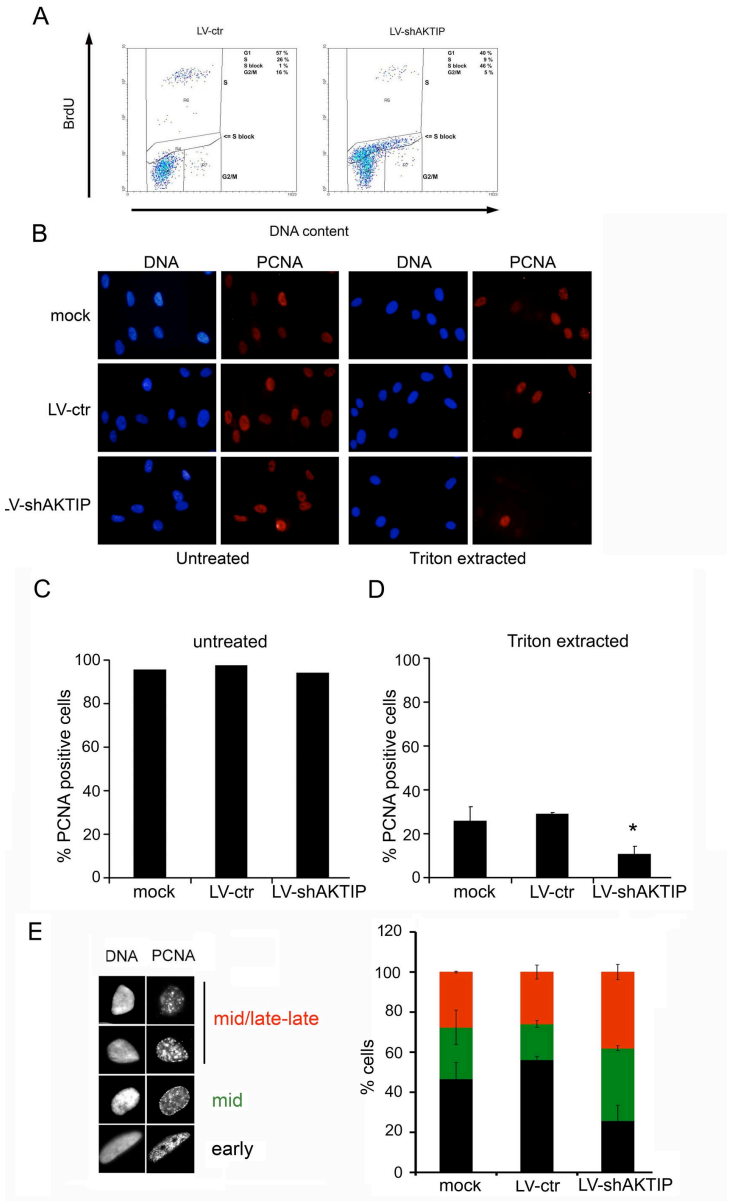


Figure 14: AKTIP downregulation impairs proper DNA replication.
(A): LV-ctr and LV-shAKTIP transduced asynchronous HPFs, 10 days p.i., were FACS analysed for PI and BrdU incorporations. Combining these two parameters, percentage of

cells in each stage of cell cycle was determined and is reported in the upper part of figure. (B): IF with anti PCNA (red) antibody of untreated (left part of the panel) or Triton X-100 extracted (right part) untransduced (mock) or transduced with the indicated recombinant lentivectors HPFs, 10 days p.i. DNA was stained with DAPI (blue). (C-D): percentage of PCNA positive cells either in untreated (C) or in Triton extracted (D) HPFs is reported. In D samples were analyzed in duplicate and the mean value is presented; p value was based on Student t-test and statistical analysis was obtained comparing sample values with both controls values ($p < 0.05$: (*)). (E): analysis of distribution of PCNA positive cells in S-phase stage (early S-stage in black, mid S-stage in green and mid/late-late S-stage in red) reported on left (for description refer to text) is reported. Samples were analyzed in duplicate and the mean value is presented; p value was based on Student t-test and statistical analysis was obtained comparing sample values with both controls values ($p < 0.05$: (*)).

Collectively these data suggest that normal replication is impaired following AKTIP downregulation, strongly enforcing its functional involvement in this cellular process.

DISCUSSION

Data presented in this work demonstrate that AKTIP is a new telomeric protein. Indeed AKTIP/Ft1 downregulation triggers the formation of a characteristic telomeric phenotype that shares some similarities with those deriving by un-functional telomeric proteins. AKTIP downregulation induces the formation of TIFs and the frequency of cells TIFs positive and also the mean number of TIFs per cell are comparable to those induced by the presence of aberrant TRF2 (Takai, 2003). Moreover, Ft1 downregulation results in the formation of different types of telomeric aberrations. Most of them were already described as part of the aberrant phenotype deriving by KD/KO either of shelterin proteins such as TRF1 (Sfeir, 2009; Martinez, 2009) or Rap1 (Martinez, 2010) and of telomeric accessory proteins such as Apollo (Ye, 2010), or ATR (Pennarun, 2010). So our data indicate that AKTIP is important for telomeres maintenance and although it remains to clarify which is the molecular function of AKTIP in telomeres metabolism, our data strongly suggest that it could be more important for their proper semi-conservative replication than for their protection. Indeed, among Ft1 KD induced telomeric aberrations, the most prominent (MTS and STA) were recently demonstrated to be caused by telomeric semi-conservative replication defects (Sfeir, 2009; Martinez, 2009; Martinez, 2010; Ye, 2010, Pennarun, 2008; Pennarun, 2010). Moreover, although we observed a significant increase of chromosomal telomeric-fusions following Ft1 downregulation, their frequency is lower than that caused by KO of TRF2 shelterin that has a principle role in telomere capping (Celli, 2005). AKTIP telomeric role is further corroborated by its interaction with telomeric DNA that we observed by ChIP assay both in HPFs and in HeLa cell line. This interaction could be direct or indirect, and further experiments are needed to determine if AKTIP binds telomeric repeats or if this interaction is mediated by the interaction of AKTIP with another telomeric protein, shelterin or nonshelterin, that can form a bridge between AKTIP and

telomeric DNA. Despite of this interaction AKTIP doesn't co-localize with TRF1, indicating that AKTIP is not stably and/or abundantly located at telomeres; its interaction, directly or indirectly, with telomeric repeats, for example, could be cell-cycle modulated, but further experiments are needed to clarify this point. This last finding, in combination with the telomeric role of AKTIP, suggests that it has to be considered as an accessory telomeric protein. Indeed, this group of proteins are not constituently associated to telomeres even if they are essential for proper telomere maintenance (Palm, 2008). Moreover also the existence of an AKTIP *Drosophila* homologue, peo, confirms its classification as a telomeric accessory protein because shelterin proteins don't have homologues in this model organism and, similarly, also the terminin proteins don't have homologues in human cells. On the contrary telomeric accessory proteins are largely conserved between human and flies and for most of them also their telomeric function is conserved (Raffa, 2011). The finding of a telomeric role for AKTIP enforces the idea that, apart from the different elongation mechanisms, *Drosophila* and human telomeres are not so different and AKTIP is the first human telomeric protein identified starting from the identification of a nonterminin protein in fly. For this reason this work confirms that *Drosophila* can be a good model organism also in telomeres biology field and the identification of other nonterminin telomeric proteins may lead to the identification of new human telomeric proteins.

In addition to its telomeric function, as the other telomeric accessory proteins, AKTIP could have a more general role in cellular metabolism and our data suggest that it's important for genomic stability maintenance. Indeed we have observed that AKTIP/Ft1 downregulation affects DNA stability also at a more general level as suggested by the increased frequency of chromosomal breaks and gaps following Ft1 reduction and by the increased sensitivity to low doses-aphidicolin treatment of AKTIP KD HPFs. Moreover, the presence of extra-telomeric chromosomal

aberrations can account for the 50% of observed DNA damage foci that don't co-localize with TRF1. These chromosomal aberrations, both telomeric and non-telomeric, trigger the DNA damage response that is activated following AKTIP downregulation and that, in turn, induces the cell cycle block and proliferation arrest observed in AKTIP KD HPFs.

We hypothesize that AKTIP downregulation could induce these chromosomal aberrations impairing proper DNA replication. We have obtained data indicating that AKTIP could be involved in DNA replication: a) among Ft1 KD induced telomeric aberrations the most prominent (MTS and STA) were recently demonstrated to be caused by telomeric semi-conservative replication defects (Sfeir, 2009; Martinez, 2009; Martinez, 2010;Ye, 2010, Pennarun, 2008; Pennarun, 2010); b) AKTIP KD increased the sensitivity to aphidicolin, a drug that, inhibiting DNA polymerases, slows down (in our experimental conditions) or arrest DNA replication; c) AKTIP downregulation impaired cell cycle progression blocking cells in S-phase, as demonstrated by FACS analysis, and affected DNA replication as shown by the reduction of the proportion of cells with fully competent replication forks and by the alteration of S-phase stage progression following AKTIP downregulation; d) AKTIP nuclear localization recalls the position that replication foci assume in mid-late S when they reach the peripheral portion of nucleus hosting most heterochromatin (Dimitrova, 2002; Chagin, 2010). Moreover, AKTIP structurally belongs to UEV proteins family and, as a member of this family, it could be involved in regulative ubiquitilation, a post-translational modification that is important for DNA replication control and regulation especially when the replication fork encounters problems (Moldovan, 2007; Branzei, 2009). Although it has to be determined if AKTIP could act mechanistically as an UEV protein and eventually which could be its target(s), its putative UEV function suggests that AKTIP could be important for the proper replication of specific regions of the genome that require particular attention. For these reasons our current hypothesis is that AKTIP could be necessary for the

stability or the progression of replication fork particularly at "replication challenging" sites, often late replicating regions, such as repetitive DNA, or at DNA with secondary structures or topological alterations to solve. The eukaryotic genome contains numerous natural impediments to replication that, during unperturbed S-phase, can induce the pausing or completely block the replication forks progression, increasing the odds for replication fork breakage events (Branzei, 2010). Telomeres are part of this group of "replication challenging" genomic regions and a lot of questions about their semi-conservative replication remains to be solved. (Gilson, 2007; Sampathi, 2011). Our data indicate that AKTIP is important for telomere replication, although further experiments are needed to understand which molecular function AKTIP could play in this process. Moreover our data suggest also that AKTIP could be important for the stability of fragile sites, that are other genomic regions characterized by a difficult replication. This last finding not only contributes to corroborate the idea that telomeres could resemble fragile site (Sfeir, 2009) but also enforces our hypothesis that AKTIP could be important for the replication not only of telomeres but also of other genomic regions sharing some features with telomeres, such as the possibility of forming secondary structures needing of unwinding during replication or the presence of proteins tightly bound to DNA.

AKTIP localization in the nucleus, with its high enrichment at nuclear periphery, could also support the hypothesis of its involvement in handling of dangerous or problematic genome regions. Growing evidences underline that chromatin is not randomly distributed in the nucleus and support the existence of a higher level of organization, known as nuclear architecture, that basing on positioning of chromatin regions and other nuclear components into 3D nuclear space, allows to organize and regulate the different nuclear functional processes (Misteli, 2011; Gonzalez-Suarez, 2008; Misteli, 2007). Different genome regions occupy specific territories in the nucleus (interphase chromosome territories); gene-poor domains are often found at the nuclear

periphery, while gene-rich territories tend to localize at the nuclear interior. However, chromosome territories are not static, but rather dynamic structures, and, for example, it has been shown that specific chromosomal regions can occupy different nuclear positions in cells from different tissues or from different developmental stages and their different positions correlate with their expression status (Misteli, 2011; Gonzalez-Suarez, 2008; Misteli, 2007). According to this model, nucleus is compartmentalized and nuclear periphery appears as a specialized zone with proper features that enable it to host and manage most of transcriptionally inactive genomic regions or most of late-replicating heterochromatin (Tam, 2004; Misteli 2011).
Several evidences indicate that telomeres interact with nuclear envelope. In budding yeast, telomeres are clustered to nuclear envelope and this localization is associated with transcriptional repression (Gartenberg, 2009; Akhtar, 2007). In yeast also unrepaired or slowly repaired ds-breaks are tethered to nuclear periphery (Nagai, 2008; Oza 2009; Gartenberg 2009; Oza, 2010) strengthens the idea that nuclear periphery is a subcompartment to handle genomic hazardous regions. Although mammalian telomeres cluster at nuclear periphery only during meiosis, abundant evidences indicate that they interact with nuclear matrix (de Lange, 1992; Luderus, 1996). Telomeres in budding yeast are replicated in concert late in S-phase (Friedman, 1995), whereas the bulk of mammalian telomere sequence is replicated through S-phase (Wright, 1999). It has been demonstrated that each telomere replicates in a specific moment of S-phase independently from its length; the replication time of each telomere correlates with its nuclear position: more peripheral is the telomere in the nucleus, later it replicates. These findings strengthen the link between replication timing and high order genome organization (Arnoult, 2010). AKTIP with its peculiar accumulation at nuclear rim could be accumulated where the “replication challenging” genomic regions were processed likely contributing to their handling. Further studying on AKTIP role in DNA replication could

contribute to enforce the nuclear architecture model and the importance of subnuclear compartmentalization for cellular metabolic processes.
The nuclear matrix in higher eukaryotes is formed essentially by nuclear lamina and by internal fibrogranular network (Nickerson, 2001) and has a crucial role in nuclear architecture maintenance (Gonzalez-Suarez, 2008; Misteli, 2011). In mammals the nuclear lamina is constituted mainly by two types of lamins: B-type lamins, which are constitutively expressed in most tissues, and A-type lamins, which are developmentally regulated and mainly expressed in differentiated cells. The main function of the nuclear lamina is to provide a scaffold for multiple protein complexes that regulate different nuclear functions, including transcription, replication, and DNA repair (Hutchison, 2004; Broers, 2006; Dechat, 2010). It also helps to maintain cell integrity and nuclear shape, in addition to serve as an anchor for chromatin at the nuclear periphery (Gonzalez-Suarez, 2008). The importance of nuclear lamins in the integration and coordination of the different nuclear processes become evident analyzing the aberrant phenotype deriving by mutations in lamin A encoding gene (LMNA). A lot of different pathologies, collectively called laminophaties, are associated with mutations in LMNA gene, some of which associated with muscular dystrophy (Goldman, 2002). Cellular and/or organismal models of these pathologies show defects in chromatin remodelling and in 3D organization of the genome, as exemplified by loss of heterochromatin from nuclear periphery (Capell, 2006; Shumacker, 2006; Coutinho, 2009). One of the most severe laminophaty is the Hutchinson Gilford Progeria Syndromes (HGPS) that it’s caused by LMNA mutations generating a truncated version of lamin A (de Sandre-Giovannoli, 2003), called progerin, which lead to premature senescence (Goldman, 2004). Although the molecular pathogenic mechanisms are still poorly understood, growing evidences underline that genomic instability and dysfunction in telomere maintenance play a crucial role in premature aging syndromes. Telomeres of HGPS

fibroblasts were shown to undergo faster telomere attrition during proliferation than normal counterparts (Allsopp, 1992; Huang, 2008) and also telomeres from a mouse model of the disease are consistently shorter than controls and exhibit an increase in signal free ends (Gonzalez-Suarez, Redwood, Perkins, 2009). Moreover also the 3D positioning of telomeres is altered in Lamin A null mice (Gonzalez-Suarez, Redwood, Perkins, 2009) and, interestingly it results affected also in tumour cells (Mai, 2006) and in senescent cells (Raz, 2008). Moreover, cellular defects associated with HGPS include a reduced lifespan in culture and premature senescence, irregular nuclear shape, altered chromatin organization, a chronic DNA damage response activation and genomic instability (Musich, 2009; Benson, 2010; Gonzalez-Suarez, Redwood, Gonzalo, 2009; Liu, 2008). Very little is known about the molecular mechanisms that underlie this complex diseases and our data suggest that AKTIP could be involved in laminopathies and in particular in progeroid syndromes. We have observed that AKTIP co-localizes with lamin B1 suggesting the possibility that it could be a new lamins interactor, and further experiments investigating AKTIP-lamins interaction, direct or indirect, can clarify this point. Moreover, some phenotypic traits of HGPS cells overlap with AKTIP KD phenotype, such as the induction of premature senescence, an high incidence of chromosomal aberrations, alteration in telomere maintenance, the activation of a DNA damage response in absence of genotoxic stress. Also for HGPS cells has been hypothesized that chromosomal aberrations observed can be linked to replication problems that may lead to stalled replication forks; their collapse could result in ds breaks formation (Musich, 2009). It will be interesting to analyze if AKTIP quantity or localization could be altered in progeroid cells and further information about AKTIP putative involvement in progeroid syndromes could come from the analysis of Ft1 KO mice that we are currently generating. Additional study on AKTIP protein could help to find a connection between aging, lamins and telomere maintenance.

MATERIALS AND METHODS

Cells and virus

Human foreskin primary fibroblasts (HPF) from healthy donors (provided by A. Orecchia, IDI, Rome, Italy) were used, unless otherwise indicated, at early passages (5-11). HPFs, $p53^{-/-}$ MEF (provided by S. Soddu, IFO, Rome Italy), HPF, HeLa (ATCC CCL-2) and 293T (ATCC CRL-11268) cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen).
Second generation recombinant lentiviruses (LV) were produced and titrated as already described (Piersanti, 2006) by co-transfection of 293T cells with the vectors pCMV-dR8.74, pMD2.G (http://www.addgene.org), and a transfer vector encoding gene specific interfering sequences (Table I). The transfer vectors were PLKO.1 (Sigma). Lentivectors produced were titrated measuring p24 concentration per ml of viral production as described (Piersanti, 2006). The moi (molteplicity of infection measured as p24 pg per cell) used for all the experiments was 3 for human cells transduction and 10 for mouse cells. Transductions were performed in complete medium supplemented with 8 μg/ml polybrene (Sigma). After viral addition, cells were centrifuged for 30' at 1800 rpm at RT, incubated 3h at 37°C, and then transferred to fresh complete medium. 72h post transduction, cells were subjected to selection in complete medium supplemented with 2μg/ml puromycin (Sigma) and kept under these conditions for further analyses.
Population doubling (pd) was calculated according to the following formula: Log (nt/n0) x 3.33, where nt is the cell number in the n day post transduction and n0 is number of cells plated; the cumulative pd are represented.
Cell irradiation with X-rays (Gilardoni MGL 200/8D, 0.2 mm copper filtration, 200KVp, 6mA) with 1Gy (42 cm, 0.28 Gy/min) was performed where indicated.

AKTIP-Flag protein was transiently expressed in 293T cells using a Calcium Phosphate transfection with the plasmid pCMV6-Entry encoding the Flag tagged protein (OriGene, NM_001012398).

Sh sequence name	Sh sense sequence	Target gene (Gene ID)	Target position
ctr	CAACAAGATGAAGAGCACCAA	**none**	**none**
ShAKTIP 09	TGAAGGTGAAGAGAAGACATT	**AKTIP (64400)**	**CDS (258-279)**
ShAKTIP 10	TCCACGAACTGCACCAAAGAA	**AKTIP (64400)**	**CDS (303-324)**
ShAKTIP 11	CACTGCTCGTTTGTTTGACCA	**AKTIP (64400)**	**CDS (870-891)**
ShAKTIP 12	TGCCCATAACTAAGCCTACAT	**AKTIP (64400)**	**CDS (353-374)**
ShAKTIP 13	CCTGTCTCTAAGTAATGCATT	**AKTIP (64400)**	**3'UTR**
ShFt1 69	GCTGTTTGATATTCCCGTCTT	**Ft1 (14339)**	**CDS (632-653)**
ShFt1 70	CCTTTCAGTAAAGAAGAGAAA	**Ft1 (14339)**	**CDS (1056-1077)**
ShFt1 73	CCTGATGAACAGCACAATAAA	**Ft1 (14339)**	**CDS (987-1008)**

Table I: Interfering sequences encoded by transfer vectors used to produce recombinant lentivectors. Name, sequence target gene and position of target sequence are indicated for each interfering sequences encoded by transfer vectors used to produce recombinant interfering lentivectors employed in this work.

Gene expression

Cells were lysed one-week post transduction by addition of TRIzol reagent (Invitrogen) and RNA extracted according to the manufacturer's instructions. After DNase treatment (Invitrogen), RNA was reverse transcribed into cDNA with oligo d(T) primer and OMNISCRIPT RT KIT (Qiagen). To quantify target gene expression, specific primers (Table I) were selected using Primer

Express software (Applied Biosystems). Reactions were performed as previously described (Piersanti, 2006).

Senescence associated β-galactoside staining

At the indicated time post transduction, 24h after plating on coverslips cells were assayed for senescence associated β-galactosidase (SA-β-GAL) as previously described (Dimri, 1995), using the Senescent cells Histochemical Staining Kit (Sigma). Images were taken using a Zeiss Axiophot microscope equipped for differential interference contrast microscopy.

Immunoblotting

Cell pellets were treated with lysis buffer [Tris–HCl 50mM pH7.4, 10% NP-40, 0.25% NaDesoxycholate, EDTA 1mM, NaCl 150mM, PMSF 1mM, protease inhibitor cocktail (Roche)]. Samples were loaded on to pre-cast 4–12% gradient acrylamide gels (NuPAGE, Invitrogen). After electro-blotting, filters were incubated with the following antibodies: anti-AKTIP (Sigma), anti-b actin-HRP conjugated (Santa Cruz Biotechnology), anti-cyclin A (Santa Cruz Biotechnology), anti-cyclin B (Santa Cruz Biotechnology), anti-cyclin E (Upstate Biotechnology), anti-p53-pSer15 (Cell Signaling Technology), anti-p53 (DakoCytomation), anti-ATM-pS1981 (Rockland Immunochemicals), anti-ATM (Genetex), anti-ChK1-PSer 345 (Cell Signaling Technology). Filters were then incubated with corresponding secondary antibodies HRP-conjugated (SantaCruz Biotechnology). Immunoreactive proteins were revealed using the enhanced chemiluminescence system (ECL plus, Amersham). Where indicated quantification was obtained valuating bands intensity with Image J software (developed by National Institute of Health (USA)); bands intensity was normalized using corresponding loading control (bactin) bands intensity.

Immunofluorescence

At the indicated time points post transduction, after plating on coverslips, cells were fixed with 3.7% formaldehyde 10' at 4°C, and permeabilized with Triton X-100 0.25% in PBS 1'. For immunoblotting with anti PCNA antibody after formaldehyde fixing the cells were treated with cold methanol for 5' at RT followed by PBS washes. Where indicated, a pre-permeabilization fixing protocol was performed as previously described (Zhu, 2000). The cells were then blocked with BSA 3% and incubated with the following primary antibodies: anti-ATM-pS1981 (Rockland Immunochemicals), anti-53BP1 (Novus Biologicals), ant-gH2AX (Upstate Biotechnology), anti-AKTIP (Sigma), anti-FLAG (SIGMA), anti-Lamin B1 (Santa Cruz Biotechnology), anti-PCNA (Santa Cruz Biotechnology) and anti-TRF1 (provided by T. de Lange, Rockefeller University NY). Cells were incubated 45' at RT with secondary antibodies FITC- (Jackson Immunoresearch) or ALEXA 555- (Invitrogen) or RODHAMINE- (Jackson Immunoresearch) conjugated. After drying, cells were mounted on glass slides with DAPI-Vectashield (Vector laboratories). Slides were analyzed with a Zeiss Axioplan epifluorescence microscope equipped with a cooled CCD camera (Photometrics); the signals recorded as grey-scale digital images were pseudo-coloured using Adobe Photoshop. In alternative, where indicated, slides were analyzed using a calibrated Prior Proscan stepping motor, with an EM-CCD camera (Cascade II, Photometrics) connected to a spinning-disk confocal head (CarvII, Beckton Dickinson) mounted on an inverted microscope (Nikon). Images were acquired using Metamorph software package (Universal Imageing). Eight fluorescence optical sections were captured at 1μm Z steps and the images of the different sections were analyzed separately or, when indicated, the images shown are a maximum-intensity projection of all the sections.

FISH and metaphase spread analyses

For mitotic index determination metaphase spreads obtained from HPFs were analyzed: 24h after plating on coverslips, cells were incubated with colchicine (Sigma) for 3h, then treated with hypotonic solution (KCl 75 mM) for 7'at 37°C and fixed with Methanol: Acetic Acid 3:1 for 15' at RT. After drying, cells were mounted on glass slides and, treated with DAPI-Vectashield (Vector Laboratories). Mitotic Index was considered as the ratio between metaphase number and the total number of cells counted. For the evaluation of aphidicolin-induced breaks and gaps, HPFs transduced as indicated, ten days post transduction were treated as described above following 24h incubation with 0.4 μm aphicolin (Sigma) in culture medium. Chromosomal aberrations were evaluated on DAPI-stained metaphases.
For FISH assay the hybridization was carried out on metaphase spreads obtained from $p53^{-/-}$ MEFs, transduced as indicated, one week post transduction and after 30' of incubation with colchicines in plates; the cells were then trypsinized and collected by centrifuge; then were treated with hypotonic solution (KCl 75 mM) for 20' at RT and fixed by treatment 3 times with Methanol: Acetic Acid 3:1 for 2h at 4°C. The suspension obtained in this way was then dropped on cold wet slides. The slides were air-dried for one week at RT; then were dehydrated by successive ethanol baths and air-dried again. Following treatment for 50' at 65°C the DNA was denatured by heat for 1'30'' at 80°C and dehydrated as described above and air-dried again. The probe was obtained by a PCR reactions carried out in absence of template using primers $(TTAGGG)_5$ and $(CCCATT)_5$ (Operon) as described (Ijdo, 1991).
The PCR reaction was performed in presence of 200 μM of dATP/dCTP/dGTP, 130 μM of dTTP and 70 μM of DIG-dUTP (PCR Dig Probe Synthesis Kit, Roche). The PCR products were fragmented by sonication (2 times for a 10'' pulse at 50% power setting using a Braun Biotec-Sartorius ultrasonicator equipped with a 2 mm tip) to reach a 500 bp-2000 bp of length. The probe was

denatured by heat for 8' at 80°C in the hybridization buffer [Formamide 50%, SSC 2X and dextran sulfate 50%] and was added to the slides; the hybridization was carried out at 37°C for 16-18h. Following 3 washes in SSC 1X at 60°C, the slides were incubated for 30' at 37°C in the blocking solution [BSA 30 mg/ml, SSC 4X, TWEEN-20 0.1 %] and then with the anti-DIG antibody (Roche, 10 mg/ml) in the detection buffer [BSA 10 mg/ml, SSC 1X, TWEEN-20 0.1 %] for 30' at 37°C. The slides were then washed 3 times in SSC 4X-TWEEN-20 0.1% and air-dried. After drying the cells were treated with DAPI-Vectashield (Vector Laboratories).The slides were analyzed using a Zeiss Axioplan epifluorescence microscope as described above. Telomeric aberrations scored were essentially: chromosomes with telomeric signal free ends, sister telomere association, multiple telomeric signals, fragments with one or more telomeric signals, chromosomes with extratelomeric signals, interchromosomal fusions with or without telomeric signal at fusion point.
For the detection of chromosomal aberrations in MEFs transduced as indicated, cells were treated as described above and breaks and gaps were observed on GIEMSA-stained metaphases.

ChIP

For ChIP assays HPFs and HeLa cells were cross-linked adding formaldehyde at a final concentration of 1% directly into the culture medium for 15' on a shaking platform; the reaction was stopped by adding glycine at a final concentration of 0.125 M. The cells were then scraped and lysed and chromatin was extracted as previously described (Benetti, 2008). Chromatin fragments obtained were incubated with 7.5 mg of mouse monoclonal anti-AKTIP antibody (Sigma) or 1 ml of mouse IgG (Sigma) at 4°C overnight on a rotating platform. Then Salmon Sperm DNA/protein agarose beads (60ml) were added and incubated for 1h at 4°C. Immunoprecipitation and following cross-linking reversal and DNA extraction were carried out as previously

described (Benetti, 2008). DNA obtained in this way was slot-blotted into a Hybond N+ and hybridized with a telomeric probe obtained from a plasmid containing a 1.6 kb of TTAGGG repeats (gift from T. de Lange, Rockefeller University, USA) and with an Alu probe obtained by genomic DNA amplification using the following oligos: sense 5'-CGCCTGTAATCCCAGCACTTTG-3', antisense 5'-ACGCCATTCTCCTGCCTCAGC-3' (MWG-Biotech). The quantification of the signal was done using a ImageQuant Software.

Southern blotting

For genomic DNA isolation ten millions of untrasduced, LV-ctr and LV-shAKTIP transduced HPFs, thirteen days post transduction and of late passage (30) untreated HPFs were harvested by trypsinization washed with cold PBS and collected by centrifugation at 1000 rpm for 5'. Then the genomic DNA was isolated with NucleoSpin Tissue Genomic DNA Isolation Kit (Clontech). Three µg of genomic DNA for each sample were cleaved with Hinf I/Rsa I (Roche) and separated in a 0.7% agarose gel. The fractionated DNA was depurinated by treatment with HCl 0.25 M for 20', denaturated by treatment with NaCl 1.5M – NaOH 0.5M for 40' and neutralized in NaCl 1.5M – TrisHCl 0.5M ph 7.5 for 40'. The DNA was then transferred in Nytran-N membrane (Whatman) in 20x SSC (NaCl 3M, sodium citrate 0.3M ph 7) over night. The membrane was backed at 80°C for 2h and then hybridization has done using DIG Luminescent detection Kit (Roche) and a TTAGGG repeats probe over night at 47.8°C. The probe was obtained as described above. After hybridization low stringency washes were performed using SSC 2X-SDS 0.1% at room temperature (two washes for 5') and high stringency washes were performed using SSC 0.2X - SDS 0.1% at 50°C. The following incubation with an anti-DIG antibody and the revelation reaction were carried out using DIG Luminescent detection Kit

(Roche) according to the manufacturer instructions. Final revelation was obtained by exposition on Hyperfilm (Amersham).

Flow cytometry analysis of cell cycle distribution.

5-bromo-deoxyuridine (BrdU) at the final concentration of 45 μM was added to the culture medium 30 min before harvesting the cells. Cells were fixed in 70% ethanol (30 min, 4°C), washed twice in PBS + 0.5% Tween 20 and incubated in 3M HCl for 45 min. Cells were then exposed to anti-BrdUrd monoclonal antibody (Dako), to the secondary Alexa-Fluor488-conjugated antibody (Jackson) and counterstained with Propidium Iodide (PI, Sigma) 20 μg/ml. Acquisition was carried out using a Beckman-Coulter Epics XL flow-cytometer and recorded data were analyzed by the WinMDI software (developed by Joe Trotter, free download at http://en.bio-soft.net/other/WinMDI.html).

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