



Sapienza, University of Rome

**“The telomeric protein AKTIP/Ft1 intercepts
lamin metabolism and is important
in mouse development”**



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INDEX	3
PUBLICATIONS	6
GLOSSARY	7
INTRODUCTION	10
Study of the determinants of ageing <i>in vitro</i>	10
<i>Telomeres are nucleoprotein structures that protect chromosome linear ends</i>	10
<i>Lamins and lamins associated proteins are important for nuclear envelope maintenance</i>	23
Study of the determinants of ageing <i>in vivo</i>	32
<i>Mouse models with impairments of the determinants of ageing display common phenotypic traits</i>	32
AKTIP/Ft1 is a novel telomeric accessory protein required for proper telomere maintenance	48
AIM OF THE PROJECT	57
RESULTS	59
Study of AKTIP interactors	59
<i>AKTIP directly interacts with shelterin TRF1, TRF2 and TRFH2 domain</i>	59
<i>AKTIP binds components of DNA replication machinery</i>	62
<i>AKTIP interacts with lamins and lamins related proteins</i>	64
Production and characterisation of Ft1 knockoutfirst mouse model	66
<i>Kof allele leads to Ft1 mRNA reduction</i>	66
<i>Ft1^{kof/kof} animals are obtained at sub-mendelian ratio and display an aberrant phenotype</i>	70

<i>Ft1^{kof/kof} animals show survival and growth defects</i>	72
<i>20.5% of Ft1^{kof/kof} animals prematurely die and display skeletal defects</i>	75
<i>Ft1^{kof/kof} animals display adipose and cardiac tissue alterations</i>	81
<i>Ft1^{kof/kof} male mice are more sensitive to Ft1 depletion</i>	85
Production and characterisation of Ft1 kof and p53 ko mouse line	87
<i>Ft1 and p53 co-depletion increases death incidence and p53 ko rescues Ft1 body weight defects</i>	87
DISCUSSION	93
<i>AKTIP/Ft1 biochemically interacts with different proteins involved in telomeric, DNA and in nuclear envelope maintenance</i>	93
<i>AKTIP/Ft1 downregulation in vivo phenocopies premature ageing traits and it genetically interacts with p53</i>	95
CONCLUSIONS	99
MATERIALS AND METHODS	103
<i>Cells</i>	103
<i>GST pull down from cell extracts</i>	103
<i>GST pull down for direct interaction with bacterially purified proteins</i>	103
<i>Immunoblotting and blue comassie staining</i>	104
<i>Production of Ft1 kof transgenic mice</i>	105
<i>Production of Ft1 kof and p53 ko double transgenic mice</i>	105
<i>Mouse manipulation</i>	105
<i>gDNA extraction from tail biopsies and genotyping</i>	106
<i>RNA extraction from tissue biopsies and Q-PCR assays</i>	107
<i>X-ray analysis</i>	108
<i>Histology</i>	108
<i>Statistics</i>	108
REFERENCES	110

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GLOSSARY

Telomere: Nucleoprotein structure that protects the linear end of the chromosome from incomplete replication, degradation and detection as DNA damage site.

Shelterin: Six-protein complex that binds TTAGGG telomeric repeats and enables mammalian cells to distinguish natural chromosome ends from DNA breaks, to repress DNA repair reactions, and to regulate telomerase-based telomere maintenance. The protein complex is formed by TRF1, TRF2, POT1, TIN2, TPP1 and Rap1.

Telomeric accessory proteins: Group of diverse proteins recruited at telomeres by shelterin proteins exploiting functions crucial for telomere maintenance. Most of these factors are involved in DNA transactions such as DNA repair [Ku70/80, XPF/ERCC1, Apollo, Mre11 complex, RAD51D, PARP1 and -2], DNA damage signaling [Mre11 complex, 9-1-1 complex], DNA replication [ORC, RecQ helicases], or chromatin structure [HP1 proteins] (Palm and de Lange, 2008).

TRF homodimerization domain (TRFH1 and TRFH2): Domain of the shelterin TRF1 and TRF2 protein involved in homodimerization and binding to accessory factors such as Apollo, SLX4, RTEL1.

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 is often found active 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.

Replication fork: the branch-point structure that is formed 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.

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.

Nuclear periphery: Term that generally refers to the nuclear-membrane bilayer, its associated proteins and the embedded nuclear-pore complexes.

Nuclear lamina: Proteinaceous meshwork that underlies the inner nuclear membrane.

Lamins: 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 that, in turn, can polymerise in a head-to-tail fashion. These linear polymers are thought to associate laterally into 10-nm lamin fibers, which form the fibrous lamina meshwork in the nuclear periphery. Mutations in the corresponding 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 associated with mutations in genes encoding lamins.

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.

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.

CRE recombinase: enzyme that mediates recombination between two loxP sites.

LoxP sites: a specific 34-base pair sequences consisting of an 8-bp core sequence, where recombination, after Cre enzyme recognition, takes place, and two flanking 13-base pair inverted repeats.

FLP: flippase recombinase: derived from the yeast *Saccharomyces cerevisiae* that mediates recombination between two FRT sites.

FRT: a flippase recombinase target composed by 34-base pair long site sequences recognised by flippase FLP

En2SA: splicing acceptor site En2

INTRODUCTION

Study of the determinants of ageing *in vitro*

Telomeres are nucleoprotein structures that protect chromosome linear ends

The end of linear chromosomes is formed by a special heterochromatic nucleoprotein structure, known as the telomere, that protects chromosome ends from degradation and DNA repair and recombination activities.

The telomeric DNA of most eukaryotes is composed of double-stranded (ds) short tandem repeats. The strand that constitutes the 3'-end is rich in guanosine and devoid in cytosine, respectively G- and C- strand (de Lange, 2009). The length of this duplex telomeric DNA per chromosome varies enormously between different organisms running from 300 base pairs (bp) of *S. Cerevisiae* and *S. Pombe*, across 10-15 kb in human, to 20 kb or more for laboratory mice and rats (de Lange, 2009; Paeschke et al., 2010; Palm and de Lange, 2008; Samassekou et al., 2010). In mammal cells, telomere DNA is constituted of tandem repeats of TTAGGG sequence. The actual end of telomere is characterised by the presence of a single stranded 3' G-strand overhang, a conserved feature of eukaryotic telomere structure that is essential for telomere function (Lu et al., 2013).

Given their nature of ss DNA region and of overhang ends, telomeres can be mistakenly recognised as damaged or broken DNA sites triggering DNA damage sensing and signalling pathways (the end-protection problem). Thus the activation of repair pathways (Non Homologous End Joining and Homologous Recombination) can eventually cause genome instability through diverse events among the other loss of chromosomal regions, end-to-end fusions and breakage-fusion-bridge during mitosis (O'Sullivan and Karlseder, 2010; Palm and de Lange, 2008).

Telomeres solve the end-protection problem following two different but interlinked strategies: the formation of higher-order DNA structures that hide its linear ends and the binding of a specialised protein complex the shelterin. The shelterin complex has either an active role in avoiding the recognition of chromosome ends as ds-breaks and facilitates also the formation and stabilisation of the protective secondary structures (Chan and Chang, 2010; de Lange, 2010; Paeschke et al., 2010). T-loops structures are presumably formed through dsDNA invasion of 3' G-overhang pairing with the C-rich strand, forming the displacement loop (D loop) (Lu et al., 2013; Palm and de Lange, 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 (de Lange 2009) (Figure 1). An alternative higher order structure proposed for telomeres is the G-quadruplex, where each G base of 3' G-rich overhang serves as both donor and as acceptor for hydrogen bond formation (Lu et al., 2013). G-quadruplex formation involves the association of four different guanines into a cyclic Hoogsteen hydrogen binding arrangement (Maizels, 2006).

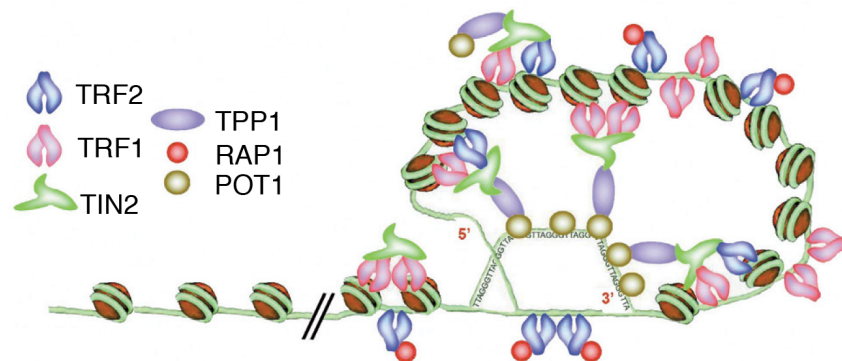


Figure 1 - Model of mammalian telomeres structure.

G-overhang folds back and invade upstream ds DNA, forming the t-loop. Shelterin components and nucleosomes, in brown, are represented. Adapted from (Pisano et al., 2008).

The second solution to the end-protection problems in mammalian cells is represented by the mentioned six-subunits protein complex, that is part of telomeres, called shelterin (Figure 1) (Palm and de Lange, 2008). The components of the shelterin complex localise to telomeres; they are abundant at telomeres throughout the cell cycle (Palm and de Lange, 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 et al., 1997; de Lange, 2010; Palm and de Lange, 2008). 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 does not have a role in anchoring the shelterin complex at telomeres (de Lange, 2010; Loayza and De Lange, 2003). POT1 binds to 5'-(T)TAGGTTAG-3' with subnanomolar affinity. POT1 recognises the 3' overhang site at the 3' end and ds-ss junction internally, moreover POT1 is able to bind the ssDNA in the D-loop (Takai et al., 2011). The mouse genome encodes two highly similar POT1 proteins, Pot1a and Pot1b, which have sequence specificity and DNA affinity similar to those of human POT1 (He et al., 2009; Takai et al., 2011). POT1 is recruited at telomeres through its interaction with another component of shelterin complex, TPP1 (known also as TINT1, PTOP, PIP1), although TPP1 has not discernible DNA binding activity by itself (Liu et al. 2004; Ye et al. 2004 a; (de Lange, 2010; Takai et al., 2011). 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 connecting also TRF1 and TRF2 (de Lange, 2005; O'Connor et al., 2006; Palm and de Lange, 2008; Ye et al., 2004). The last shelterin component is Rap1, the human ortholog of the yeast Repressor/Activator Protein 1, which is recruited at telomeres through its interaction with TRF2 (Chan and Chang, 2010; Li et al.,

2000). Shelterins form a stable complex also in the absence of telomeric DNA (Palm and de Lange, 2008). 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 et al., 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 (de Lange, 2010; Takai et al., 2010). The shelterin complex is highly conserved in mammals, including mice that are frequently used for gene targeting studies. The only distinctions in rodents, as previously mentioned, is the presence of two POT1 genes, Pot1 a and b, both interacting with Tpp1 and approximately equally abundant at telomeres, but with different functions (Hockemeyer et al., 2006).

In addition to shelterin complex, mammalian telomeres contain a large number of non-shelterin telomeric proteins, referred as accessory telomeric proteins, that give an important and essential contribution to chromosome ends maintenance and protection. The accessory-telomeric proteins are typically less abundant at telomeres than shelterin and some of them are only transiently associated to telomeres; moreover these proteins have non telomeric functions. Most of them are involved in DNA transactions, such as DNA repair, DNA damage signalling, chromatin structure organisation and maintenance, and often are recruited to telomeres by interaction with shelterin components, in particular TRF2 and TRF1 (Palm and de Lange, 2008). TRF1 and TRF2 share a common domain structure consisting of the TRF homodimerization (TRFH) domain and a C-terminal SANT/Myb DNA-binding domain, which are connected through a flexible hinge domain (Palm and de Lange, 2008). While the Myb domain recruits the two proteins to telomeric DNA as already mentioned, TRFH domain provides protein interaction surfaces for recruiting other telomeric partners in the formation of the protective telomeric complex. Sequence conservation between the two TRFs protein is restrained to the DNA binding core probably because of their common role in recognising the same conserved

telomeric sequence (TTAGGG). On the contrary TRFH domain sequence divergence reflects the different role of the two TRFs at telomeres exploited with the interaction with different telomeric partners (Fairall et al., 2001).

Since accessory telomeric proteins normally function in DNA processing, at one hand, they facilitate shelterin tasks and take part in chromosome ends protection, but in the other hand, these factors are potentially harmful for telomeres. This paradox suggests that shelterin must carefully controls and regulates accessory telomeric proteins actions, although mechanisms used for this control have not yet be fully elucidated (de Lange, 2005).

Telomeres, as well as the other DNA regions, are maintained by conventional semiconservative replication and, which accounts for the bulk of telomeric DNA synthesis, by additional mechanisms in order to extend G-strand, which shortens each replication round. It has been demonstrated that 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 synthesised 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 and Norbury, 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 synthesised 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 eukaryotes counteract telomere attrition thanks to telomerase (Greider and Blackburn, 1985, 1987). This is a ribonucleoprotein enzyme with two components: a highly conserved reverse transcriptase (TERT) (Lingner et al., 1997; Nakamura and Cech, 1998) and an associated template RNA (TERC)

(Feng et al., 1995). 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 and de Lange, 2004).

Robust telomerase expression is a feature of pluripotent stem cells and early stages of embryonic development, although telomerase activity is also present in adult stem cell compartments (Blasco, 2005b). Telomerase activity in adult stem tissues, such as ovary, testes and other high proliferative tissue, however, is not sufficient to prevent telomere shortening associated with ageing (Cong et al., 2002; Donate and Blasco, 2011). Thus, progressive and cumulative loss of chromosome end protection occurs both in stem and in somatic cells leading to activation of DNA damage response. The resulting accumulation of genetic damages activates cell cycle checkpoint that blocks cell proliferation and triggers cellular senescence (Blackburn et al., 2015).

Different mouse lines knockout (ko) for individual shelterin component have been created. Among these transgenic mouse lines, only mice depleted for shelterin Rap1 and Pot1b result viable (Takai et al., 2011). Rap1 is dispensable for telomere protection from ATM and NHEJ activation at telomeres because deletion of Rap1 in TRF2 proficient cells does not lead to telomere fusions or Telomeric Induced Foci (TIFs) formation (Martinez et al., 2009; Sfeir et al., 2009). Rap1 seems to have an important role in repressing HR; indeed in Ku70 Ko (KO) context, Rap1 depletion induces high frequency of exchanges between sister telomeres (TSCEs) (10% of chromosome ends) (Martinez et al., 2009; Sfeir et al., 2009). How Rap1 protects telomeres from HR is not clear; it's likely that Rap1 has an interacting partner that blocks HR (de Lange, 2010). Moreover, Rap1 has a role in telomere length regulation. Transgenic mouse line

that presents mutation in Rap1 gene that encode a Rap1 fragment which lacks TRF2-binding domain results viable and fertile. Mouse Embryonic Fibroblast (MEF) cells, obtained from this model, grow with rates similar to control cells and do not present TIFs formation, suggesting that Rap1 deletion does not result in major telomere dysfunctions, which is known to be lethal (Sfeir et al., 2009). Moreover the absence of Rap1 does not lead to telomere fusions events, telomere length is maintained over three generations of mouse breeding and in cultured cells and telomeric nucleosomal organisation results unvaried. However, Rap1 was critical for the repression of homology-directed repair (HDR), which can alter telomere length (Sfeir et al., 2009). Similar to Rap1 ko, the depletion of Pot1b results in mice viable and fertile but male Pot1b depleted mice become sterile due to high apoptosis levels in proliferative tissue such as testes and intestinal crypt epithelia; this happens because the absence of Pot1b diminished telomerase recruitment (enzyme necessary for telomere elongation, discuss later) at telomeres and this could result in accelerated telomere shortening and downregulation of 3' overhang length (He et al., 2009; Takai et al., 2011). Depletion of one of the other shelterin components results in generation of not viable mice (Takai et al., 2011). Indeed deletion of Pot1a in mice results in early embryonic lethality, increased cytogenetic abnormalities, and activation of an ATR-dependent DNA Damage Response (DDR) at telomeres, initiating both NHEJ and HR-dependent repair pathways. These results suggest that Pot1a is primarily required to protect telomeres from an ATR-dependent DNA damage checkpoint emanating from the single-stranded overhang, while Pot1b appears to be required for formation of the 3'overhang (He et al., 2009). Contemporaneously removal of both Po1a and Pot1b from murine telomeres results in rapid accumulation of RPA, induction of TIFs and phosphorylation of Chk1. As for Pot1a depletion, this DDR, which occurs in G1 and S/G2 phases, is ATR kinase dependent. Additional phenotype features of Pot1a/b double ko (DKO) cells include polyploidization due to endoreduplication, metaphases with diplochromosomes, and a low frequency of

sister telomeric fusions (Takai et al., 2011). Moreover 4 hours after removal of Pot1a from Pot1b deficient cells results in accumulation of RPA at telomere (Gong and de Lange, 2010).

The conventional deletion of the shelterin protein TRF1 in mice is embryonically lethal, embryos die very early during the blastocyst stage, and for this reason the characterisation of TRF1 role in differentiated cells has remained impractical (Donate and Blasco, 2011). TRF1 deleted MEFs do not show changes in telomere length, but TRF1 depletion leads to a rapid induction of p53/Rb-dependent cellular senescence concomitant with TIFs formation, abundant end-to-end telomere fusions, abundant multi-telomeric signals, also known as fragile telomeres, and activation of the ATM/ATR downstream checkpoint kinases CHK1 and CHK2 (Martinez et al., 2009). To study the role of TRF1 in telomere biology and diseases in the context of mammalian organism and cells, mice in which TRF1 was conditionally deleted specifically in epithelial tissues have been generated. According with severe telomere dysfunction, observed in MEFs, these mice die prenatally and show reduced skin thickness and stratification as well as hyperpigmentation plus a series of pathologies accompanied by persistent activation of DDR, p53/p21 and p16 pathways (Martinez et al., 2009). Suppression of p53 in mouse with epithelial ko for TRF1 rescues perinatal survival and the functionality of epidermal stem cells. Long-lived mice, however, develop epithelial abnormalities similar to those of human diseases linked to mutations in the component of shelterin and/or telomerase that will be discussed later. Furthermore these mice show also development of spontaneous squamous carcinomas, showing that TRF1 acts as a tumour suppressor by preventing telomere-related genomic instability (Martinez et al., 2009). Moreover TRF1 ko model results to be the first mouse model of ageing induced by telomere dysfunction in absence of critical telomere shortening (Martinez et al., 2009).

TRF2 ko animals result not viable; embryonic lethality occurred early in development, before embryonic day 13.5, and is not rescued by p53 deficiency.

Because of TRF2 deficiency results in embryonic lethality, cellular consequence of TRF2 deficiency were determined using p53 ko MEFs conditionally downregulated for TRF2. As a consequence of TRF2 absence the telomere loses the 3' overhang and are processed by NHEJ pathway. Deletion of TRF2 leads to extensive telomeric fusions dependent on DNA ligase IV activity. Both events mediated by NHEJ and telomeric fusions were abrogated in DNA ligase IV deficient cells and, in these cells, telomeres persist without undergoing DNA degradation. In this condition, telomeres retain the 3' overhangs but they are recognised as site of DNA damage; indeed DDR mediated by ATM kinase results active and chromosomes are subjected to endoreduplication (Celli and de Lange, 2005; Takai et al., 2011).

Concerning to TPP1 shelterin protein, its conditional KO results in no live-born mice, they die presumably before 14.5 days of embryo life. MEFs derived from 13.5 embryonic TPP1 depleted mice were analysed and shown growth defect and signs of senescence. TPP1 deletion results in the release of Pot1a and Pot1b from chromatin and loss of these proteins from telomeres, indicating that TPP1 is required for Pot1a and b association to telomere. The telomere dysfunctional phenotype associated with TPP1 depletion is identical of those of Pot1a and Pot1b DKO cells: TIF formations, Chk1 and Chk2 phosphorylation where Chk1 activations is mediated by ATR signalling and Chk2 phosphorylation is likely due to secondary activation of ATM signalling after the breakage of dicentric chromosomes formed during occasional telomere fusions. TPP1 depletion also produces endoreduplication phenotype, also observed in Pot1a and Pot1b DKO cells (Kibe et al., 2010).

The last shelterin that remains to analyse is TIN2 that is a central component of shelterin that not only connects TPP1/POT1 to the other shelterin components but also stabilises TRF1 and TRF2 on the telomeric duplex repeats array. Deletion of TIN2 in mice results in embryonic lethality (embryos die before 7.5 days of their embryonic development) through a mechanism that is telomerase independent (Chiang et al., 2004). Generation of mice lacking TIN2

exon 3-7, that produces a severely truncated TIN2 that lacks most of the protein, including the TRF1 binding site, allows to estimate the importance of TIN2 for a proper organism development. TIN2 deletion mimics the loss of TPP1 or Pot1a/b and including the same combination of phenotypes: ATR signalling activation, endoreduplication, postreplicative telomere fusions involving both leading- and lagging-ends. Therefore, TIN2, like Tpp1, is required to allow POT1 protein to fulfil its functions. In addition to the phenotypes typical of Pot1a/b loss previously described, TIN2-deficient cells show ATM kinase signalling activation and a modest level of chromosome-type telomere fusions (Takai et al., 2011).

Table 1. Phenotypes Associated with the Deletion of Individual Shelterin Components

Gene	Telomere Fusions									
	Viable	DDR		Endoreduplication	Chromosome-Type	Chromatid-Type		3' Overhang	Fragile Telomeres	T-SCEs (in Ku Null)
		ATR	ATM			Leading	Lagging			
POT1a	−	+	−	+	+	+	+	−	−	−
POT1b	+	−	−	−	−	−	−	+	−	−
POT1a/b	−	++	−	++	+	+	+	+	−	+
TPP1	−	++	−	++	+	+	+	+	−	?
TIN2	−	++	+	++	+	+	+	+	−	?
TRF1	−	^a	−	+	−	−	−	−	+	?
TRF2	−	−	++	+	++	^b	−	−	−	+
Rap1	+	−	−	−	−	−	−	−	−	+

^a ATR signaling requires progression through S phase.

^b Leading-end chromatid fusions occur only in absence of MRN or ATM.

Table 1 - Phenotype associated with the deletion of individual shelterin component.

Adapted from (Takai et al., 2011).

Telomeres, like other DNA regions, are maintained by conventional semiconservative replication and, which accounts for the bulk of telomeric DNA synthesis, by additional mechanisms in order to extend G-strand, which shortens each replication round. The replication of eukaryotic genomes initiates

bidirectionally at defined origins. At chromosome ends, the last origin is expected to be responsible for the replication of telomeric sequences (Gilson and Geli, 2007). Currently, except in few organisms, there has been little evidence of initiation of DNA replication within the telomeric repeats themselves (Makovets et al., 2004), so telomeric replication is thought to start from an origin located in subtelomeric region (Liew and Norbury, 2009). 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 synthesised 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 and Norbury, 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 synthesised 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.

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 and Chai, 2011).

Most eukaryotes counteract telomere attrition thanks to telomerase (Greider and Blackburn, 1985, 1987). This is a ribonucleoprotein enzyme with two components: a highly conserved reverse transcriptase (TERT) (Lingner et al., 1997) and an associated template RNA (TERC) (Feng et al., 1995). TERT is related to the reverse transcriptase of non-LTR retrotransposons and group II introns and like these, it extends the 3' end of a DNA, rather than RNA, primer (Nakamura and Cech, 1998; Smogorzewska and de Lange, 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 (Chen et al., 2000; Romero and Blackburn, 1991). 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 and de Lange, 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 and de Lange, 2004).

Robust telomerase expression is a feature of pluripotent stem cells and early stages of embryonic development, although telomerase activity is also present in adult stem cell compartments (Blasco, 2005b). Telomerase activity in adult stem tissues, such as ovary, testes and other high proliferative tissue, however, is not sufficient to prevent telomere shortening associated with ageing (Cong et al., 2002; Donate and Blasco, 2011). Telomerase 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, immortalising the cells (Smogorzewska and de Lange, 2004). Tumour cells require a telomere maintenance system for long-term proliferation, and in the majority of cases, 85%, this is provided upregulating hTERT (Henson et al., 2002). However telomerase activity per se does not induce transformation (Morales et al., 1999; Smogorzewska and de Lange, 2004), and, although telomerase is necessary for immortalisation, hTERT is not an oncogene (Hahn et al., 1999; Hahn et al., 2002; Smogorzewska and de Lange, 2004). Moreover, cancer cells can activate also alternative mechanisms to counteract telomeres

attrition (Reddel et al., 2001). These alternative mechanisms, collectively termed ALT (Alternative Lengthening of Telomeres), have the ability to activate telomerase-independent telomere maintenance pathways. ALT cells, differently from telomerase positive cells, are characterised by heterogeneous telomeres 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 et al., 2002). Moreover ALT cells show frequent sequence exchanges between sister telomeres (Londono-Vallejo et al., 2004). Another alternative mechanism of telomere elongation based on retrotransposition is present in *Drosophila* and some related Dipteran species (Biessmann et al., 1990), where telomeres are maintained by telomere-specific non-long terminal repeats (LTR) (Mason et al., 2008).

Lamins and lamins associated proteins are important for nuclear envelope maintenance

The nuclear lamins are type V intermediate filament proteins and are the major structural proteins of the nuclear lamina, which is located in the peripheral nuclear region between the inner nuclear envelope membrane and chromatin; they are also distributed, with a lower concentration, throughout the nucleoplasm (Butin-Israeli et al., 2012; Dechat et al., 2010). Genetic analyses have revealed that these genes are highly evolutionarily conserved in metazoans, but are not found in plants or unicellular organisms (Dittmer and Misteli, 2011). These nuclear intermediate filaments are named lamins A, B and C, according to their molecular weight (Dechat et al., 2010). Similar to most intermediate filament proteins, lamins have a conserved α -helical coiled-coil rod domain flanked by variable amino-terminal head and carboxy-terminal tail domain. The lamins tail domain contains an immunoglobulin-like fold motif and a nuclear localisation signal (Zuela et al., 2012).

The two major isoforms of vertebrate A-type lamins, lamin A and C, derive both from the LMNA gene by alternative splicing. Beyond these two isoforms, in some vertebrates LMNA alternative splicing can also produce other two less abundant isoforms: lamins AD10 and C2. Concerning the B-type lamins, lamins B1 and B2 are the two major B-type proteins in most vertebrate. The LMNB1 and LMNB2 genes encode them, respectively. The latter gene also encodes for the minor isoform, lamin B3 (Dechat et al., 2010).

Throughout development, at least one B-type lamin is expressed in all cells; on the contrary, the expression of A-type lamins is developmentally regulated (Dechat et al., 2010). During mouse development, lamins A and C are not expressed until days 10-12 of mouse embryogenesis and then primarily in primordial muscle cells. The lamin A expression in other organs begins only after mouse birth (Rober et al., 1989). Lamin A, B1 and B2 are expressed as pre-lamins that require extensive modifications to become mature lamins (Figure 2). The first post translational modification concerns the carboxy-

terminal domain -CAAX box. This modification process starts with farnesylation of the cysteine residue, by a farnesyltransferase (Davies et al., 2009). After that, a sequence of processing steps begin that, first of all, include the removal of the -AAX by an endopeptidase, most likely Rce1 and/or Zmpste24. Subsequently the farnesylated cysteine residue is carboxymethylated, process catalysed by the enzyme isoprenylcysteine carboxyl methyltransferase (Icmt). While the maturation of B-type lamins is terminated at this step, resulting in their permanent farnesylation and carboxymethylation, lamin A is further processed by Zmpste24/FACE1 to form mature lamin A. This step removes 15 amino acids from the carboxyl terminus of farnesylated/carboxymethylated prelamins A (Dechat et al., 2010; Dechat et al., 2008). Lamin C, which is 74 residues shorter than mature lamin A, does not present a -CAAX box and therefore is not farnesylated or otherwise modified (Dechat et al., 2010). The pre-lamin A, B1 and B2 modifications of the -CAAX box are thought to play a role in targeting the lamins to the inner nuclear membrane and also in establishing protein-protein interactions (Rusinol and Sinensky, 2006). Interestingly the lamin farnesylation inhibition does not impair their incorporation into the nuclear lamina. Moreover also lamin C that contains a unique C terminus becomes incorporated into the nuclear lamina (Dechat et al., 2008).

The three-dimensional supermolecular organisation of lamin filaments was analysed in *C. elegans*; for the formation of higher-order lamins structure, the basic building block is the lamin dimer. The first step in this assembly involves head-to-tail polymerisation of the lamin dimers. These polymers associate laterally in an antiparallel fashion to form the protofilament, and then between three and four protofilaments to form the lamin filament (Ben-Harush et al., 2009). Recent evidence, obtained by *in vitro* analysis on human lamin A/C and B, indicates that lamins can form both homodimers and heterodimers, suggesting that lamin filaments could assemble into protofilaments composed of both A- and B-type lamins (Kapinos et al., 2010). Alternatively, it is possible that protofilaments formed only by A-type or B-type lamins could associate laterally

to form mixed higher order lamin structures (Butin-Israeli et al., 2012). Now there are no evidence of lamin assembly and structure within nuclei *in vivo*, although it has been suggested that the formation of lamin network in cells is a self-organisation process (Volkova et al., 2011).

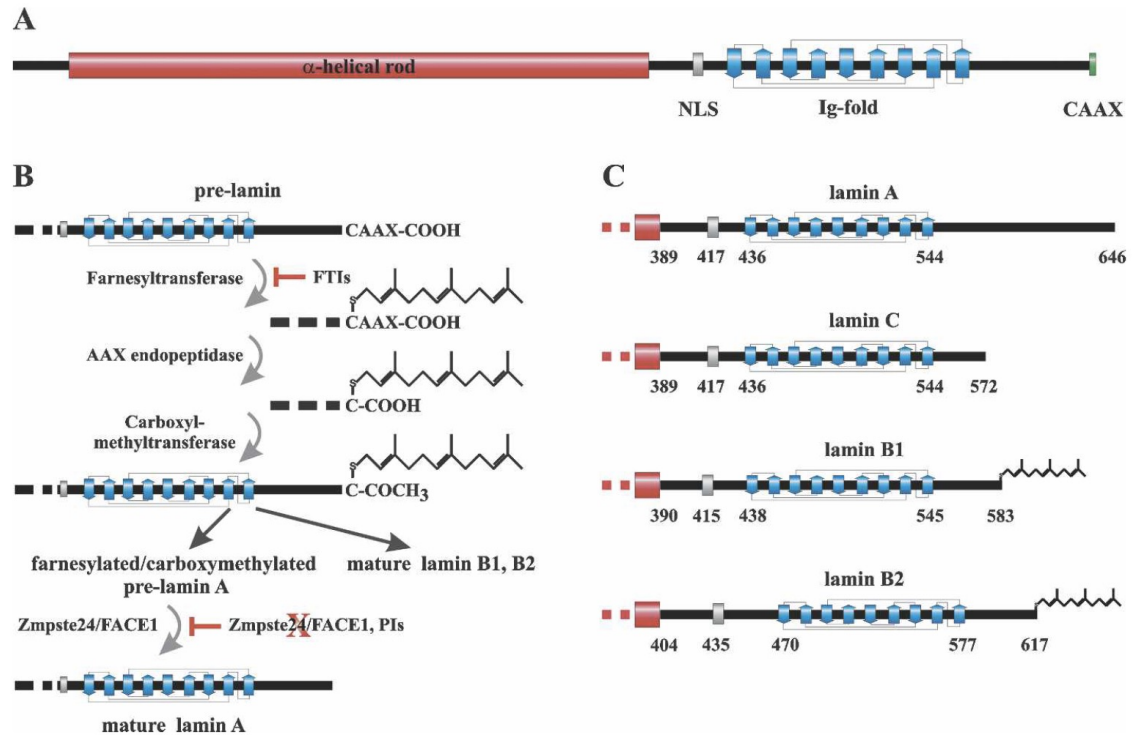


Figure 2 - Lamins structure.

(A) Schematic representation of prelamin polypeptide chain. (B) Post-translational processing of pre-lamin A, B1 and B2. (C) Schematic drawings of the C-terminal tails domains of mature lamins A, C, B1 and B2. Adapted from (Dechat et al., 2008).

As members of nuclear envelope, both A- and B-type lamins are disassembled during mitosis in late prophase. Depolymerization of the lamins is regulated by mitotic kinases, and reassembly during telophase/G1 into newly forming daughter cells requires dephosphorylation by protein phosphatase 1a (PP1) (Moir et al., 2000; Thompson et al., 1997). Due to the nuclear envelope disaggregation during late prophase, A-type lamins become dispersed throughout the cytoplasm in an apparently freely diffusible state whereas the majority of B-type lamins remain associated with the nuclear membrane, which appears to be mainly dispersed into the endoplasmic reticulum. This association of B-type lamins with membrane during mitosis appears to be mainly attributable to their permanently farnesylated state (Dechat et al., 2008; Rusinol and Sinensky, 2006). While lamin B1 accumulates along the entire periphery of the decondensing daughters chromosomes and assembles into a relatively stable polymer by mid- to late- telophase, exclusively at the periphery of reassembling daughter nuclei, A-type lamins accumulate in the nucleoplasm at the end of mitosis and in early G1 (Dechat et al., 2010; Dechat et al., 2008). Nucleoplasmic lamin A/C are present throughout interphase, prevalently in G1 phase (Dechat et al., 2010). Lamin B1 is also present in the nucleoplasm forming stable structure, compared to those of lamin A/C, and intranuclear lamin B foci are associated with sites of mid-late S phase replication (Moir et al. 2000).

Lamins appear to play a role in maintaining the mechanical properties of the entire cell, not only the nuclear structure. Indeed the nuclear lamins are connected to the cytoskeleton through a complex of proteins called the LINC complex (linker of nucleoskeleton and cytoskeleton) (Figure 3). In this complex, the integral proteins of the inner nuclear membrane (INM), Sun1 and Sun2, interact with the outer nuclear membrane (ONM) proteins, nesprin-1, nesprin-2 and nesprin-3, in the luminal space between the inner and outer nuclear membranes. Sun proteins interact on one side with lamins and in the other side

Lamins not only organise nuclear architecture and maintain the entire cell mechanical properties, but they may also regulate transcription by organising chromatin into active and inactive domains. It is known that there is a close association between peripherally located heterochromatin and the nuclear lamina (Fawcett, 1966). This suggests that lamins may be involved in the anchoring or organisation of interphase chromosomes. The lamins interaction with chromatin could be mediated by the rod domain, which requires a higher order lamin structure, and/or by the C-terminal tail domain, which is independent of lamin polymerisation and requires the proteins nuclear localisation signal. The interaction between lamins and chromatin could be mediated by chromosome-associated proteins, or could be direct through the scaffold/matrix attachment regions (S/MAR) (Dechat et al., 2009).

For all of these reasons, research on the INM and lamins is relevant to human health, indeed mutations in genes that encode for lamins and/or for lamins INM interacting proteins cause a wide range of diseases referred to as laminopathies (Mendez-Lopez and Worman, 2012). Fewer diseases are caused by mutations in genes that encode B-type lamins: LMNB1 duplication, which leads to increased lamin B1 expression, is reported to cause adult-onset autosomal-dominant leukodystrophy, a slowly progressive neurological disorder characterised by symmetrical widespread myelin loss in the central nervous system similar to that occurring in chronic progressive multiple sclerosis (Padiath et al., 2006); mutation in LMNB2 gene instead lead to susceptibility to acquire partial lipodystrophy (Hegele et al., 2006).

While mutations in B-type lamins genes caused a restricted diseases number, mutations in LMNA gene origin diverse disease pathologies and phenotypes, here we will discuss the major diseased characterised till now.

The first human disease identified to be caused by LMNA mutation was autosomal dominant Emery-Dreifuss muscular dystrophy (EDMD) (Bonne et al., 1999). Patients with EDMD classically have early contractures of the elbows,

Achilles tendons, and posterior neck, rigidity of the spine, slowly progressive muscle weakness in the upper arms and lower legs, and dilated cardiomyopathy with an early onset atrioventricular conduction block (Emery, 2000; Muchir and Worman, 2007). Mutations in LMNA gene were shown to cause other dominantly inherited diseases affecting primarily striated muscle, including dilated cardiomyopathy 1A and limb girdle muscular dystrophy type 1B (Muchir et al., 2000). Mutations in LMNA gene also cause diseases affecting adipose tissue, leading to a partial lipodystrophy or other disorders with insulin resistance as a common feature. Familial partial lipodystrophy type 2 or Dunningan-type familial partial lipodystrophy is an autosomal dominant disease in which patients born with normal fat distribution but after the onset of puberty there is regional loss of fat from the extremities and an excess of fat accumulation in the neck and face (Worman et al., 2009). An amino acid substitution in LMNA gene is responsible for the mandibuloacral dysplasia, which is a rare autosomal recessive lipodystrophy disorder characterised by marked acral loss of fatty tissue with normal or increased fatty tissue in neck and trunk. Mandibuloacral dysplasia is a multisystemic disorder with numerous abnormalities in addition to partial lipodystrophy. There are also present features involving bone formation such as undersized jaw, underdeveloped clavicles, mandibular hypoplasia, osteolysis of the distal phalanges and clavicles, and pigmentary skin changes; furthermore are present symptoms of premature ageing (Mendez-Lopez and Worman, 2012; Novelli et al., 2002). Among the laminopathies the most investigated and analysed is the Hutchinson-Gilford Progeria Syndrome (HGPS) that is a rare childhood disease with features of accelerated or premature ageing (Hutchinson, 1886). Individuals with this autosomal dominant sporadic syndrome generally die in the second decade of life due to myocardial infarction or stroke. During the first five years of life the affected subjects present signs and symptoms that include sclerotic skin, joint contractures, craniofacial abnormalities (including small face, small jaw and pinched nose), other bone abnormalities, alopecia, and growth

impairment (Merideth et al., 2008). Almost all cases result from a de novo dominant amino acid substitution (cysteine to thymine). This mutation activates a cryptic splice donor site resulting in the synthesis of a protein with 50 amino acids deleted near the carboxyl terminus of prelamin A. Because the deletion removes the site for endoproteolysis catalysed by ZMPSTE24, a truncated prelamin A variant called progerin, which retains its farnesylated cysteine, is expressed (Mendez-Lopez and Worman, 2012). On a cellular level the average telomere length in fibroblasts from HGPS patients is shorter than in age-matched controls, and this shortening is likely a consequence of lamin A mutation. Indeed hematopoietic cells, which do not express LMNA or express it at lower levels, do not present telomere shortening, suggesting that progerin expression leads to short telomeres (Decker et al., 2009).

As previously reported, mutations in genes encoding integral proteins of the INM and proteins associated with this proteins or lamins, have been recently linked to a number of ageing diseases. The first described disease connected to the INM was X-linked EDMD, which has a very similar phenotype to autosomal EDMD caused by LMNA mutation; it is caused by mutations in the EMD gene that codifies for emerin protein. The clinical spectrum of diseases resulting from mutations in EMD is actually wider than the classical EDMD phenotype and includes a limb-girdle muscular dystrophy, cardiomyopathy with minimal muscle or joint involvement, and various intermittent forms (Astejada et al., 2007). Some cases of EDMD and related phenotypes have also been associated with heterozygous missense mutation in the SYNE1 and SYNE2 genes, which respectively encode nesprin-1 and nesprin-2 isoforms (Zhang et al., 2007). Moreover homozygous mutations in SYNE1 have been shown to cause an autosomal recessive cerebellar ataxia, specifically affecting a part of the brain. Not only the lamin A interacting proteins can be involved in disease induction, but there are also evidences of diseases caused by mutation in LBR gene. LBR gene encodes for the lamin B receptor that is an integral protein of the INM that binds to B-type lamins; it has a basically charged nucleoplasmic amino-terminal

domain that binds lamin, DNA and chromatin proteins (Ye and Worman, 1994). Homozygous mutations of the LBR gene leads to hydrops-ectopic calcification-“moth-eaten”- of Greenberg skeletal dysplasia (abnormal chondro-osseous calcification begins in the second or third trimester of gestation, followed by fetal hydrops and death (Waterham et al., 2003). In contrast, heterozygous mutations in LBR give rise to Pelger-Huët anomaly, a condition with hyposegmentation and abnormal chromatin organisation in granulocyte nuclei (Hoffmann et al., 2002).

There are also evidences of laminopathies caused by mutations in proteins involved in prelamin A maturation, such as ZMPSTE24. This protein, as previously described, is a zinc metalloprotease, which removes 15 amino acids from the carboxyl terminus of farnesylated/carboxymethylated prelamin A. Loss-of-function mutations in ZMPSTE24 cause autosomal recessive restrictive dermopathy, a neonatal lethal progeroid disorder (Navarro et al., 2005). Compound heterozygous mutations in ZMPSTE24 also cause progeroid disorders with some cases being diagnosed as mandibuloacral dysplasia (Shackleton et al., 2005).

It appears clear that researches on this group of rare diseases indicate that the INM subcellular structure and the subset of proteins involved in nuclear envelope formations have critical roles in physiological process. Furthermore defects in lamins production have been linked to premature ageing process underlining their probable role on physiological ageing. For these reasons it results interesting to study in depth diseases linked to proteins of the INM, even if they are rare, in order to achieve a better understanding of many aspects of human health.

Study of the determinants of ageing *in vivo*

Mouse models with impairments of the determinants of ageing display common phenotypic traits

For most species, ageing promotes a host of degenerative pathologies that are characterised by debilitating losses of tissue or cellular function. However, especially among vertebrates, ageing also promotes hyperplastic pathologies, the most deadly of which is cancer. Ageing is accompanied by the functional decline of cells, tissues, and organs, as well as a striking increase in a wide range of diseases (Lopez-Otin et al., 2013). Scientists have long tried to identify pathways that are relevant for ageing. In this paragraph it will be discussed the telomere and lamins related ageing, starting from modulation of telomerase activity and arriving to mouse models of lamins dependent progeroid syndromes.

Looking at the telomerase enzyme, in addition to catalytic telomerase subunit TERT and telomerase RNA component TERC, telomere elongation needs other two components (Figure 4). One of this is Dyskerin protein that is a pseudouridine synthase and has a central function in assembly and stability of ribonuclear proteins (RNPs), including telomerase; mutational analysis and knockdown experiments show that dyskerin depletion impairs TERC and telomerase activity (Mitchell et al., 1999). The other telomerase component is TCAB1 protein (Telomerase Cajal Body Protein 1) that is involved in TERC localisation into Cajal bodies and telomere synthesis. It has been suggested that Cajal bodies coordinate the distribution of telomerase to the telomeres and that TCAB1 functions as a molecular link in this process (Venteicher and Artandi, 2009) (Figure 4).

As reported previously, telomerase activity is repressed in most somatic tissues due to the suppression of TERT expression. It results active only in a subset of adult cells, including stem cells, progenitor cells, and germ cells and activated lymphocytes. In contrast most differentiated cells show a complete

suppression of TERT expression and do not exhibit telomerase activity. The inactivation of telomerase in somatic tissue is a tumour-suppressor mechanism inhibiting the immortal growth of transformed cells.

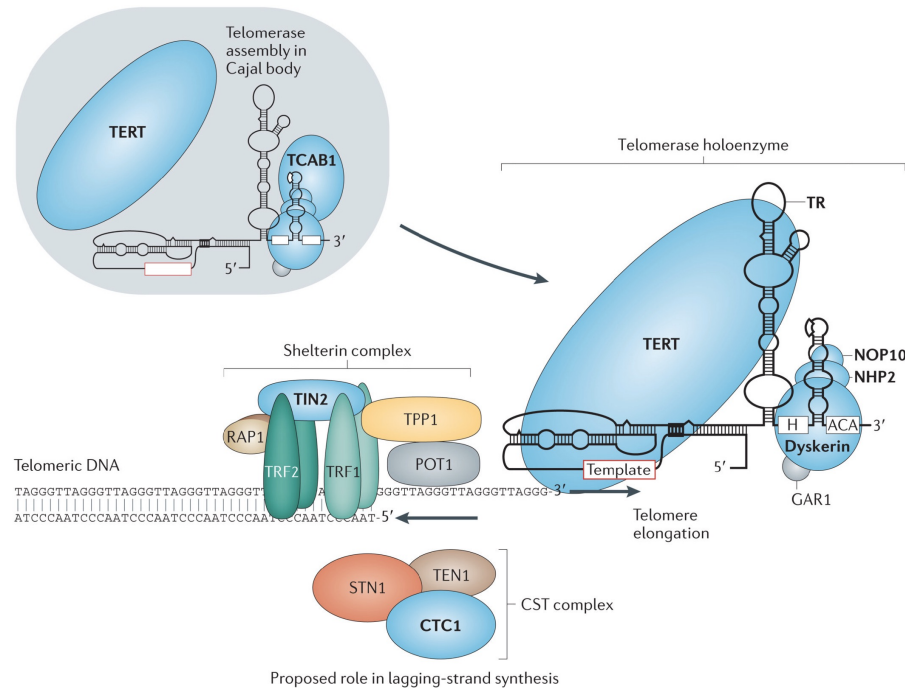


Figure 4 - Telomerase and telomere components.

The telomerase enzyme complex is composed by TERT (catalytic subunit) and TERC (RNA component that contain a template for telomere repeat addition). TERC interacts with dyskerin protein, which is part of a larger dyskerin complex. TCAB1 binds a Cajal body localisation motif in TERC. In the Cajal body, TERC and TERT assemble into a functional holoenzyme complex. Adapted from (Armanios and Blackburn, 2012).

There are several human diseases that have been associated with mutation in dyskerin, TERC, TERT or TCAB1 including dyskeratosis congenita (DC), aplastic anemia, idiopathic pulmonary fibrosis and liver cirrhosis (Tumpel and Rudolph, 2012). DC is an inherited disease associated with nail dystrophy, abnormal skin pigmentation, oral leukoplakia, bone marrow failure and a predisposition to cancer. Patients with DC have very short germ-line telomeres compared with normal individuals due to a defect in telomere maintenance. To date, mutations in six genes encoding for components of telomerase and shelterin have been identified in patients with DC. In addition several chronic diseases have been shown to associate with accelerated telomere shortening in the affected organs, including liver cirrhosis, hematopoietic stem cell-associated disorders, chronic HIV infection, myelodysplastic syndromes, and ulcerative colitis. These studies imply a functional role of telomere shortening in disease progression and tissue ageing. Because stem cells regulate tissue homeostasis, the impairment of stem cell function has been implicated as one of the factors contributing to ageing and disease progression (Tumpel and Rudolph, 2012).

In contrast to humans, telomerase activity is found in most somatic tissues of laboratory mouse strains, and it is not restricted to stem and progenitor cell compartments. Moreover, most laboratory strains have very long telomere reserves (about 50–70 kb) compared to humans (about 10 kb). Therefore, telomerase ko mice with shortened telomeres are used to delineate molecular causes of stem cell dysfunction and the evolution of diseases that are associated with telomere dysfunction; moreover recently wild-derived castaneous mice were introduced that are highly relevant for the study of age-related disease because their telomere length mimics those of humans (Tumpel and Rudolph, 2012).

Among the mouse model systems of ageing, mice in which Terc gene is disrupted exhibit a decline in telomere length that increase along successive

mouse generations and these mice are viable through at least six generations (Blasco et al., 1997). Complete survival curves of increasing generation telomerase-null mice from generation one to two indicate that telomere shortening along successive mouse generations was paralleled by a progressive decrease in both median and maximum longevity (Herrera et al., 1999a). The limited number of generations achieved in mTERC ko mice can be due to infertility and to progressive anticipation of pathologies that are associated with loss of telomeric repeats. These pathologies include loss of fertility, heart failure, immuno-senescence-related diseases, various tissue atrophies and decreased tissue regeneration (of the digestive system, skin and hematopoietic system). Interestingly these pathologies recapitulate disease states of different aetiology that also occur during human ageing, which are characterised by cells with short telomeres, possibly as the consequence of excessive proliferation (Blasco, 2005b). The breeding of late generation mTERC depleted mice with wild type (wt) animals creates healthy heterozygote offspring. This means that re-introduction of telomerase activity in late generation mTERC depleted mice is sufficient to elongate short telomeres, rescue chromosomal instability and prevent to a large extent the severe phenotypes associated with telomere shortening in these mice (Samper et al., 2001). Similar to mTERC deficient mice, in strains with long telomeres (>60 kbp), animals that are heterozygous for mTERT undergo telomere erosion for nine generations and remain phenotypically normal. To examine the phenotypes of mTERT haploinsufficiency and determine whether loss of function of TERT has effects other than those associated with telomere shortening, mTERT deficient mice on the CAST/EiJ genetic background have been characterised (Hemann and Greider, 2000). Phenotypic analysis showed a loss of tissue renewal capacity with progressive breeding of heterozygous mice that was undistinguishable from that of mTERC deficient mice: later generations show degenerative phenotypes in the testes, intestine, and bone marrow (Strong et al. 2011). On the contrary, *in vitro* analysis in embryonic stem cells shows that

overexpression of mTERT leads to enhanced proliferation, resistance to apoptosis and oxidative stress and improved differentiation. However aged mice with constitutive telomerase overexpression are more sensitive to spontaneous and induced tumours, but the mice that remain tumours free exhibit decreased incidence of degenerative diseases and increased life span (Tumpel and Rudolph, 2012). Telomerase seems to be a longevity gene in the context of the organism by preventing premature telomere attrition, but, at the same time, overexpression of TERT is a common feature of human cancers and can increase cancer incidence in transgenic mouse. Experimental analyses of mTERT overexpression in mice engineered to be cancer resistant (Sp53/Sp16/SArf) demonstrate that telomerase activity is sufficient to decrease telomere damage with age, delay ageing and increase median longevity by 40% (Donate and Blasco, 2011). Moreover reactivation of mTERT in adult mice (1 years old) and old mice (2 years old) using adeno associated virus (AAV) expressing mTERT, had remarkable beneficial effects on health and fitness, including insulin sensitivity, osteoporosis, neuromuscular coordination and several molecular biomarkers of ageing; furthermore these mice have an increase in median life span. Importantly these mice do not develop more cancer than their control littermate. This is likely to be related to the fact that AAV vectors are non-integrative, and therefore mTERT over-expression will be lost in highly proliferating cells. In addition, AAV preferentially targets post-mitotic cells from peripheral tissues, which are considered more resistant to cancer than the highly proliferative ones, and could explain the tissue contribution for healthspan amelioration (Bernardes de Jesus et al., 2012).

Not only telomerase activity could influence ageing process, but also mutations in LMNA gene could lead to premature ageing. For this reason and in order to understand how mutations in LMNA result in different diseases, mouse model carrying some of the mutations found in humans with laminopathies have

been created. In this section some of this ageing mouse model will be discussed.

The first lamin A mouse model created was lamin A and C null ($Lmna^{-/-}$) (Figure 5B) that presents deletion of a region extending from *Lmna* exon 8 to the middle of exon 11, eliminating both polyadenylation signal of lamin C and 152 codons from the lamin A coding region (Sullivan et al., 1999). At birth, these mice were indistinguishable from their heterozygous or wt siblings. Mice $Lmna^{-/-}$ show growth reduction starting from 2/3 weeks, and a completely growth arrest at 4 weeks. At 3/4 weeks $Lmna^{-/-}$ begin to display gait abnormalities characterised by stiff walking posture. Their overall posture become progressively more hunched, exhibiting distinct scoliosis/kyphosis. All homozygous mice die by 8 weeks, whereas heterozygous did not exhibit any premature mortality compared to wt siblings (Sullivan et al., 1999). Homozygous mice present thymic atrophy and a reduction in spleen size; there is also absence of white fat and presence of muscular dystrophy affecting perivertebral muscles and muscles surrounding the femur. $Lmna^{-/-}$ mice develop a cardiac and skeletal myopathy bearing a striking resemblance of human EDMD (Sullivan et al., 1999).

The discovery of a link between prelamin A processing and progeroid syndromes has led to investigation of the physiological importance of lamin A biogenesis. For this reason series of mice were generated: lamin A only mice ($Lmna^{LAO/LAO}$) able to directly express only a mature form of lamin A because of elimination of intron 10 (that abolish lamin C production) and sequences between the last codon of mature lamin A and the prelamin A stop codon (Figure 5E); prelamin A only ($Lmna^{PLAO/PLAO}$) that express exclusively prelamin A due to elimination of intron 10 (which abolishes lamin C synthesis) that follow the normal prelamin A processing leading to the production of a mature lamin A (Figure 5D); and mice able to codify only non farnesylated prelamin A ($Lmna^{nPLAO/nPLAO}$), that are identical to $Lmna^{PLAO/PLAO}$ mice except that the cysteine in the CaaX motif of the $Lmna^{nPLAO/nPLAO}$ allele was changed to serine

(Figure 5F) (Coffinier et al., 2010; Davies et al., 2010). Mice that synthesise mature lamin A directly ($Lmna^{LAO/LAO}$) exhibit no disease phenotypes or histopathological abnormalities, even after more than 24 month of observation. The mature lamin A reaches the nuclear envelope normally, despite the absence of prelamin A synthesis and processing. Similar $Lmna^{PLAO/PLAO}$ mice do not exhibit any phenotype and result viable. Both $Lmna^{LAO/LAO}$ and $Lmna^{PLAO/PLAO}$ fibroblasts show a higher frequency of nuclear blebbing than $Lmna^{+/+}$ wt mice fibroblasts, which implies that cellular abnormalities do not alter normal nuclear mechanisms and correlate to the severity of tissue pathologies. In addition in $Lmna^{LAO/LAO}$ mice there was increased amount of mature lamin A in the nucleoplasm compared to $Lmna^{+/+}$ and $Lmna^{PLAO/PLAO}$ mice, implying a potential role of prelamin A processing in nuclear lamin targeting (Coffinier et al., 2010).

The situation is strongly different in $Lmna^{nPLAO/nPLAO}$ mice. These mice have no Progeria phenotypes of any kind; they maintain virtually normal body weights and lack osteolytic lesion. Even if they did not exhibit progeroid-like phenotypes, they were not phenotypically normal. $Lmna^{nPLAO/nPLAO}$ male mice die prematurely, with a median survival of 38.5 weeks; $Lmna^{nPLAO/nPLAO}$ female mice lived slightly longer but still had a markedly reduced median survival of 49.5 weeks (Davies et al., 2010). $Lmna^{nPLAO/nPLAO}$ mice have a dilated cardiomyopathy, not recorded in $Lmna^{PLAO/PLAO}$ mice. Two hypotheses were postulated to explain this situation: one possibility is that non-farnesylated prelamin A is a toxic molecule that leads to cardiac dysfunctions; the second possible explanation is that the non-farnesylated prelamin A is less effective than wt lamin A (Davies et al., 2010). To discriminate between the two hypotheses $Lmna^{nPLAO/-}$ (crossing $Lmna^{nPLAO/nPLAO}$ mice with $Lmna^{+/-}$ mice) were generated. $Lmna^{nPLAO/-}$ show greatly shortened survival (14.6 weeks) suggesting that the cardiomyopathy in $Lmna^{nPLAO/nPLAO}$ mice is caused by functionally hypoactive lamin (Davies et al., 2010).

Due to the absence of phenotype in $Lmna^{LAO/LAO}$ mice and the tragic phenotype obtained in $Lmna^{-/-}$ mice, a transgenic mouse line able to synthesise only lamin C was generated in order to evaluate the consequences of lamin A, but not lamin C, absence. Lamin C only mice ($Lmna^{LCO/LCO}$) were produced deleting $Lmna$ gene last 150 nucleotides of exon 11 and all intron 11; this deletion creates an 11-12 exons fusion that prevents the formation of a prelamin A transcript, obtaining only lamin C production (Figure 5C) (Fong et al., 2006). $Lmna^{LCO/LCO}$ mice were entirely healthy for over 2 years, fertile, with normal growth rates and no evidence of bone or muscular diseases (Figure 5C). The targeting of lamin C at the nuclear envelope was normal in $Lmna^{LCO/LCO}$ fibroblast. Moreover only minor alterations in fibroblast nuclear shape and nuclear deformability were detected (Fong et al., 2006). These analyses revealed that lamin A appears to be dispensable for mice healthy life and for targeting lamin C to the nuclear envelope (Fong et al., 2006).

In order to better understand the HGPS phenotype and the importance of prelamin A that retains its farnesylated cysteine for the pathogenesis of HGPS, knock-in mouse of mutant $Lmna$ allele, $Lmna^{HG/HG}$ that exclusively produce progerin was created. In this allele were deleted intron 10, 11 and the last 150 nucleotides of exon 10 (Figure 5H). Both homozygous and heterozygous $Lmna^{HG}$ mice show phenotype similar to children with HGPS. In particular heterozygous mice show evidence of progeria syndrome starting from 6-8 weeks characterised by slow growth, weight loss, loss of adipose tissue, and osteolytic lesions in ribs and other bones and die by 28 weeks of age. Homozygous mice are severely affected and die by 3 weeks of age. No cardiovascular defects were reported in both heterozygous and homozygous mice (Davies et al., 2011). The evidence that mice carrying a single copy of $Lmna^{HG}$ allele live significantly longer than mice with a double copy of $Lmna^{HG}$ suggests that the production of progerin results toxic (Davies et al., 2011).

The last mouse model related with nuclear lamina defects that will be analysed presents abolition of Zmpste24 function. Two different $Zmpste24^{-/-}$

mouse lines were created, in both models no mature lamin A was produced, leading to accumulation of farnesylated prelamin A (Figure 5G). First model presents replacement of exon 8 with a neomycin resistance cassette, subsequently eliminated in order to not compromise *in vivo* phenotype analysis. For the first 12 months of life, heterozygous mice appear normal; at 15 months mice appear smaller and weaker than wt littermate and lose hair. Homozygous mice were smaller than wt starting from 3 weeks of age, by 6-8 weeks mice develop kyphosis of spine, multiple spontaneous bone fractures, alopecia and appear very malnourished, moreover the phenotype was progressive and mice die by 6-7 months (Bergo et al., 2002; Zhang et al., 2013). In the second model, Zmpste24 sequence was interrupted after codon 39 (Pendas et al. 2002). At birth there were no differences between wt littermate and both heterozygous and homozygous mice. Subsequently, within 4-6 weeks, homozygous mice were smaller compared to wt, at 7 weeks they stop growing, despite normal feeding habits. After 2 months of age Zmpste24^{-/-} mice began to progressively lose weight, showing abnormal posture characterised by a hunched position and scoliosis. Homozygous mice also present dilated cardiomyopathy, muscular dystrophy, lipodystrophy and premature death (Zhang et al., 2013).

The phenotype of both Zmpste24^{-/-} mice confirms the hypothesis that accumulation of farnesylated A-type lamin (prelamin A in Zmpste24^{-/-} mice or progeroid Lmna^{HG/HG} mice) results in toxicity and, at least, partially contributes to HGPS phenotype (Zhang et al., 2013).

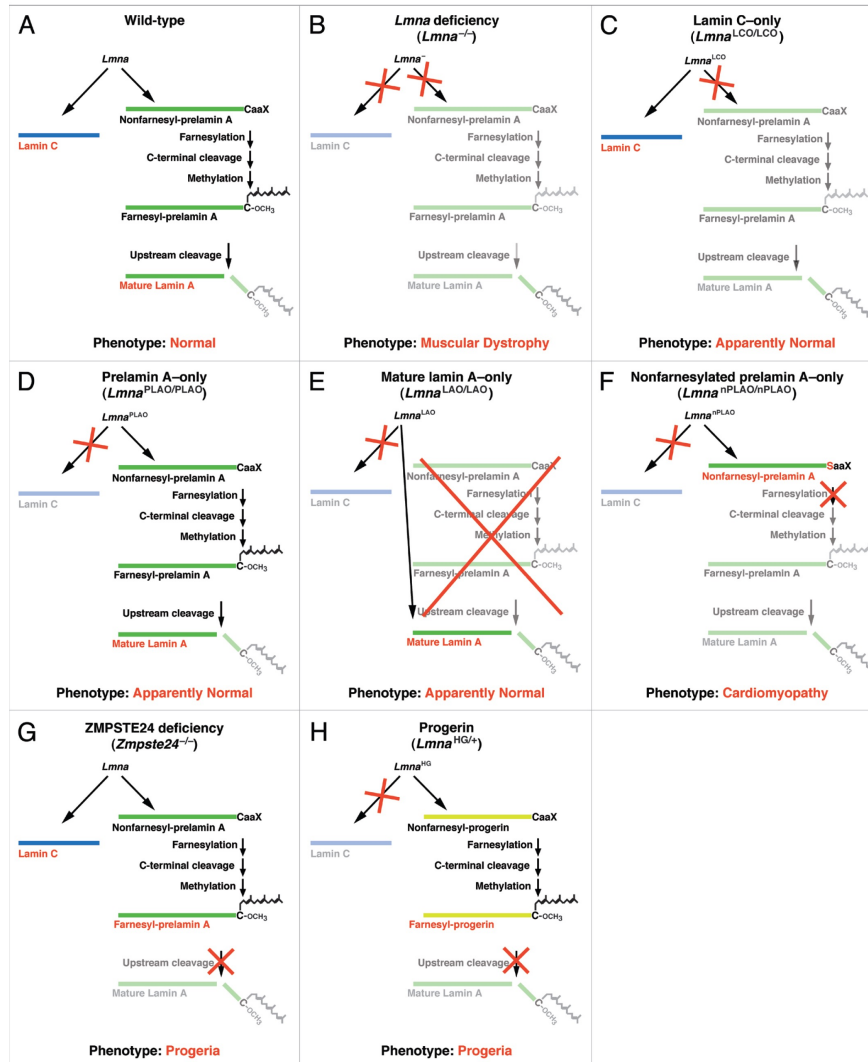


Figure 5 - A-type alterations in mouse model summary.

For each mouse model of laminopathy lamin A/C biogenesis, alteration in protein synthesis due to mutation in *Lmna* generated in mouse transgenic lines and the main phenotype obtained were represented. (A) Wt *Lmna* expression and proteins maturation. (B) *Lmna*^{-/-} mice produce neither lamin A nor lamin C. (C) *Lmna*^{LCO/LCO} mice produce only lamin C. (D) *Lmna*^{PLAO/PLAO} mice do not produce lamin C, but prelamin A that is efficiently converted to mature lamin A. (E) *Lmna*^{LAO/LAO} mice produce lamin A directly. (F) *Lmnan*^{PLAO/nPLAO} mice produce exclusively non farnesylated prelamin A. (G) *Zmpste24*^{-/-} produced both lamin C and prelamin A, but the final endoproteolytic step does not occur, resulting in the accumulation of farnesylated prelamin A. (H) *Lmna*^{HG} allele is able to produce the truncated form of prelamin A called progerin. Mice *Lmna*^{HG/+} result able to produce both lamin A and C plus progerin protein. Adapted from (Davies et al., 2011).

Analysis of genes upregulated in the liver of *Zmpste24*^{-/-} mice revealed that among the genes which expression was most upregulated, there were some that have been characterised as downstream targets of the p53 tumour suppressor or associated with p53 signalling pathway. Indeed co-depletion of *Zmpste24* and p53 partially rescues the premature ageing phenotype. p53 depletion partially rescues body weight defects, survival impairment and at the cellular level p53 abrogation rescues the cell senescence features observed in *Zmpste24*^{-/-} cells. Indeed it has been demonstrated a genetic interaction between *Zmpste24* and p53 (Varela et al., 2005).

Besides mutations in telomerase and in nuclear lamina components there are also mouse models in which mutation in genes involved in DNA metabolism result in premature ageing phenotypes. In this last session it will be discussed the contribution to the premature ageing phenotype given by CST, Brca1 and XPF/ERCC1 proteins.

The first animal model is the ko for the CST complex. CST complex is composed by CTC1-STN1-TEN1 and it is involved in DNA replication, especially at telomeres. It has been shown that the complex binds to the ss G-overhang, modulates telomerase activity and interacts with DNA polymerase α (Pol α) to couple leading- and lagging-strand DNA synthesis (Grossi et al., 2004). It has been shown that this complex is involved in genomic stability maintenance. CTC1 null mouse model has been generated through the LoxP-Cre system, engineering the CTC1 locus by flanking the exon 6 with loxP sites. Deletion of exon 6 leads to the production of a frameshift mutation, resulting in premature termination of the CTC1 open reading frame. Indeed CTC1 truncated protein inhibits the formation of the stable CST complex (Gu et al., 2012). CTC1 ko animals appeared overtly normal at birth, although they became to show growth defects and die almost after 24 days after birth. Indeed MEFs obtained from CTC1 null animals show activation of the DNA damage response, accumulation of ATM/ATR, Chk1, Chk2 proteins, with concomitant block of cell

cycle progression and acquisition of a cellular senescence phenotype. The most affected organs are the highly proliferative ones, in fact cells derived from spleen, bone marrow and liver show proliferation impairments with p21 hyperactivation, which is the major effector of p53-mediated DNA damage response, progressively loss of telomere length and sister telomeric fusions with loss of telomeric signal (Gu et al., 2012). On the other hand it has been demonstrated that CTC1 overexpression in CTC1 null MEFs and in cells derived from highly tissues from CTC1 ko animals is able to restore the loss of the telomere signals restoring the proliferative defects (Gu et al., 2012). It has been postulated that when CST complex is not correctly localised at telomeres, there is the formation of critically long G-overhangs on which TPP1-POT1 complex is no more capable to protect the ssDNA. Indeed the recruitment of the damage sensing protein RPA, recognises the ssDNA as DNA damage site with concomitant activation of the DDR mediated by ATM/ATR (Flynn et al., 2011).

Brca1 is a gene involved in DNA metabolism. It has been shown that it has a role in the repair of damaged DNA, upon the induction of DDR it has been shown that it is hyperphosphorylated and it is relocated to sites of replication forks marked with PCNA (Scully et al., 1997). After its relocation to DNA damaged sites, it interacts with numerous proteins to modulate DNA repair, transcription and more in general the cell cycle (Yoshida and Miki, 2004). At telomeres it has been shown that it regulates their length and stability by acting on the telomerase. It has been shown by Brca1 overexpression that it blocks TERT expression by inhibiting c-Myc binding to TERT DNA sequence (Xiong et al., 2003). Indeed in breast tumour cell lines ko for Brca1 leads to the induction of chromosomal aberrations such as dicentric chromosomes and end to end fusions, which are hallmarks of uncapped telomeres (Cabuy et al., 2008; Rosen, 2013). In mouse complete abrogation of Brca1 is lethal since embryo dies at around E7-8 (Gowen et al., 1996), also loss of function mutations of Brca1 in mouse results in embryonic lethality accompanied by growth

retardation, apoptosis, defective DNA damage repair, centrosome amplification, loss of G2/M cell cycle checkpoint, and genetic instability (Deng and Scott, 2000; Scully and Livingston, 2000). While mice carrying a hypomorphic mutation of Brca1 ($Brca1^{\Delta11/\Delta11}$) with haploid loss of p53 survive to adulthood but they show premature ageing defects. $Brca1^{\Delta11/\Delta11};p53^{+/ko}$ animals shows growth and size defects, thickness of dermis and loss of subcutaneous fat layer and bone defects such as osteoporosis and kyphosis (Figure 6 A-D). Brca1 hypomorphic mice display also defects in fur regeneration as well as defects in wound healing capacity (Cao et al., 2003). Indeed it has been shown that Brca1 and p53 genetically interact since p53 haploinsufficiency is able to rescue the detrimental effects of Brca1 hypomorphic loss, while p53 abrogation does not prevent the premature ageing phenotype. It has been observed indeed that in $Brca1^{\Delta11/\Delta11};p53^{+/ko}$ animals there is the induction of tumourigenesis. This might be due to the fact that p53 rescue is established on the relaxed G1–S checkpoint and attenuated apoptosis that are caused by Brca1 loss of control, therefore enabling mutant cells to propagate without being properly checked (Cao et al., 2003).

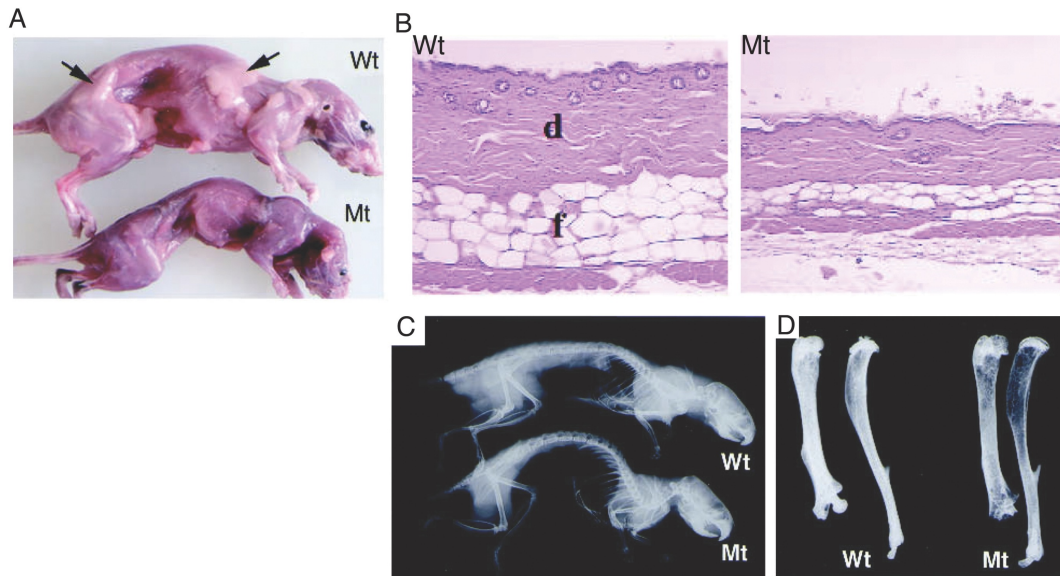


Figure 6 - Representative age-related phenotypic aberration in $Brca1^{\Delta11/\Delta11};p53^{+/ko}$ animals. (A) Arrows indicate the fat deposition that is absent in $Brca1^{\Delta11/\Delta11};p53^{+/ko}$ (Mt) animals; (B) Hematoxylin-eosin-stained skin section from Mt and wt animals. Note the reduction in dermis thickness and in subcutaneous fat layer in Mt animals; (C) X-Ray radiograph of mutant mouse showing kyphosis; (D) Decreased bone density in long bones obtained from Mt animals. Adapted from (Cao et al., 2003).

The last mouse model that it will be described that show traits of premature ageing is the XPF/ERCC1 null mouse. The ERCC1/XPF complex is a specific endonuclease involved in the repair of damaged DNA. ERCC1/XPF has a role in nucleotide excision repair (NER), and is also involved in the repair of DNA interstrand crosslinks (ICLs) and some double-strand breaks (DSBs) (Gregg et al., 2011). It has been also shown that XPF/ERCC1 could be needed for the correct recruitment of the shelterin TRF2 at telomeres (Zhu et al., 2003). XPF/ERCC1 mouse model has been generated by the insertion the human mutation that has been found in Xeroderma Pigmentosum patients (XP23OS), that are affected by sensitivity to light and increased incidence of skin cancer. Indeed the insertion of an A between the nucleotide 1330 and 1331 leads to frameshift mutations and the appearance of a stop codon that leads to the production of a dysfunctional XPF protein and resulting in the dysfunctional complex (Tian et al., 2004). XPF mutant animals were overtly normal at birth while by P15 they show dramatic reduction of body weight and they prematurely die at around three weeks of age, which are hallmarks of premature ageing (Tian et al., 2004). ERCC1^{-/-} in mice results upon the insertion of the neomycin expressing gene into Ercc1 mouse gene that interrupts exon 7, thus aborting the essential carboxy-terminal 74 amino acids of ERCC1 protein. ERCC1 null animals show premature ageing traits such as growth defects, premature death, hearing loss, loss of subcutaneous fat, tremors, ataxia (Gregg et al., 2011).

To summarise the diverse premature ageing mouse models described show mostly overlapping phenotypic traits such as growth defects, premature death, loss of subcutaneous fat, heart impairments. Indeed some of the disorders described including Brca1, TRF1, Zmpste24 null animals are linked to p53 since its abrogation is able to rescue the observed premature ageing traits. For some of them p53 rescue in mutant cells enable them to propagate without being properly checked leading to higher tumour induction.

AKTIP/Ft1 is a novel telomeric accessory protein required for proper telomere maintenance

AKTIP gene is located on long arm of chromosome 16 (16q12.2), and encodes for a 33 kDa protein. It has been firstly identified as an interactor of Ser/Thr kinase B (PKB)/AKT1 by a cDNA screening using a GFP-AKT as bait (Remy and Michnick, 2004), from which its name has been derived. 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 and Michnick, 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 et al., 2008). In this study AKTIP is proposed as a member of a complex that could be involved in vesicle trafficking and/or fusion via the HOPS complex (Xu et al., 2008).

Later 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 et al., 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 et al., 2011). More recently AKTIP was identified as a tumour suppressor candidate gene within a work of integrated system-biology approach to identify novel cancer genes contributing to endometrial tumorigenesis (Liang et al., 2012).

Murine homologue of AKTIP is Ft1. Ft1 gene is located on chromosome 8, D region and it was identified in 1997 as one of 6 genes deleted in a mouse strain

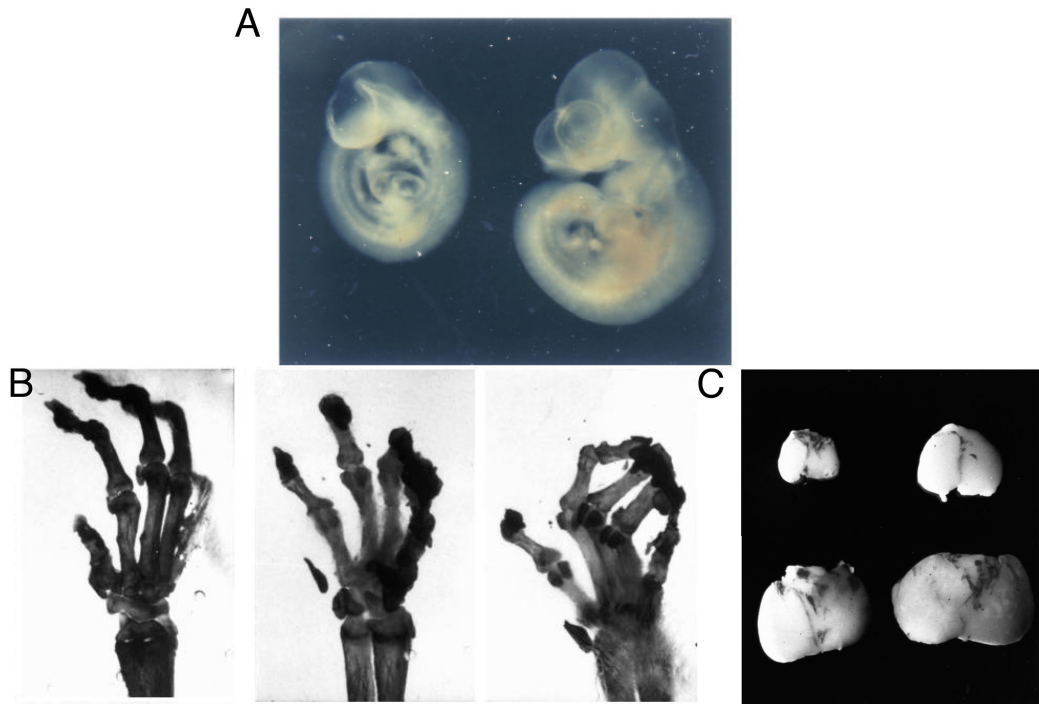


Figure 7 - Phenotypic aberrations of Ft mutant strain.

(A): Ft/Ft (left) 9.5 day old embryo is represented in comparison with stage matched wt embryo (right.) (B): skeletal stain of a wt right fore limb (left) is compared to right fore limbs of Ft/+ mice (right.) (C): Thymus from 6 weeks old wt 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 et al., 1994).

obtained by insertional mutagenesis, Fused Toes (Ft) (Lesche et al., 1997). Ft/Ft homozygous embryos die at 9-10 day of embryonic development and display several abnormalities in the head development characterised by the absence of telencephalon and mesencephalon; moreover, they show a phenotype described as situs inversum viscerum characterised by the absence of right-left symmetry. Ft/+ heterozygous animals are characterised by thymic hyperplasia and fusions of fore limb digits (Figure 7).

These phenotypic features are linked to alteration of programmed cell death during morphogenesis (van der Hoeven et al., 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 et al. 2002). It is 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 spatiotemporal 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 et al., 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 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 analysed. However, the expression level and the ratio of the Ft1 transcripts seems 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 et al., 1997). It has not been defined which is Ft1 molecular function and which is its contribute to Ft phenotype.

It has been recently reported that AKTIP is a novel telomeric accessory protein (Burla et al., 2015b). It has been demonstrated that AKTIP/Ft1 downregulation in p53^{ko/ko} MEFs leads to the increase of telomeric aberration such as multiple telomeric signals (MTS) and sister telomeric associations (STA), which are hallmarks of replication defects at telomere defined as fragile telomere phenotype. The same telomeric aberrations have been observed upon the impairment in other protein that have roles at telomeres including TRF1 (Martinez et al., 2009; Sfeir et al., 2009) the BLM and RTEL1 helicases (Sfeir et al., 2009; Vannier et al., 2012) the Apollo nuclease (van Overbeek and de Lange, 2006; Wu et al., 2010) and the components of the mammalian CTC1-STN1-TEN1 (CST) complex (Gu et al., 2012; Kasbek et al., 2013; Stewart et al., 2012). Interestingly it has been found that AKTIP/Ft1 and TRF1 genetically interacts since there is no an increase in the percentage of fragile telomeric aberrations in cells co-depleted for the two proteins respect of the single depleted ones, implying that the two proteins act in the same pathway. The role of AKTIP at telomeres has been further supported by the its biochemical interaction with the two shelterin protein TRF1 and TRF2 (Burla et al., 2015b).

AKTIP is involved also in proper DNA metabolism maintenance. In HPFs AKTIP depletion leads to cell cycle block in S-phase. In synchronised depleted AKTIP HeLa cells, pulse labeled with BrdU the amount of telomeric DNA immunoprecipitated with TRF1 is lower respect of that obtained from wt cells. Furthermore the amounts of chromatin bound PCNA in HPFs is strongly reduced upon AKTIP downregulation (Burla et al., 2015b).

Moreover it has been shown that AKTIP has a peculiar cellular localisation at the nuclear rim (Burla et al., 2015b).

More recently it has been demonstrated that AKTIP co-localises at the nuclear rim with A-type lamins and lamin B1 in interphase nuclei (Figure 8 A). Moreover AKTIP depleted cells recapitulate progeroid traits such as loss of correct chromatin distribution and increase of distorted nuclei (Burla, 2015; Scaffidi and Misteli, 2006). Indeed in early and mid-late passage HPFs, AKTIP

depleted cells displayed a higher frequency of nuclei with a diffused distribution of trimethylation of K9 in histone 3 (trimet H3K9) compared with passage-matched control cells (Figure 8 B). In late passage HPFs depleted for AKTIP, the frequency of morphologically altered nuclei was significantly higher than in passage-matched controls (Figure 8 C).

It has been demonstrated that AKTIP signal at the nuclear rim is reduced and mislocalised in HPFs expressing mutant versions of lamin A which have been linked to human progeroid disorders (Figure 8 D). Altogether these data suggest a functional link between lamin A and AKTIP. This hypothesis is further supported by the fact that AKTIP binding to telomeric DNA is impaired upon the expression of mutant lamin A (progerin) in synchronised HeLa cells (Figure 8 E) (Burla et al., 2015a).

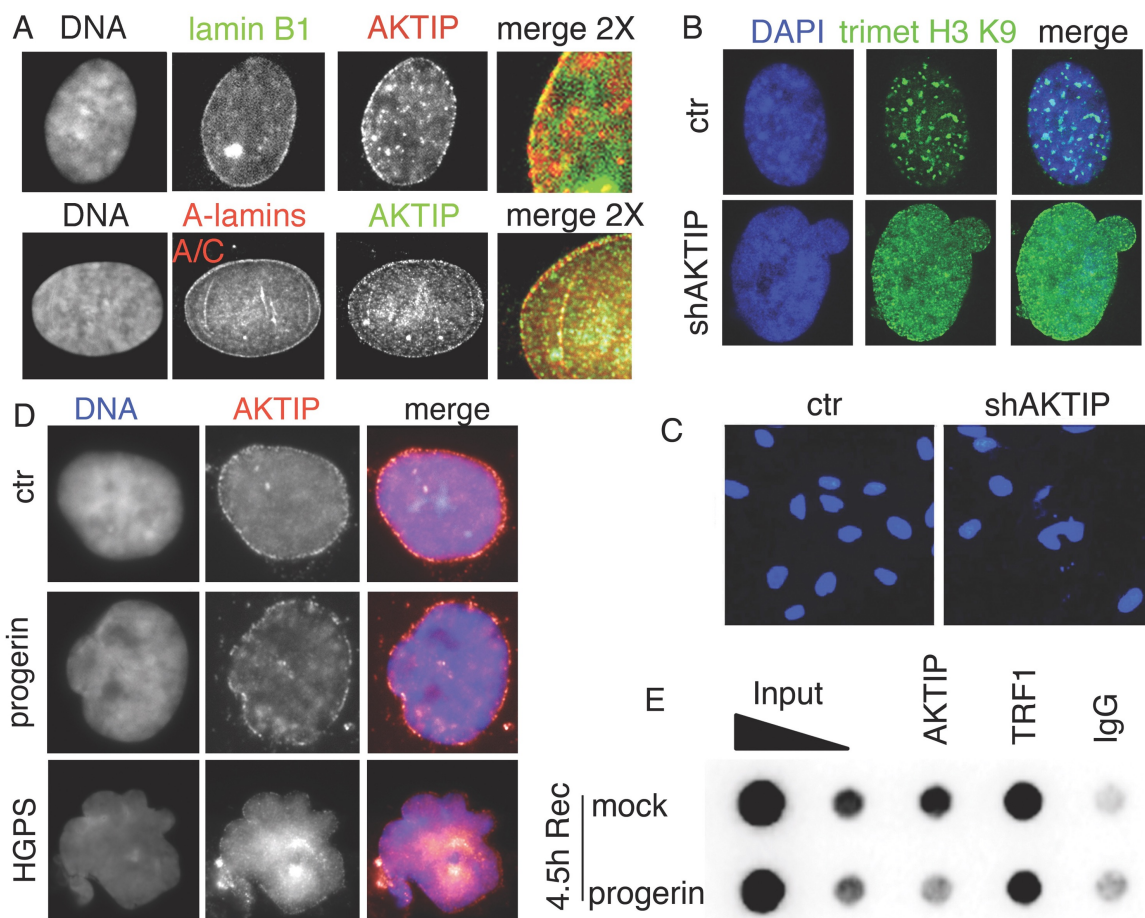


Figure 8 - AKTIP co-localises with the nuclear lamina and it functionally interacts with lamin A.

(A) Detergent treated HPFs immunostained with anti-AKTIP antibody and anti-lamin B1 (up) and anti-lamin A/C. DNA stained with DAPI. Merging of the AKTIP and lamins co-localisation spots at the nuclear periphery. (B) Controls or AKTIP depleted (shAKTIP) p6 HPFs stained with anti-trimeth H3K9 antibody. DNA stained with DAPI. In control cells, trimeth H3K9 formed clustered signals, while in AKTIP depleted cells nuclei displayed diffused distribution. (C) HPFs at different passages (p6 and 22) AKTIP depleted (shAKTIP) or control cells stained with DAPI, showing nuclear abnormalities in AKTIP depleted cells. (D) HPFs derived from HGPS (1824C>T LMNA) or infected with LV-progerin (progerin), or mock treated HPFs were immunostained with anti-AKTIP antibody. DNA was stained with DAPI. HGPS, and LV-progerin cells display an overall AKTIP signal that is weaker and more discontinuous at the nuclear rim than in controls. (E) Mock and LV-progerin HeLa cells synchronised with double thymidine block. AKTIP and TRF1 localisation at telomeres assessed by ChIP at 4.5h post recovery when cells are in S phase. Progerin expression significantly reduced the accumulation of AKTIP at telomeres. Adapted from (Burla et al., 2015a).

Collectively these data indicate that the telomeric accessory protein AKTIP/ Ft1 is important for proper telomere maintenance, that have a role in DNA replication. That its depletion results in cellular phenotypic traits that are linked to premature ageing disorders and that it is functionally linked with lamin A (Figure 9).



Figure 9 - Schematic representation of the mechanisms in which AKTIP is involved.

AKTIP is involved in telomere metabolism, in DNA metabolism and in processes of nuclear envelope maintenance. These mechanisms are involved in normal and pathological premature ageing. Adapted from (Lopez-Otin et al., 2013).

AIM OF THE PROJECT

A new accessory human telomeric protein named AKTIP (Ft1 in mouse) has been recently identified (Burla et al., 2015). This protein intercepts mechanisms involved in cellular homeostasis, such as DNA and telomere metabolism and nuclear envelope maintenance (Burla et al., 2015). Impairments in these mechanisms lead to premature ageing phenotypes both *in vitro* and *in vivo* (Lopez-Otin et al., 2013).

The aim of this project was that to investigate on the role of AKTIP in these mechanisms both *in vitro* and *in vivo*.

The project design included *in vitro* GST pull down experiments on TRFs, RPA70, PCNA and components of the nuclear lamina to analyse the biochemical properties of AKTIP in order to define its molecular partners and to investigate on its role in the biological pathways that it intercepts.

Along with this, Ft1 hypomorphic mouse were produced to understand whether AKTIP/Ft1 could have a role at the organismal level. Given that upon AKTIP/Ft1 loss at the cellular level premature ageing traits were detected, the focus of the analysis of Ft1 hypomorphic mice was on those organs and tissues that have been found to be affected in mouse models of premature ageing, including bone, heart and fat (Cao et al., 2003; Martinez et al., 2009; Tejera et al., 2010; Varela et al., 2005).

Finally, in order to assess whether AKTIP/Ft1 interacted *in vivo* with p53 related pathways we crossed our Ft1 hypomorphic mice with p53 ko animals to analyse the phenotypic effect of the double depletion.

Altogether this work is expected to give further insights on the linkage among AKTIP, lamins, DNA metabolism and the premature ageing phenotype, contributing to the knowledge in this field at the biochemical and organismal level.

RESULTS

Study of AKTIP interactors

AKTIP directly interacts with shelterin TRF1, TRF2 and TRFH2 domain

The novel telomeric accessory protein AKTIP interacts with telomeres and it is required for efficient telomere replication (Burla et al., 2015b). Previous GST pull down experiments with total 293T cell extracts have shown that AKTIP interacts with the two shelterin proteins TRF1 and TRF2 (Burla et al., 2015b). In order to further investigate on the role of the human AKTIP protein in telomere metabolism, we were interested in understand the nature of this interactions. To this purpose we expressed and purified the human AKTIP with GST fusion tag protein and the two human shelterin TRF1 and TRF2 expressed in bacteria cells. GST pull down experiments performed in this work with the purified proteins demonstrate that AKTIP directly interacts with the two shelterins. This interaction is specific since no TRFs pull down is observed with a protein containing only GST fusion tag (empty-GST) (Figure 10). Here we asked whether AKTIP direct binding to TRFs could be mediated by TRFH domains (TRFH1 and TRFH2), that have been shown to interact with different proteins such as RTEL1, TIN2, hRap1 and Apollo (Chen et al., 2008; Kim et al., 1999; Li et al., 2000; Vannier et al., 2012). We performed GST pull down experiments with bacterially purified AKTIP-GST and TRFH1 and TRFH2. We found that AKTIP is able to specifically pull down TRFH2 since no binding is observed with empty-GST alone indicating that AKTIP directly binds TRFH2. No specific binding is observed with TRFH1 domain since almost the same amount of protein level is detected in AKTIP-GST and empty-GST lanes in comassie stained SDS gel (Figure 11).

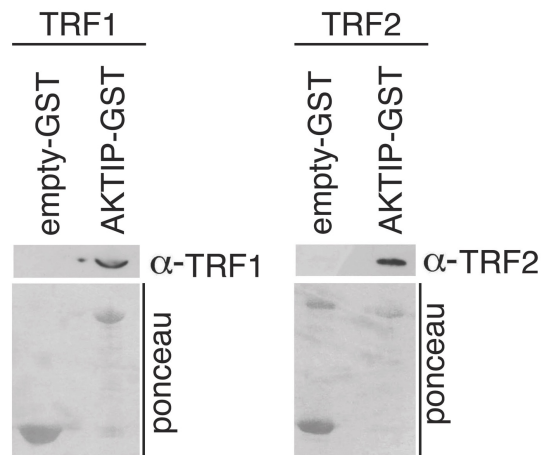


Figure 10 - AKTIP directly interacts with shelterin TRF1, TRF2

GST pull down assay with bacterially purified AKTIP-GST and the shelterin TRF1 and TRF2 showing that AKTIP directly interacts with the two proteins. Empty-GST bindings with the TRFs has been used as a negative controls.

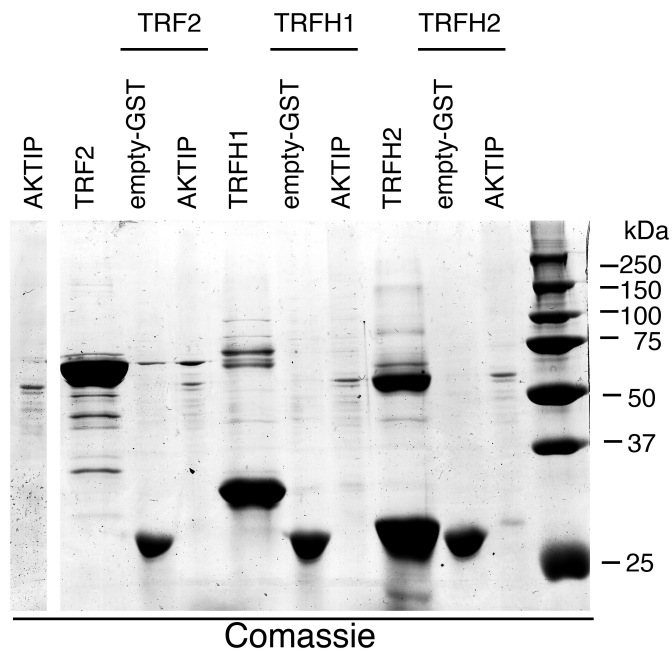


Figure 11 - AKTIP directly interacts with TRF2 homodimerization domain.

GST pull down assay with bacterially purified AKTIP-GST and the TRF1 and TRF2 homodimerization domains. AKTIP-GST is able to pull down TRFH2 but not TRFH1. Empty-GST pull downs were used as a negative control, while TRF2 with AKTIP-GST was used as a positive binding control.

AKTIP binds components of DNA replication machinery

AKTIP depletion impairs PCNA binding to the chromatin and causes telomere replication impairment (Burla et al., 2015b). These data suggest that AKTIP has a role in DNA metabolism in particular in DNA replication. We then asked whether AKTIP physically interacts with PCNA (proliferating cell nuclear antigen) and RPA70 (Replication Protein A), which are components of the DNA replication machinery.

PCNA is an evolutionarily well-conserved protein found in all Eukaryotic species as well as in Archaea that belongs to the DNA sliding clamp family (Strzalka and Ziemienowicz, 2011). PCNA acts as a processivity factor of DNA polymerase δ , which is required for DNA synthesis during replication (Prelich et al., 1987), moreover it takes function in chromatin remodelling, DNA repair, sister-chromatid cohesion and cell cycle control (Maga and Hubscher, 2003).

RPA is a heterotrimeric single-stranded DNA-binding protein that is highly conserved in Eukaryotes (Wold, 1997). Human RPA is a stable complex of three subunits of 70, 32 and 14 KDa. The three subunits form a stable complex when ss-DNA is present (Wold, 1997). As a complex, RPA binds ss-DNA and it results essential in DNA replication, recombination, and repair including nucleotide excision, base excision, mismatch and double-strand repairs (Fan and Pavletich, 2012; Zou et al., 2006).

GST pull down experiments using AKTIP-GST fusion protein and total protein extracts from 293T cells, revealed that AKTIP GST specifically precipitates both PCNA and RPA70 (Figure 12 A). These data indicate that AKTIP is able to bind PCNA and RPA70.

To further investigate on the nature of AKTIP and PCNA bindings, we purified AKTIP fused with GST tag and PCNA proteins from bacteria cells. We found that AKTIP specifically pulls down purified PCNA (Figure 12 B). This finding indicates that the binding of AKTIP and PCNA is not mediated by other components but it is a direct binding. Pull down of purified AKTIP-GST and

TRF2 was used as a positive control and pull down of purified TRF2 and PCNA with empty-GST were used as negative controls.

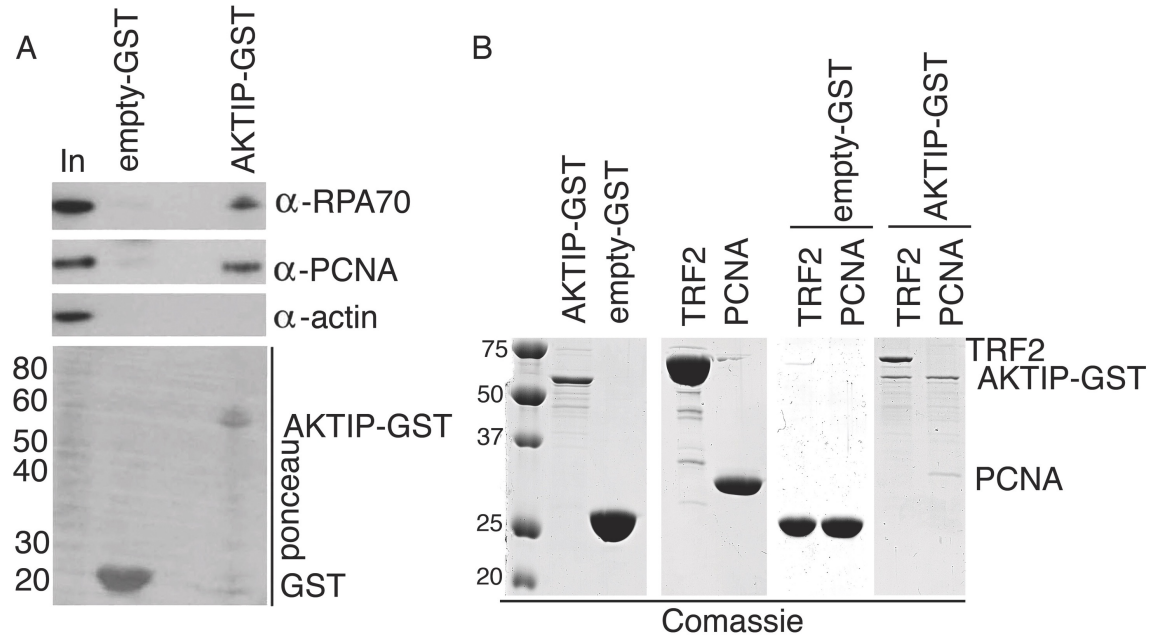


Figure 12 - AKTIP binds PCNA and RPA70 and it directly interacts with PCNA.

(A) AKTIP-GST pulls down PCNA and RPA70 from 293T cells extracts. In, input. (B) GST pull down assay with bacterially purified AKTIP-GST, TRF2, PCNA. AKTIP-GST directly binds PCNA (TRF2 has been used as a positive control, empty-GST as negative controls, In as for inputs).

AKTIP interacts with lamins and lamins related proteins

AKTIP mainly localises at the nuclear rim in interphase nuclei (Burla et al., 2015b). This peculiar localisation recalls the one of the lamins proteins, lamin A/C and B1. Indeed AKTIP co-localises with A-type lamins and lamin B1 and its nuclear signal and localisation are respectively reduced and disrupted upon the presence of aberrant lamin A protein as described into the introduction (Burla et al., 2015a). These data suggest a genetic interaction between AKTIP and A-type lamins. We thus asked whether AKTIP physically interacts with lamins.

In order to answer this question, we performed GST pull down assays using GST-AKTIP fusion protein. Using this approach we demonstrated that GST-AKTIP is able to pull down both lamin A/C and lamin B1 from 293T cell extracts (Figure 13 A), indicating that AKTIP interacts with lamin A, lamin C and lamin B1.

Matrin 3 is a highly conserved nuclear matrix protein and is able to interact with lamins (Maraldi et al., 2011). Matrin 3 is a nucleoskeleton constituent and it has been involved in chromosome anchorage and gene expression regulation (Zeitz et al., 2009). More recently it has been connected to mRNA stabilisation function; in fact this protein presents both DNA and RNA binding domains (Salton et al., 2011).

We performed GST pull down experiments using a GST-AKTIP fusion protein and 293T total cell extracts. This assays showed that AKTIP is able to precipitate matrin 3 (Figure 13 B), demonstrating that AKTIP is able to interact also with matrin 3.

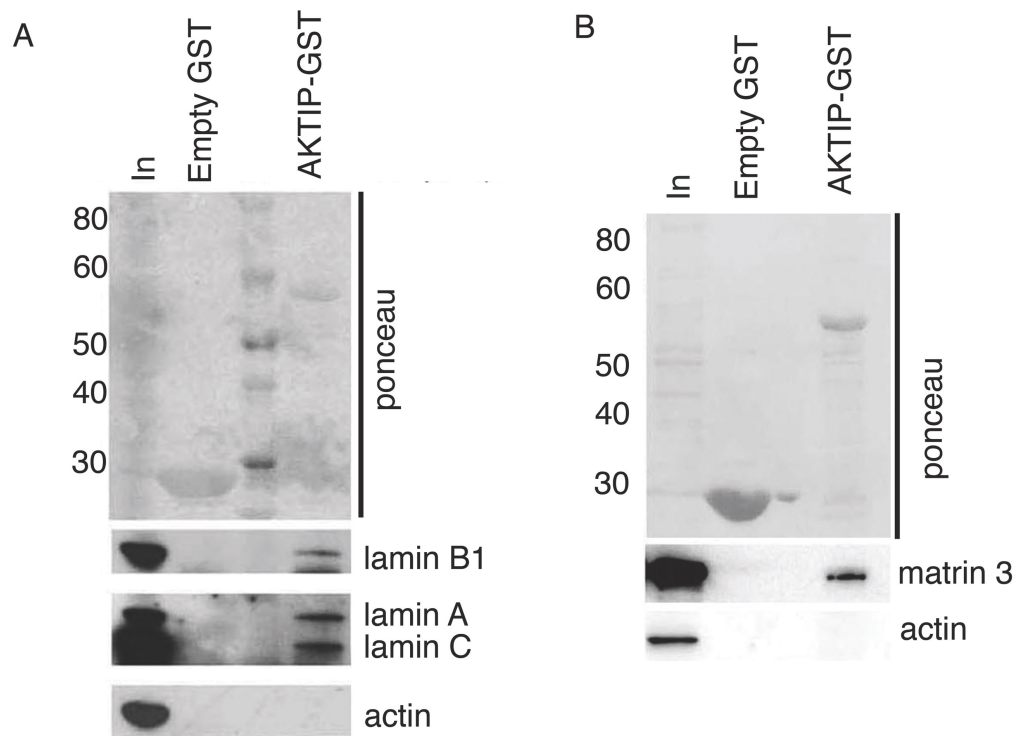


Figure 13 - AKTIP interacts with lamin B1, lamin A/C and matrin 3.

(A) AKTIP-GST pulls down lamin B1, lamin A and lamin C from 293T cells extracts. (B) GST pull down assay with bacterially purified AKTIP-GST protein and 293T cells extracts. AKTIP-GST binds matrin 3. Actin was used as a control for the goodness of the cellular extracts and for specificity, empty-GST was used as negative control. In, input.

Production and characterisation of Ft1 knockoutfirst mouse model

AKTIP/Ft1 has a crucial role in proper telomere maintenance, DNA metabolism (Burla et al., 2015b) and nuclear envelope maintenance, as described into the introduction (Burla et al., 2015a). It is able to interact with most of the determinants of the mechanisms that play essential roles in physiological functions and prevent ageing syndromes. Indeed in this work we showed that AKTIP binds TRFs proteins, PCNA, RPA70 and nuclear lamina components. So AKTIP/Ft1 seems to be important in mechanisms that are fundamental for preventing age related disorders.

Here we were interested in assess whether AKTIP/Ft1 depletion could result in a specific phenotype *in vivo* that would be related to one or more of the mechanisms that it intercepts at the cellular level.

Kof allele leads to Ft1 mRNA reduction

To generate Ft1 ko animals we choose the knockoutfirst (kof) strategy which is based on the insertion into mouse ES cells of the β geok cassette (L1L2_Bact_P Cassette) referred as kof allele (Figure 14 A) (Testa et al., 2004) (Figure 14 A). The presence of the splicing accepting site, from *engrailed-2* (En2 SA) (Gossler et al., 1989) and the polyadenylations sites (pA) trap and truncate Ft1 nascent transcript, that it is expected to lead to the production of a dysfunctional and truncated Ft1 protein. Indeed the activity of this cassette would lead to Ft1 expression reduction instead of a full Ft1 ko. Chimeric mice were generated by the injection of targeted Ft1 kof (IKMC project 46319) ES cells (HEPD0589_6_H06) into C57Bl host blastocysts subsequently injected into a pseudopregnant foster C57Bl female mice. From blastocysts injections eight male and four female chimeric mice were obtained and backcrossed with C57Bl wt animals in order to obtain kof allele germ line transmission. Two male chimeric mice were able to give germ line transmission. Ft1^{+/*kof*} (heterozygous)

animals were then crossed with C57Bl mice or intercrossed in order to respectively stabilise the line or to obtain Ft1^{kof/kof} (homozygous) animals (Figure 14 B). Mice were screened by PCR using primers specific for exon 4 (F) and exon 6 (R), in order to discriminate from Ft1^{+/+} (wt), Ft1^{+/kof} and Ft1^{kof/kof} animals (Figure 14 C).

Given that the kof cassette is predicted to lead to Ft1 expression reduction instead of a full ko we wanted to confirm Ft1 mRNA reduction. To this purpose Ft1^{+/kof} and Ft1^{kof/kof} animals, were tested by Q-PCR in order to evaluate the mRNA reduction. Q-PCRs were performed using two primers pair, one pair specific for Ft1 exon 3 mRNA, and the other specific for Ft1 exon 6 mRNA. The En2 splicing acceptor site is placed after exon 3, so the transgenic cassette is able to form mRNA molecules that include only the first three exons. Primer pair on exon 3 is expected to amplify both wt and transgenic allele; on the contrary primer pair on exon 6 is expected to amplify only wt allele (Figure 14 A - blu arrows). Q-PCR analyses on mice tail biopsies show that Ft1 expression is significantly reduced by 80% (Student t-test, ***p<0.0001) in Ft1^{kof/kof} animals and by 40% in Ft1^{+/kof} mice when compared to Ft1 expression in wt animals (Student t-test, ***p<0.001) (Figure 14 D), demonstrating that kof cassette is able to reduce Ft1 expression.

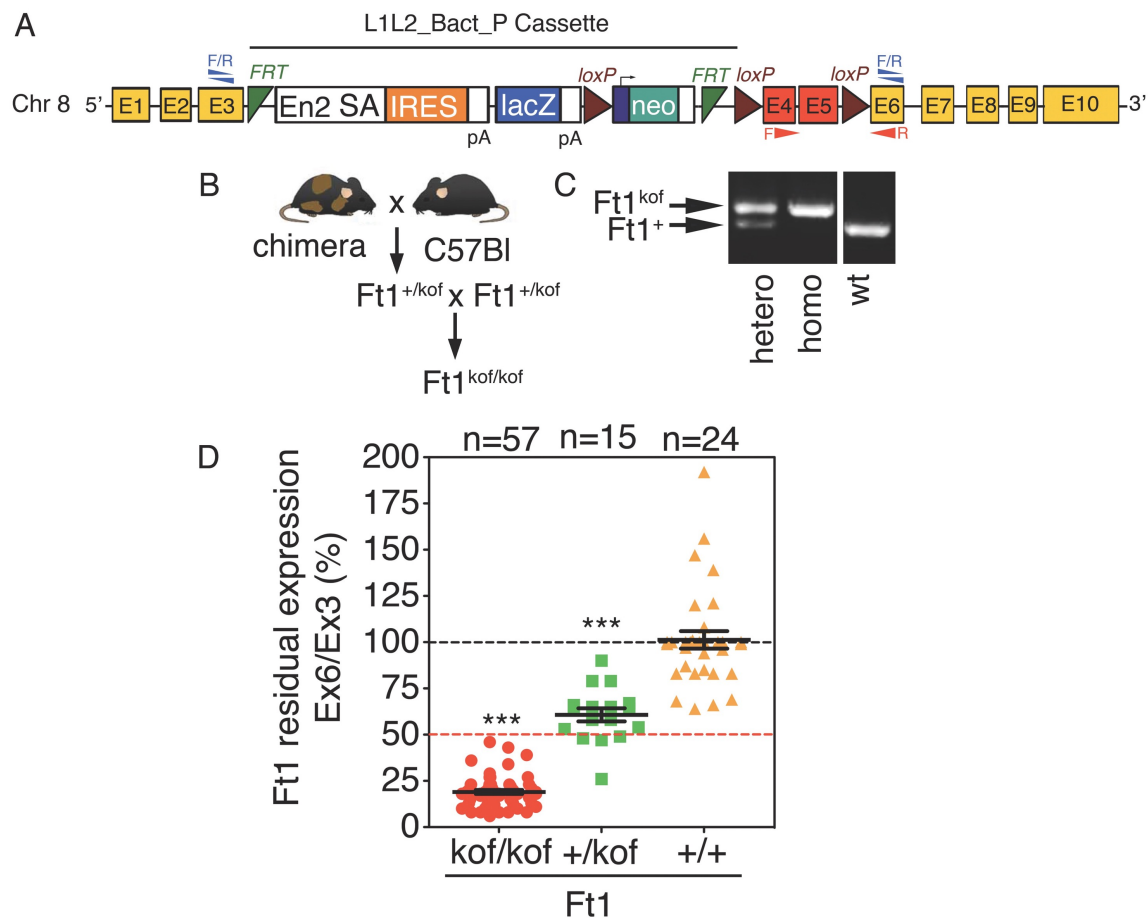


Figure 14 - Kof cassette leads to Ft1 mRNA reduction.

(A) Schematic representation of knock out first cassette used to generate Ft1 knockdown mouse model. Splice acceptor site (En2 SA, splice acceptor element from engrailed-2) and polyadenylation elements (SV40 Poly-Adenylation signal - pA) trap and truncate the nascent Ft1 transcript. Red and blue arrows indicate primers respectively used for genotyping and Q-PCR analysis. E, Ft1 exons; FRT, target site for FLP recombinase; loxP, target site for Cre recombinase; IRES, internal ribosomal entry site from the encephalomyocarditis virus; lacZ, gene encoding β -galactosidase; neo, neomycin phosphotransferase, selectable marker. Adapted from (Testa et al., 2004) (B) Schematic representation of the strategy used to obtain Ft1^{kof/kof} animals. Chimeric mice obtained after kof targeted ES cells blastocyst injection were backcrossed with C57Bl animals in order to obtain germ line transmission of kof allele. Ft1^{+/kof} mice were intercrossed to obtain Ft1^{kof/kof} animals. (C) Examples of PCR amplification of Ft1 targeted allele on genomic DNA (gDNA) extracted from Ft1^{+/+}, Ft1^{+/kof} (hetero) and Ft1^{kof/kof} (homo) animals to discriminate the three genotypes. Amplicon derived from wt allele has a length of 692 bp and amplicon derived from transgenic allele has a length of 755 bp. (D) Q-PCR analysis on total RNA extracted from mice tail biopsies. Results are presented as the ratio between the exon 6 and exon 3 normalised to GAPDH. Ft1 expression is significantly reduced up to 80% in Ft1^{kof/kof} mice (n=57) and up to 40% Ft1^{+/kof} animals (n=15) compared to Ft1^{+/+} controls (n=24) (Student t-test analysis, ***p<0.001). n, number of analysed mice for each genotype.

Ft1^{kof/kof} animals are obtained at sub-mendelian ratio and display an aberrant phenotype

It has been described that among the different mouse line ko for individual shelterin component that have been created, only mouse models ko for Rap1 and Pot1b result viable (Takai et al., 2011), instead the others result in embryonic lethality in diverse stages. Given that AKTIP is important for proper telomere maintenance (Burla et al., 2015b) we followed kof allele segregation in Ft1^{+/kof} matings, since an unbalanced mendelian ratio could reflect embryonal lethality. We analysed genotype distribution of the offsprings obtained in Ft1^{+/kof} animals matings from the first generation (F1) to the fifth (F5) (Figure 15 A). We obtained 28%, 52%, 20% of respectively wt, Ft1^{+/kof} and Ft1^{kof/kof} animals over the total 573 animals analysed. Since the expected mendelian ratio is 1:2:1 we performed χ^2 test analysis in order to understand if expected and observed genotype distributions were significantly different. Indeed we obtained that the observed Ft1^{kof/kof} animals were significantly obtained at sub-mendelian ratio than expected (χ^2 test analysis, **p=0.006). This result could reflect embryonic lethality of Ft1^{kof/kof} animals (Figure 15 A).

We next macroscopically observed Ft1^{kof/kof} animals starting from the first generation (F1) to the fifth (F5) obtained from Ft1^{+/kof} intercross in order to understand whether Ft1 downregulation could lead to an aberrant phenotype. Indeed we observed that 100% of Ft1^{kof/kof} animals display an aberrant phenotype. 20.5% of Ft1^{kof/kof} animals shows a strong phenotype while the leftover 79.5% is affected by a milder one (Figure 15 B).

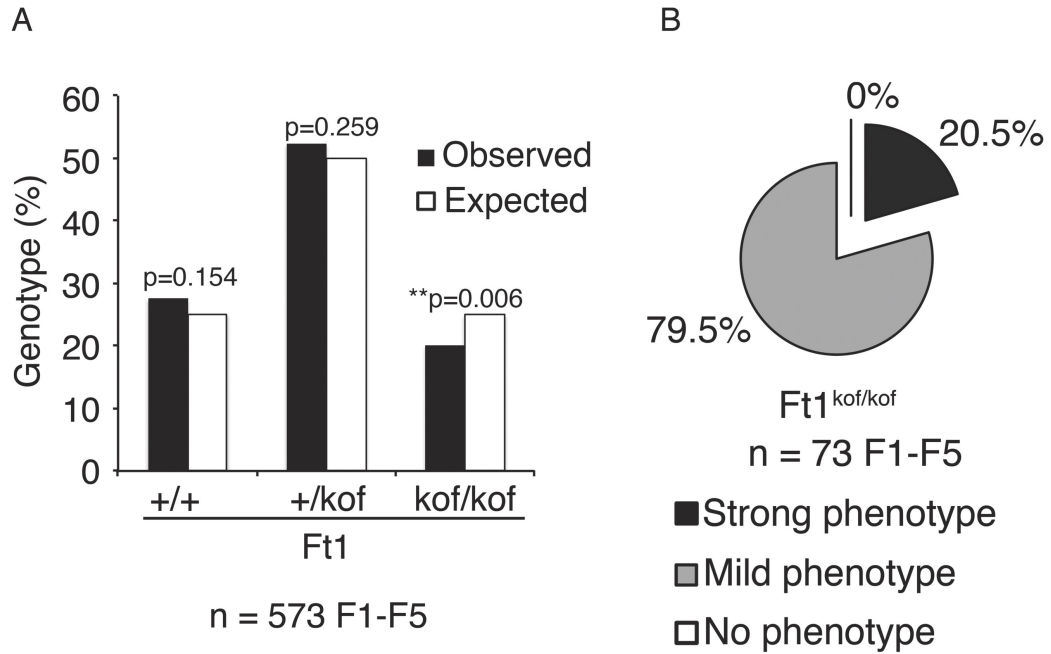


Figure 15 - $Ft1^{ko/ko}$ animals are obtained at submendelian ratio. 100% of $Ft1^{ko/ko}$ animals display an aberrant phenotype.

(A) Genotype distribution in $Ft1$ ko mouse colony. The difference between the observed and the expected $Ft1^{ko/ko}$ animals is significant in the χ^2 test (**p=0.006). $Ft1^{ko/ko}$ mice are obtained with submendelian ratio possibly reflecting embryonic lethality. (B) Phenotype distribution in $Ft1^{ko/ko}$ animals. 100% of $Ft1^{ko/ko}$ animals shows a phenotype, 20.5% of $Ft1^{ko/ko}$ animals display a strong phenotype, the leftovers (79.5%) are affected by a milder one. n, total number of analysed mice, F1-F5, generation 1 to 5.

Ft1^{kof/kof} animals show survival and growth defects

It has been shown that proteins involved in DNA damage repair (Cao et al., 2003), in telomere (Martinez et al., 2009) and nuclear envelope maintenance (Mounkes et al., 2003) play an essential role in lifespan determination. Since AKTIP/Ft1 intercepts these pathways we wanted to investigate on its role in mice survival. We followed 496 wt and 119 Ft1^{kof/kof} animals for a total range of 140 weeks of age.

Kaplan-Meier survival curve analysis shows that Ft1^{kof/kof} animals display a reduced lifespan compared to wt animals (log-rank – Mantel-Cox test, ***p<0.001). In particular we observed that 20.5% of Ft1^{kof/kof} animals prematurely die at around 12 postnatal day (P12). While the leftovers that survive the critical age display a reduced median survival of 113 weeks of age compared to wt animal of which 70% survives up to 140 weeks (Figure 16 A).

We also analysed body weight of Ft1^{kof/kof} animals since growth impairment is a well characterised premature ageing phenotypic trait (Mounkes et al., 2003). We weekly measured body weight on five Ft1^{kof/kof} and five age matched wt animals for 120 weeks starting at weaning. Ft1^{kof/kof} animals are affected by a significant body weight reduction compared to the age matched wt counterparts (Student t-test, ***p<0.001 - Figure 16 B). We also analysed the differences between the two growth curves (Δ Body weight), dividing mice lifespan in 4 major intervals: young starting at weaning until 20 weeks of age (3<W<20); juvenile between 21 and 60 weeks of age (21<W<60); adult between 61 and 100 weeks of age (61<W<100) and old between 101 and 120 weeks of age (101<W<120) (Figure 16 C). The differences between the two growth curves significantly increase as mice age, indicating that growth defect in Ft1^{kof/kof} animals is exacerbated with ageing (Student t-test).

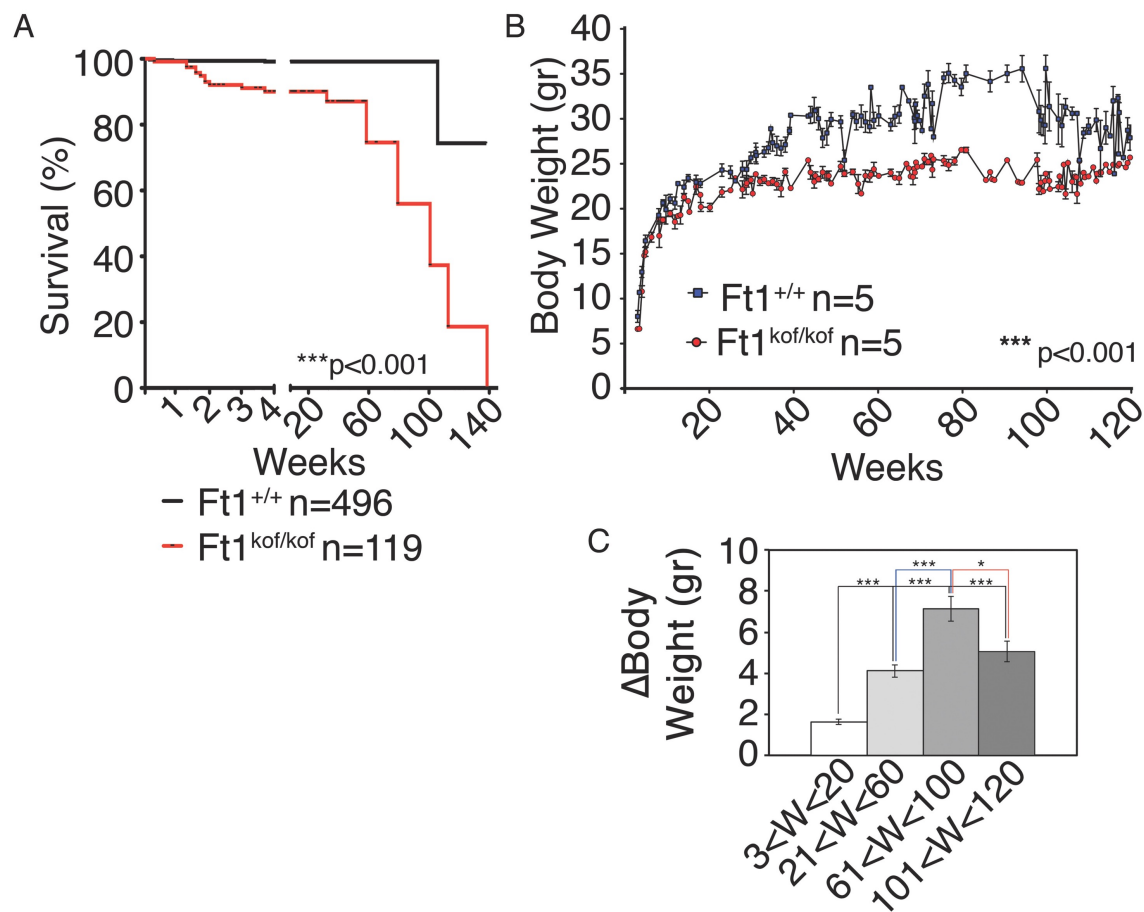


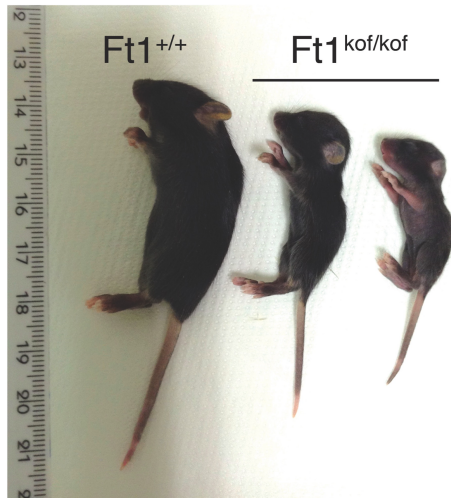
Figure 16 - Ft1^{kof/kof} animals show survival and growth defects exacerbated with ageing.

(A) Kaplan-Meier survival curve of Ft1^{kof/kof} and wt animals. Survival of Ft1^{kof/kof} mice is significantly decreased compared with that of wt (**p<0.001 – log-rank – Mantel-Cox test). (B) Body weight analysis of Ft1^{kof/kof} animals and age matched wt controls. Ft1^{kof/kof} growth curve is significantly different in Student t-test (**p<0.001) respect of that of aged matched wt animals. (C) Analysis of the differences of the two curves (Δ Body weight) in different interval of mice lifespan. The difference between the two curve increase as mice age (** and * indicate p<0.001 and p<0.05 in the Student t-test). n, total number of mice analysed; W, weeks.

20.5% of $Ft1^{kof/kof}$ animals prematurely die and display skeletal defects

20.5% of $Ft1^{kof/kof}$ animals does not survive to the critical age of P12. At the age of death this mice are smaller in size respect of wt littermate (Figure 17 A). $Ft1^{kof/kof}$ animals have an average body weight of 3.3 gr (n=10), while age matched wt controls weight in average 7.9 gr (n=13), we found that this difference is significant in Student t-test analysis (**p=0.0024) (Figure 17 B). We collectively refer to these mice as strong phenotype mice.

A



B

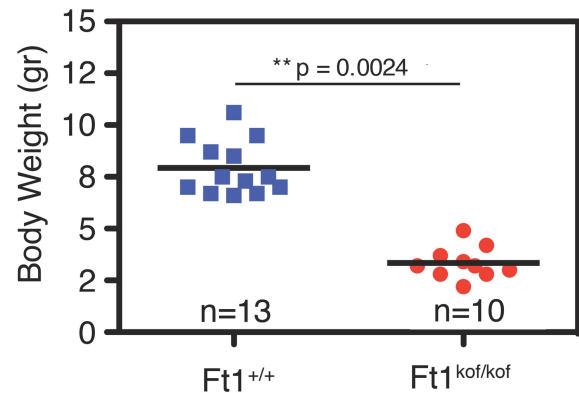


Figure 17 - 20.5% of $Ft1^{kof/kof}$ animals prematurely die at P12 and display growth defects.

(A) Representative pictures of $Ft1^{kof/kof}$ animals at P12 day and age-matched wt mice showing size differences of $Ft1^{kof/kof}$ animals respect of wt littermate. Strong phenotype $Ft1^{kof/kof}$ mice prematurely die at around P12 (B) Body weight analysis performed on $Ft1^{kof/kof}$ animals and age matched wt counterparts. $Ft1^{kof/kof}$ animals are significantly smaller than wt animals (**p=0.0024, Student t-test analysis).

In order to characterise the phenotype of strong $Ft1^{kof/kof}$ animals we have analysed bone, fat, heart and skin that are mostly affected in premature ageing disorders (Cao et al., 2003; Martinez et al., 2009; Tejera et al., 2010; Varela et al., 2005).

We analysed bone of strong phenotype $Ft1^{kof/kof}$ animals affected by premature death. We performed X-ray analysis at the age of death (P12) on eight strong phenotype mice and on three wt age matched animals. X-radiographs on total body of $Ft1^{kof/kof}$ animals highlight craniofacial dysmorphisms absent in age matched wt animals, such as a more curved skull compared to that of wt counterparts (Figure 18 A - white arrowheads). We also noted that $Ft1^{kof/kof}$ animals displayed an abnormal curvature of the spinal column that would be consistent with a kyphosis defects (Figure 18 A - white bars) that is considered a premature ageing trait (Cao et al., 2003). In order to verify this hypothesis we analysed the amplitude of the angle comprised between cervical and thoracic vertebrae on eight $Ft1^{kof/kof}$ animals with strong phenotype and three age matched wt controls. Indeed $Ft1^{kof/kof}$ animals with strong phenotype display a more acute angle comprised between cervical and thoracic vertebrae respect of wt controls. This difference is significant in Student t-test analysis ($***p<0.001$) (Figure 18 B), indicating that $Ft1$ knockdown in strong phenotype mice leads to kyphosis.

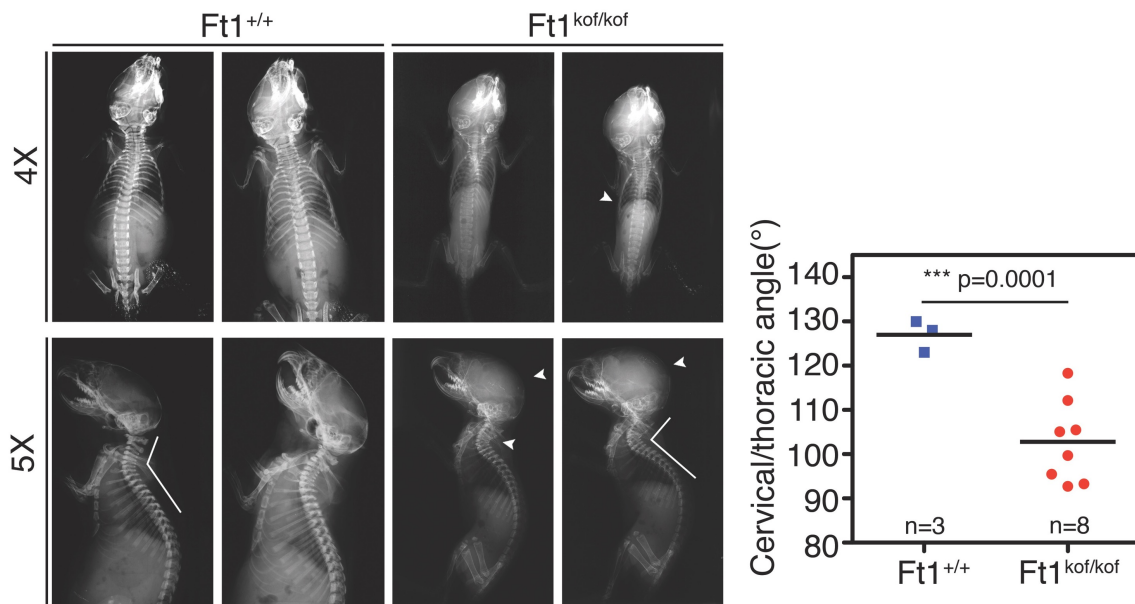


Figure 18 - Strong phenotype *Ft1*^{kof/kof} mice show craniofacial dysmorphisms and kyphosis.

(A) X-ray analysis (Faxitron X-ray Corp., Wheeling, IL) on total body of P12 *Ft1*^{kof/kof} animals and aged matched wt littermate. Arrowheads indicates spine defects and craniofacial dysmorphisms in *Ft1*^{kof/kof} animals. (B) Quantification of the angle formed by cervical and thoracic vertebrae that is significantly more acute in mutant animals than wt controls (angle analysed with Photoshop CS6 analysis plug in and ***p<0.001 – T-test analysis).

X-ray analysis on lower body part from Ft1^{kof/kof} animals show differences in long bones sizes that correlates with whole body reduction in size of Ft1^{kof/kof} animals compared to age matched wt controls. This difference is more evident in the magnification of femur and tibia (Figure 19 A-B) obtained from Ft1^{kof/kof} animals compared to the same bones from wt controls. X-ray analysis also show that femur and tibia from Ft1^{kof/kof} animals are less reflective than in wt matched bones; this difference suggests a different X-ray absorption and could underlie bone composition differences between Ft1^{kof/kof} and wt controls that need to be further investigated.

Total tail ray analysis shows different bone size corresponding to whole body size impairment of Ft1^{kof/kof} strong phenotype animals (Figure 20 A). We histologically analysed sections from caudal vertebrae obtained from P12 Ft1^{kof/kof} and age matched wt animals, in order to identify morphological defects linked to Ft1 knockdown. Hematoxylin and eosin staining shows differences in structure and cellular composition between Ft1^{kof/kof} and wt samples (Figure 20 B). In order to identify the nature of these differences further study are needed.

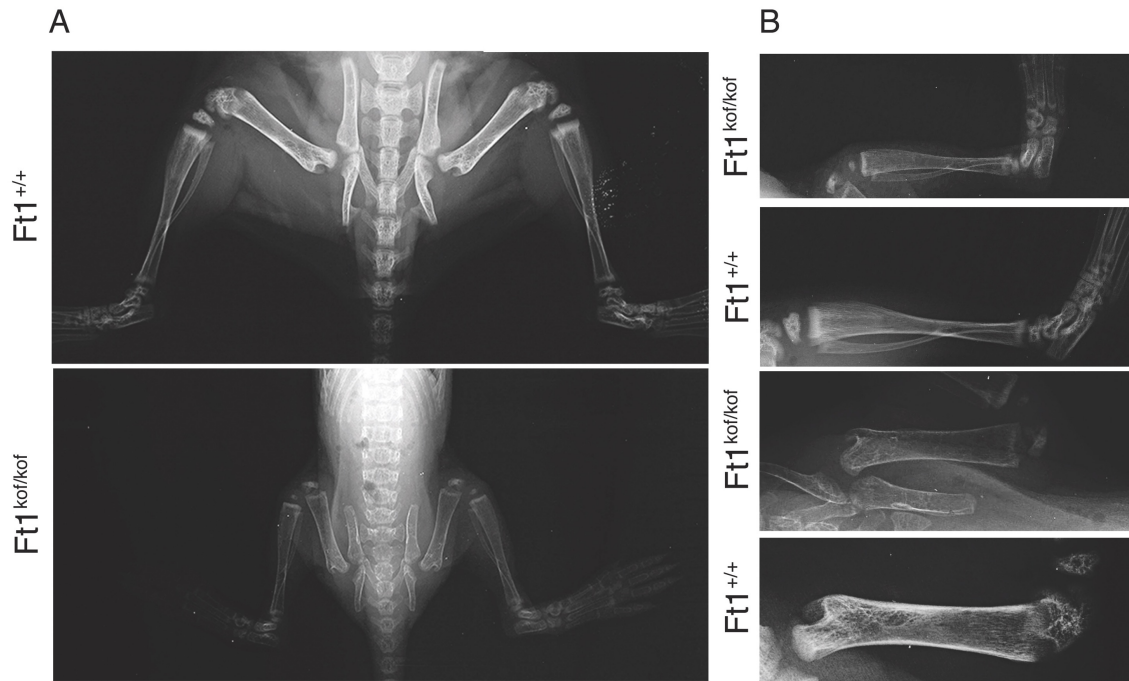


Figure 19 - $Ft1^{kof/kof}$ strong phenotype mice show bone defects: femur and tibia.

(A) X-ray analysis (Faxitron X-ray Corp., Wheeling, IL) on lower body part and magnification of femur (upper right) and tibia (bottom right) radiographies from P12 $Ft1^{kof/kof}$ animals and aged matched wt. All bones radiographies show differences in size and in X-ray absorption, reflecting possible differences in bone composition of $Ft1^{kof/kof}$ animals respect of wt animals.

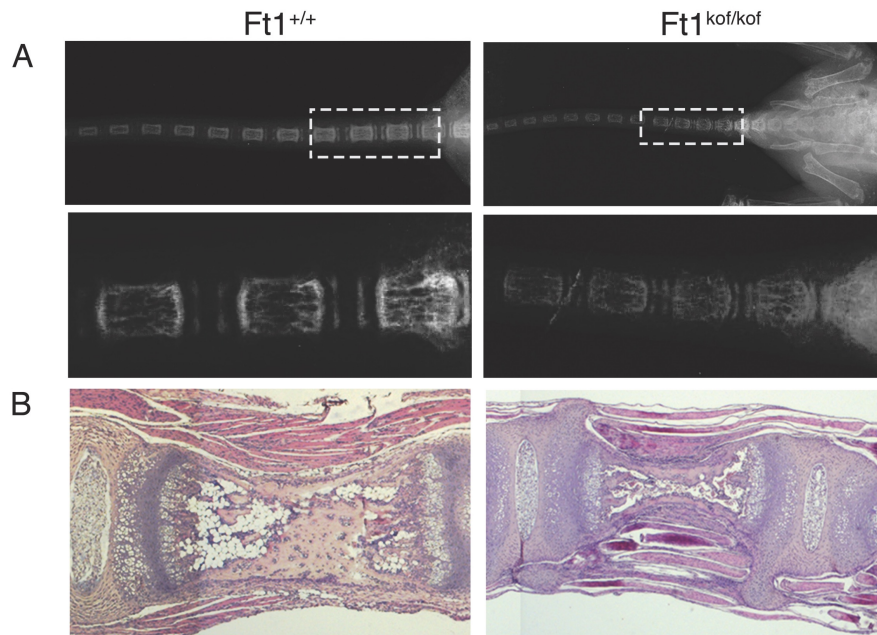


Figure 20 - *Ft1*^{ko/ko} strong phenotype mice show bone defects in caudal vertebrae.

(A) Radiography of total tail (top – Faxitron X-ray Corp., Wheeling, IL) and magnification of caudal vertebrae (bottom). *Ft1*^{ko/ko} mice vertebrae are smaller respect age matched wt animals in accordance to whole body reduction in *Ft1*^{ko/ko} animals. X-ray absorption is different in *Ft1*^{ko/ko} mice respect wt, reflecting possible differences in bone composition. (B) Hematoxylin-Eosin stained section of caudal vertebrae of P12 of *Ft1*^{ko/ko} and wt aged matched animals showing structural and cellular compositions differences between transgenic animals and wt controls.

Ft1^{kof/kof} animals display adipose and cardiac tissue alterations

Adipose and cardiac tissues are often impaired in premature ageing disorders linked to genome instability, nuclear integrity loss or telomere defects (Bergo et al., 2002; Cao et al., 2003; Mounkes et al., 2003; Watson et al., 2013). We wanted to investigate whether Ft1 knockdown could induce adipose tissue impairment. Skinning of P12 mice demonstrated that adipose tissue deposits of Ft1^{kof/kof} strong phenotype were overtly smaller than those of age matched wt animals, indicating that Ft1 knockdown impairs correct adipose tissue deposit (Figure 21 A).

Pathological analysis on hematoxylin and eosin stained skin sections, obtained from strong phenotype animals (P12) and age matched wt counterparts, highlights reduction of intradermal fat in mutant animals (Figure 21 B, black arrowheads). This result indicates that also intradermal fat deposit is affected upon Ft1 downregulation in mice.

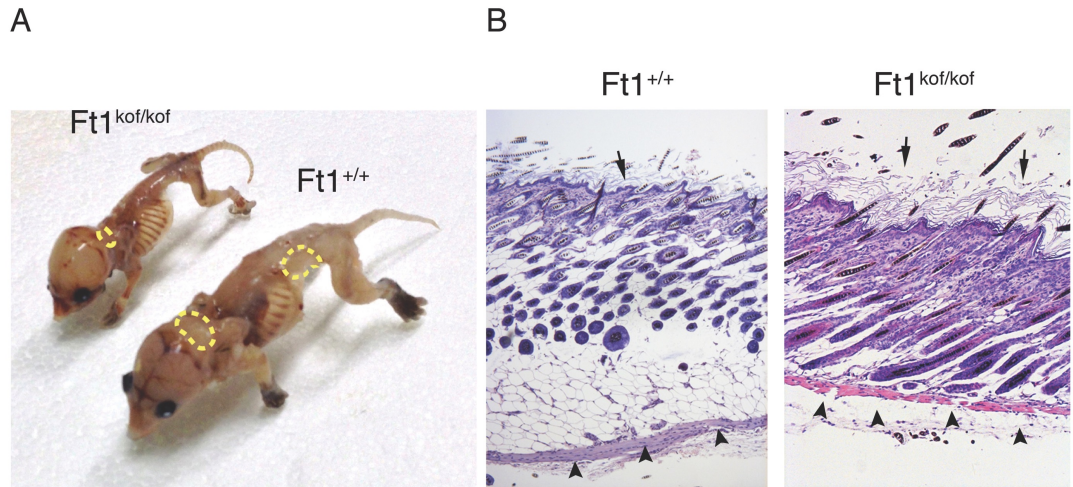


Figure 21 - $Ft1^{ko/ko}$ strong phenotype mice show total body and intradermal fat loss.

(A) Representative picture of P12 day skinned $Ft1^{ko/ko}$ mouse and age matched wt littermate. Mutant animals have total body fat reduction (yellow dot circle lines) respect wt controls. (B) Hematoxylin-eosin stained skin sections of $Ft1^{ko/ko}$ and wt age matched control. Arrowheads indicate intradermal fat, of which there is an almost complete absence in the mutant.

We took the heart from Ft1 transgenic animals at the age of death (P12) and from age matched wt littermate. Histological analysis on hematoxylin and eosin stained heart sections show that no obvious macroscopical changes occurs in Ft1^{kof/kof} observing total hearts sections (Figure 22 upper panel). Indeed a more detailed analysis on the enlargements of the same sections reveals that the cellular structure of the heart differs in Ft1^{kof/kof} animals compared to wt controls. Myocytes seems to be smaller in Ft1^{kof/kof} animals compared to wt myocytes (Figure 22 bottom panel). It has been shown that impairment of the heart is a consequence of lamin A mutation. In this mutant animals the myocytes appear to be smaller than wt myocytes, in particular there is an unbalance in cell populations with fewer myocytes in mutant tissue with a compensatory increase in the fibrocyte population, that is a hallmark of hypoplasia and/or atrophy of heart muscle (Mounkes et al., 2003). Indeed further analyses are required to assess whether also Ft1^{kof/kof} hearts shows the same heart defects that are suggested by macroscopical analysis.

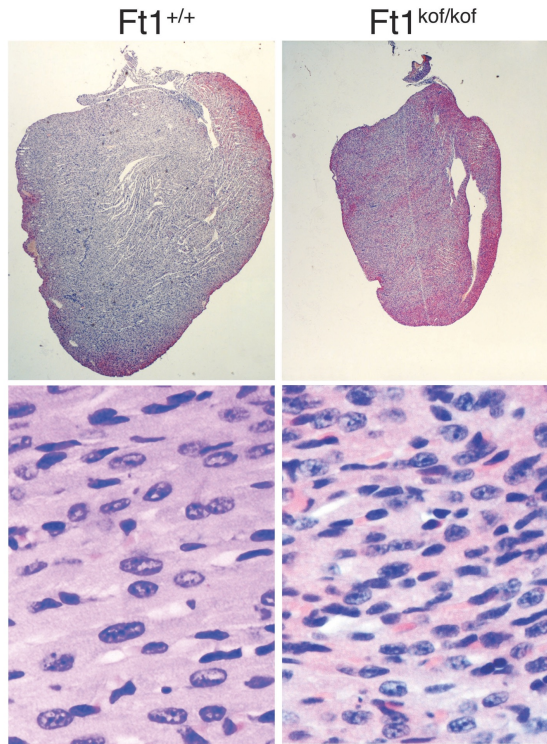


Figure 22 - Heart differences between strong phenotype $Ft1^{kof/kof}$ animals and wt age matched animals. Hematoxylin-eosin stained total heart from wt and $Ft1^{kof/kof}$ mice (upper panel). Magnification of hematoxylin-eosin stained heart areas from mutant (right below panel) and age matched wt animals (left below panel). Myocytes in $Ft1^{kof/kof}$ animals are smaller than in wt counterparts.

Ft1^{kof/kof} male mice are more sensitive to Ft1 depletion

We analysed strong phenotype occurrence in female and male Ft1^{kof/kof} animals. Phenotype distribution in male Ft1^{kof/kof} animals is 16% of strong phenotype versus 84% of mild phenotype over the total Ft1^{kof/kof} male animals. On the other hand in female the occurrence of the strong phenotype is 2% the leftovers (98%) are affected by the mild phenotype over the total Ft1^{kof/kof} female mice. It is possible to note the percentage of the strong phenotype in male animals (16%) is higher than the one observed in female mice (2%) (Figure 23 A). These data suggest that male mice are more sensitive to Ft1 depletion.

Intriguingly we found that Ft1^{kof/kof} mild male mice are sterile. We followed breedings of different genotype combinations and we analysed pups average obtained in different litters. In breedings of males Ft1^{kof/kof} with Ft1^{+ /kof} females we did not observed any pregnancies. We analysed six male mice and we kept the mating for more than 50 days. On the contrary female Ft1^{kof/kof} mice are fertile, since the three female bred with Ft1^{+ /kof} male mice gave an average of 4.4 pups in different litters (Figure 23 B).

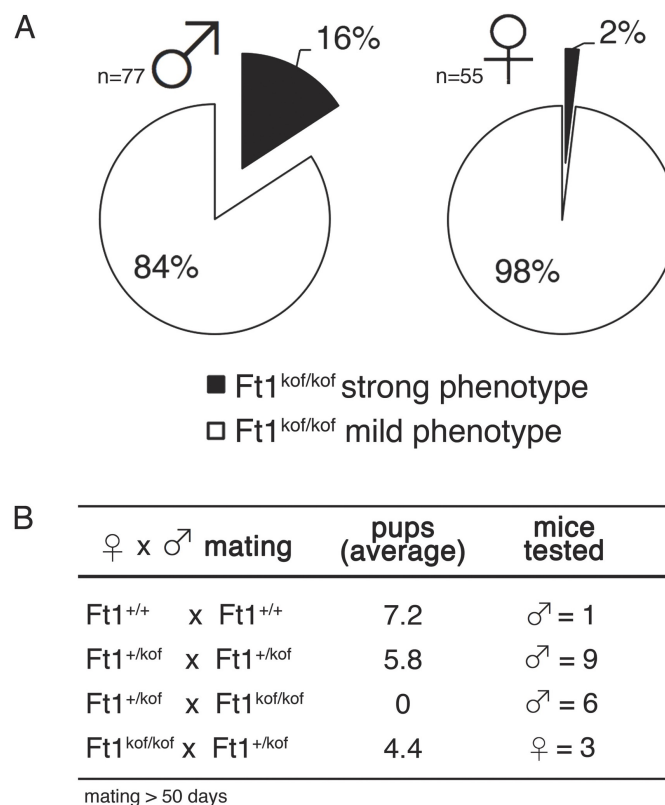


Figure 23 - Male mice are more sensitive to Ft1 depletion.

(A) Strong phenotype occurrence in male and female $Ft1^{kof/kof}$ mice. 2% of $Ft1^{kof/kof}$ female animals are affected by the strong phenotype, on the contrary 16% of $Ft1^{kof/kof}$ male are affected by the strong phenotype. (B) Pups average obtained from Ft1 matings. $Ft1^{kof/kof}$ male mice mated with $Ft1^{+/-}$ female do not gave any litter when kept in mating for more than 50 days (n indicates total mice number).

Production and characterisation of Ft1 kof and p53 ko mouse line

Ft1 and p53 co-depletion increases death incidence and p53 ko rescues Ft1 body weight defects

p53 genetically interacts with most of the actors involved in telomere and DNA metabolism and nuclear envelope maintenance. It has been described that p53 abrogation in mouse models of premature ageing disorders ameliorates or even rescues the organismal impairment (Cao et al., 2003; Martinez et al., 2009; Varela et al., 2005).

It has been also shown for TRF1 ko mouse model, that while p53 abrogation can rescue TRF1 loss lethal phenotype, their co-depletion leads to an increase in tumour incidence and a consequent increasing in mice death. The genomic instability that it is derived upon the shelterin loss acts at the cellular level without the control of the cell cycle regulator p53 leading to loss of the proper cellular maintenance (Martinez et al., 2009).

Given that AKTIP/Ft1 has a role in the same mechanisms that have been linked to premature ageing and that in particular it genetically (Burla et al., 2015b) and physically interacts with TRF1 we asked whether p53 could rescue Ft1-dependent phenotypic traits and that it would affect mice death. To this purpose we generated a double Ft1 kof and p53 ko (ko) mouse line. We crossed Ft1^{+/kof} with p53^{+/ko} animals in order to obtain double transgenic animals in the generation zero (F0). Ft1^{+/kof};p53^{+/ko} animals were then intercrossed to obtain Ft1^{kof/kof};p53^{ko/ko} animals in the first generation (F1) (Figure 24 B). Mice were screened by PCR analysis using the same PCR primers described previously for Ft1 kof allele, and, for p53 allele, a triplex of primers pairing on exon 6 (F), neomycin cassette (F) and exon 7 (R). Examples of PCR analysis is shown in figure 24 C, where it is possible to discriminate different genotype combinations obtained from Ft1^{+/kof};p53^{+/ko} intercross (Figure 24 C).

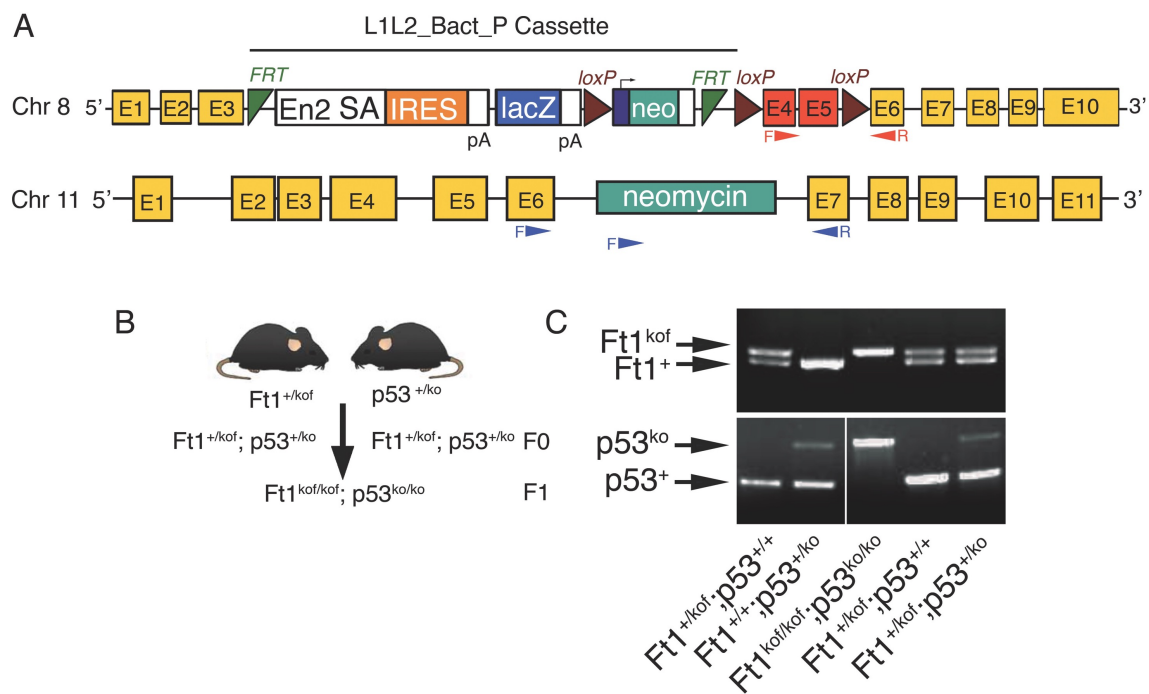


Figure 24 - Ft1 kof; p53 ko mouse line production. (A) Schematic representation of Ft1 kof allele (up) and p53 ko (bottom) allele. The insertion of the neomycin gene between exon 6 and exon 7 disrupts p53 expression on 11th mouse chromosome. Adapted from (Jacks, Remington et al. 1994). (B) Schematic representation of the strategy used to obtain double Ft1^{kof/kof} and p53^{ko/ko}. Briefly Ft1^{+/-kof} and p53^{+/-ko} mice were mated in order to obtain double heterozygous animals in the generation zero (F0). Ft1^{+/-kof};p53^{+/-ko} were intercrossed in order to obtain Ft1^{kof/kof};p53^{ko/ko} mice in F1. (C) Representative PCR analysis used to detect Ft1 kof allele (up) and p53 ko allele (bottom) on genomic DNA obtained from pups at weaning. The picture shows different genotype combination obtained in Ft1^{+/-kof};p53^{+/-ko} intercross. Amplicon derived from wt Ft1 allele has a length of 692 bp and amplicon derived from transgenic Ft1 kof allele has a length of 755 bp. As for p53 gene, amplicon derived from wt allele has a length of 400 bp and amplicon derived from transgenic p53 ko allele has a length of 587 bp.

It has been already reported that p53^{ko/ko} mice develops tumours by 10 months of age, while roughly 50% of p53^{+/-ko} mice develop tumours by 18 months (Donehower, 1996).

We analysed death incidence in double transgenic mouse line (Figure 25 A). The results obtained show that Ft1^{+/-kof};p53^{+/-ko} animals display 15.6% (n=64) of death incidence while p53^{+/-ko} do not display any death event (n=8), this difference is significant in χ^2 test, ***p<0.001. On the other hand the difference between Ft1^{kof/kof};p53^{+/-ko} is not significant in χ^2 test but this might be due to the low number of the animals analysed so far. We also observed that in a p53 null background the differences between the Ft1^{+/-kof} animals and Ft1^{+/-+} animals is not significant in χ^2 test. These data indicate that Ft1 loss has an effect on p53-dependent death induced phenotype.

We asked whether p53 depletion could rescue Ft1 phenotype. We analyse body weight of Ft1^{kof/kof} animals in p53^{+/-ko} background. We weekly took body weight on wt, Ft1^{kof/kof}, and Ft1^{kof/kof};p53^{+/-ko} along with p53^{+/-ko} animals and we compared the resulting growth curves. While Ft1^{kof/kof} animals display growth defects (Figure 25 B green triangle), p53^{+/-ko} can rescue body weight defects caused by Ft1 depletion. The observed body weight rescue after p53 haploinsufficiency, in Ft1 knockdown background, implies that p53 genetically interacts with Ft1 *in vivo*.

So far we obtained few Ft1^{kof/kof};p53^{ko/ko} animals so we are unable to analyse the effect of the double co-depletion *in vivo*.

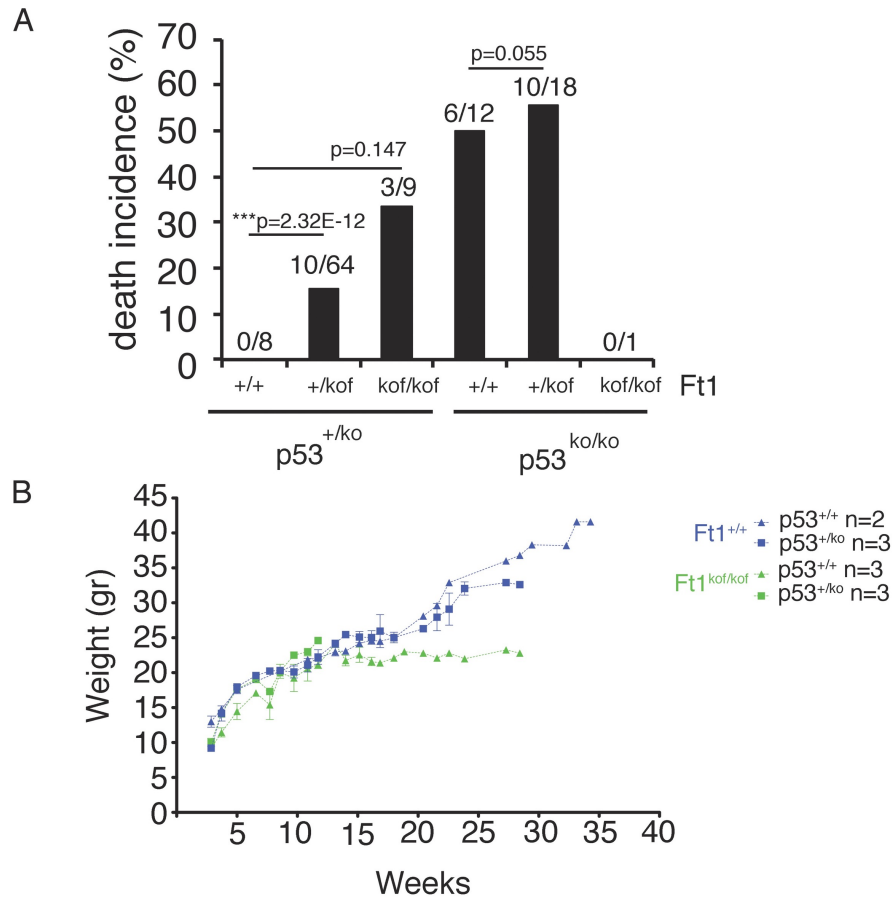


Figure 25 - Ft1 and p53 co-depletion increases death incidence. p53^{+/ko} rescues Ft1^{kof/kof} growth defect. (A) Death incidence analysis in p53 ko (ko);Ft1 kof mouse colony. Death incidence in double Ft1^{+/kof};p53^{+/ko} mice is significantly higher in the χ^2 test ($***p < 0.0001$) respect of the single p53^{+/ko} animals. Fractions on each column indicate the number of dead over total alive animals of each genotype. (B) Body weight analysis of Ft1 kof; p53 ko animals. Growth impairment in Ft1^{kof/kof} animals (green triangles) are rescued in Ft1^{kof/kof};p53^{+/ko} (green squares). n, total mice number analysed for each genotype.

DISCUSSION

AKTIP/Ft1 biochemically interacts with different proteins involved in telomeric, DNA and in nuclear envelope maintenance

It has been shown that AKTIP/Ft1 is able to interact with the telomeric DNA and with TRFs proteins and that its depletion *in vitro* induces the fragile telomere phenotype related to replication impairments. AKTIP/Ft1 is important for PCNA tethering to the chromatin and its depletion leads to lower efficiency of telomeric DNA replication. Altogether these data reveal that AKTIP/Ft1 is important for proper telomere maintenance and more in general it is involved in mechanisms of DNA metabolism, such as replication (Burla et al., 2015b). AKTIP/Ft1 mainly localises at the nuclear rim and, as it has been described in the introduction, that it co-localises with A-type lamins and lamin B1 (Burla et al., 2015a). In cells that either endogenously or ectopically express mutated versions of lamin A, AKTIP/Ft1 localisation at the nuclear rim along with its interaction with telomeric DNA are disrupted, as it has been described into the introduction (Burla et al., 2015a).

But how can AKTIP/Ft1 act in these diverse mechanisms? In order to answer to this question we choose the biochemical approach to understand whether AKTIP could exploit its role by interacting with the diverse actors of the mechanisms in which it is involved.

Firstly we analysed the nature of AKTIP interactions with the TRFs protein and we found that AKTIP is able to directly interact with both TRF1 and TRF2 (Figure 10) reinforcing the role of AKTIP in telomere metabolism. We also analysed its binding with the TRFH domains of the two TRFs proteins. Intriguingly we found that AKTIP directly binds TRFH2 but not TRFH1 (Figure 11). We also analysed AKTIP interaction with components of the replication machinery. We found that it is able to interact with both RPA70 and PCNA (Figure 12 A), for the latter we also found a direct interaction (Figure 12 B).

These data reinforce the role of AKTIP in telomere metabolism, especially during replication.

Replication is a well fine-tuned process and in complex DNA regions, such as telomeric region, it represents a real challenge. For this reason the replication machinery requires the recruitment of a wide range of proteins that play different crucial roles. The two TRFs shelterins, for example, play a crucial but different role during telomeres replication. It has been demonstrated that they can recruit different accessory proteins during replication events or that they can even recruit the same components but during different phases of the replication (Arnoult and Karlseder, 2015). One example is the recruitment of the helicase RTEL1 that, when it is recruited from TRF1 and PCNA during replication, it unfolds the G-quadruplex structure on the lagging strand of telomeric DNA (Vannier et al., 2013), and then, when it is recruited by TRFH2, it unfolds the telomeric T-loop allowing the end of the replication (Arnoult and Karlseder, 2015; Vannier et al., 2012). Indeed AKTIP could be recruited or could recruit itself components that are required for different replication phases, but further studies are needed to dissect its mechanism of action. For example by defining the biochemical interaction sites with TRF1 and PCNA and subsequently look at the phenotypic effects that arise when the known interactions (TRF1, PCNA and TRFH2) are disrupted.

Finally we described that AKTIP interacts with A-type lamins and lamin B1 (Figure 13 A) and lamin related matrin 3 protein (Figure 13 B). 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 (Broers et al., 2006; Dechat et al., 2010; Hutchison and Worman, 2004). It has been shown that the lamin A Ig fold domain interacts with PCNA (Shumaker et al., 2008), which in turn localises at the nuclear periphery during heterochromatin replication, that represents another challenging DNA region to be replicated (Dimitrova and Berezney, 2002).

Here we have demonstrated that AKTIP is able to interact with different components of the mechanism that it functionally intercepts, in particular TRF1, TRFH2, PCNA, RPA70, and component of the nuclear lamina. Our hypothesis is that AKTIP/Ft1 could be able to form a multi-protein hub that is composed by telomeric protein and components of the replication machinery and that the hub could be necessary for proper telomeric replication or more in general for replication in complex DNA region.

The hub hypothesis is further supported by the fact that AKTIP localisation at the nuclear rim is lost upon the expression of aberrant lamin A, as it has been described into the introduction (Burla et al., 2015b). Considering the role of the nuclear lamina in regulating diverse DNA metabolism events its role into the hub could be to orchestrate in a time- and a spatial- dependent manner the functional role of the multi-protein hub.

Indeed it would be of interest to assess whether AKTIP interaction with the nuclear lamina proteins is direct, or if it could interact with the mutated forms of the lamin A (progerin) in order to assess whether the dysfunctional protein could have detrimental contribution to the hub functionality. Moreover, to define the time and spatial properties of the hub, it would be interesting to look at the co-localisation of AKTIP, lamins, PCNA and telomeric proteins.

In this scenario major open questions requires further investigations:

- Are the AKTIP interactions, here described, mutually exclusive?
- Could AKTIP mediate different interactions with different binding partners in a cell cycle dependent manner?
- Which is the role of AKTIP inside of the hub?

AKTIP/Ft1 downregulation *in vivo* phenocopies premature ageing traits and it genetically interacts with p53

As we discussed into the introduction AKTIP intercepts *in vitro* mechanisms fundamental in telomere and DNA metabolism and in nuclear envelope

maintenance. For these mechanisms it has been proved that the experimental perturbation leads to premature ageing cellular phenotypes (Blasco, 2005a; Burla et al., 2015b; Burla, 2015; Lopez-Otin et al., 2013; Munoz et al., 2005; Tejera et al., 2010). Here we have demonstrated that AKTIP interacts with major components of those mechanisms and we have introduced the concept of a multi-proteins hub.

So we asked whether Ft1 depletion could result in a specific phenotype *in vivo*, that would be ascribable to one or more of the mechanisms that it intercepts at the cellular level. To this purpose we produced and characterised a hypomorphic Ft1 mouse model by the insertion of a kof cassette into the mouse Ft1 gene locus (Figure 14 A) (Testa et al., 2004). Among the *in vivo* ko of the individual shelterin only mice depleted for Rap1 and Pot1b result viable (Takai et al., 2011), moreover either mouse with deletions in lamins components or in gene involved into general DNA metabolism display major survival impairment (Cao et al., 2003; Mounkes et al., 2003; Munoz et al., 2005). Given that AKTIP/Ft1 has a role in those mechanisms, we would not know whether its full ko could be lethal, so we decided to generate a hypomorphic knockdown mouse model instead of a full ko. We choose the kof cassette approach to obtain Ft1 knockdown because of its high plasticity, since a wt condition can be restored after crossing these mice with Flp-deleter animals, thanks to the presence of the FLP recognition sites flanking the kof cassette. From restored wt animals conditional or full ko animals can be also derived by crossing those animals with tissue-specific or ubiquitously Cre-expressing animals, since two critical exons, outside of the kof cassette, are flanked by loxP sites.

We obtained 80% of Ft1 downregulation in Ft1^{kof/kof} animals (compared to wt Ft1 expression – Figure 14 D) and this reduction impairs in 100% of Ft1^{kof/kof} mice survival and growth rate; these mice were also obtained at a submendelian ratio (Figure 15 A) suggesting a possible embryonic lethality. In 20.5% of Ft1^{kof/kof} animals Ft1 downregulation induces a strong phenotype (Figure 15 B) leading to premature death at around P12 and to body weight

reduction at death (Figure 17 A-B). The leftover 79.5% (Figure 15 B) of Ft1^{kof/kof} animals survive the critical age of P12 but the median survival is around 113 weeks (Figure 16 A), which is significantly reduced from what we observed in wt animals. These animals display body weight reduction, that we started to analyse at weaning (Figure 16 B), which is exacerbated with ageing (Figure 16 C). In particular we observed that the differences between the growth curves are significantly different, from the young to the adult age (3 to 100 weeks). Indeed the difference between the wt and Ft1^{kof/kof} animals is no more significant in the old age (101 to 120 weeks – Figure 16 C); this could be most likely due to the fact that also wt animals age and reduction in body weight is a characteristic also of physiological ageing.

Pathological examination of strong phenotype mice, performed at the premature age of death (P12) highlights impairments in bone structure and composition (Figures 18-20), general (Figure 21 A) and intradermal fat loss (Figure 21 B), and reduced myocytes size (Figure 22). This last observation is in line with what has been observed in a lamin A mutant mouse models (Lmna^{L530P/L530P}). In particular it has been described that reduced myocytes size, accompanied by unbalance in cellular population, could be linked to hypoplasia and/or atrophy of heart muscle (Mounkes et al., 2003). Although further analysis are required to assess whether also Ft1^{kof/kof} hearts shows the same heart defects, as it has been suggested by the macroscopical analysis.

Altogether these phenotypic traits have been linked to premature ageing disorders linked to A-type lamins mutation. In fact mice that expresses a mutated version of the lamin A (Lmna^{L530P/L530P}) display growth defects along with premature death, bones defects associated with osteoporosis, mild to moderate degeneration mixed with hypoplasia and/or atrophy of both heart and skeletal muscle. Skin of these mice shows a complete absence of the subcutaneous fat layer (Mounkes et al., 2003). Some of these phenotypic traits have been described also for progeroid mouse models with other mutations in nuclear lamina components (Bergo et al., 2002; Burke et al., 2001; Roux and

Burke, 2007) or in genes involved in general DNA metabolism (Cao et al., 2003; Munoz et al., 2005).

Our results suggest that AKTIP/Ft1 loss *in vivo* phenocopies premature ageing traits. Although in nuclear lamina related disorders, the macroscopic phenotypic traits of premature ageing have been deeply characterised, the path that links the molecular and cellular phenotypes to the organismal alterations is still ill defined. Given that AKTIP/Ft1 physically and functionally interacts with components of DNA metabolism, telomeres and nuclear envelope maintenance, which impairment converge in overlapping premature ageing disorders, dissecting its role could help to elucidate the molecular mechanisms that underlie ageing.

Intriguingly we also found that male mice are more sensitive to Ft1 depletion (Figure 23). Indeed we observed an increased occurrence of strong phenotype in male mice respect of female animals (Figure 23 A). One hypothesis, of this sex-linked effect, could be that the hormonal set, that differs from female and male, could be protective in female animals or detrimental in male, but more analyses are required to assess this hypothesis.

Another sex linked aspect that we observed in mild animals is male sterility (Figure 23 B). This is not surprising since telomere defects, studied through diverse mouse models, result in male sterility, for example in Pot1b and late generation of Terc depleted animals. In these animals sterility is caused by failure of the proliferative capacity of male gonads due to the high genomic instability observed upon telomere metabolism impairments (He et al., 2009; Herrera et al., 1999b; Takai et al., 2011). It would be of interest to analyse male gonads in Ft1 depleted animals in order to assess whether there is the same failure in the proliferative capacity.

Most of the determinants of ageing genetically interacts with p53. It has been demonstrated, in fact, that its depletion in premature ageing mouse models ameliorate or rescue the detrimental age-related effects at the organismal level (Cao et al., 2003; Martinez et al., 2009; Siegl-Cachedenier et al., 2007; Tejera et

al., 2010; Varela et al., 2005). In order to understand if AKTIP/Ft1 genetically interacts with p53 we produced a double transgenic Ft1 kof and p53 ko mouse line (Figure 24). Indeed we observed a rescue of the growth defects due to Ft1 downregulation when we analysed Ft1^{ko/kof};p53^{+/-ko} animals (Figure 25 B). But on the other side Ft1^{+/-kof};p53^{+/-ko} mice display significantly higher death percentage (15.6% n=64) compared to p53^{+/-ko} animals (0% n=8) (Figure 25 A). The same effect has been described for TRF1 and Brca1, where p53 abrogation leads to the rescue of detrimental *in vivo* effects due to loss of the individual protein. It has been also reported that there is an increase in mouse death, due to higher percentage of tumour induction. It has been hypothesised that the high genomic instability observed upon TRF1 loss or, upon to the loss of proper cell cycle checkpoint G1/S in Brca1 deficient animal models, could lead to tumour induction, when it is abrogated also p53 activity (Cao et al., 2003; Martinez et al., 2009). These results suggest that, in Ft1 depleted animals, the higher percentage of death could be due to higher tumour induction, so it would be of interest to analyse the cause of death in double transgenic mouse line. Altogether these data suggest that AKTIP/Ft1 genetically interact with p53. Furthermore the described TRF1 and p53 genetic interaction (Martinez et al., 2009) is similar to what it has been observed in our mouse model reinforcing the biochemical and genetic interaction between AKTIP/Ft1 and TRF1 described *in vitro*.

CONCLUSIONS

In this work we analysed the biochemical interactions of the novel telomeric accessory protein AKTIP with the components of telomere and DNA metabolism, along with nuclear envelope components. Indeed we found that AKTIP directly interacts with TRF1, TRF2 shelterin proteins, and we found the domain of TRF2 that directly interacts with AKTIP (TRFH2). AKTIP binds also replication fork components such as RPA70 and PCNA, and it directly interacts

with PCNA. Moreover AKTIP binds nuclear envelope components such as A-type lamins, lamin B1 and matrin 3, which is a lamin related protein.

The major question that arises from these findings is: why AKTIP binds all these partners? Our hypothesis is that AKTIP creates a hub of multi-proteins. Keeping in mind that AKTIP is involved in replication events, especially in challenging DNA regions such as telomere are (Burla et al., 2015b), and that it binds components that are involved in DNA replication (TRFs, RPA70 and PCNA), this hub might be functional during replication events.

Another question that arises from these findings is what is the role of the nuclear lamina in the multi-protein hub? It has been described that the nuclear lamina orchestrates diverse DNA metabolism events, including DNA replication (Broers et al., 2006; Dechat et al., 2010; Hutchison and Worman, 2004). It is possible that lamin controls AKTIP hubs that could then recruit other proteins and contribute to structurally challenging DNA replication steps.

It would be also of interest to understand what happens when the hub is impaired. As it has been described into the introduction, AKTIP depletion *in vitro* phenocopies nuclear envelope cellular premature ageing, moreover AKTIP is delocalised upon lamin A mutations, which are responsible for progeroid syndrome in humans (Burla et al., 2015a). One hypothesis is that the impairment of one of the hub components (lamin A or AKTIP) would then generate hub dysfunction and/or mislocalisation and, in turn, this would induce DNA metabolism alterations and progeroid cellular traits.

Given such important role of AKTIP at the cellular level and that its impairments leads to premature ageing cellular traits we asked whether AKTIP/Ft1 would be important at the organismal level.

In this work we report on the production and characterisation of an AKTIP/Ft1 hypomorphic mouse model, using the novel and powerful gene trap based kof technology (Testa et al., 2004). We found that AKTIP/Ft1 is crucial for mice survival, downregulation of its expression *in vivo* ($\leq 80\%$ of wt Ft1 expression) causes, in 20.5% of Ft1^{kof/kof} mice, premature death at P12 and an overall

reduction of lifespan in mice that survive the critical age (79.5%). Premature death animals displayed gross defects in bone, in fat along with a strong reduction in body weight. This strong phenotype is sex-linked, in fact male mice are more affected respect females, although the causes that underlie this difference have to be studied, one hypothesis is that the differences in hormones sets could play an important role. Mild phenotype animals showed male sterility and growth defects exacerbated during lifespan. We also found that AKTIP/Ft1 and p53 genetically interacts since we observed a rescue of Ft1 growth defects in Ft1^{kof/kof};p53^{+/-ko} animals and an increase in death incidence in Ft1^{+/-kof};p53^{+/-ko}. The rescue of the phenotype is in accordance to what it has been observed in progeroid mouse models (Cao et al., 2003; Martinez et al., 2009; Siegl-Cachedenier et al., 2007; Tejera et al., 2010; Varela et al., 2005) while the higher incidence of mice death is in line with what it has been reported in TRF1 or in Brca1 and p53 double ko animals (Cao et al., 2003; Martinez et al., 2009). Although it remains to be assessed the cause of death in Ft1^{+/-kof};p53^{+/-ko} animals, it is highly probable that this animals dies because of tumour induction. If this hypothesis will be confirmed by further studies, then it is possible that this might be due to the fact that p53 rescue is established in a genomic instability background induced by Ft1 loss, therefore enabling mutant cells to propagate without being properly checked, as it has been described for both Brca1 and TRF1 (Cao et al., 2003; Martinez et al., 2009).

AKTIP/Ft1 hypomorphic mouse model, here described, shows an overlap of the phenotypic traits that have been observed in mouse models with impaired telomeric metabolism, DNA metabolism or nuclear envelope maintenance (Behrens et al., 2014; Beier et al., 2012; Bergo et al., 2002; Gu et al., 2012; Martinez et al., 2009; Watson et al., 2013), which altogether are bonafide hallmarks of ageing. Nowadays the premature ageing phenotypic traits, both at the cellular and at the organismal level, are well defined. Nevertheless there is a missing connection path among telomerie, DNA metabolism and nuclear envelope maintenance and the overlapping premature ageing phenotype that

arises from their impairments. The study of AKTIP/Ft1 multi-protein hub properties *in vitro* and *in vivo* could help to unravel this path.

MATERIALS AND METHODS

Cells

293T (ATCC CRL-11268) cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen).

GST pull down from cell extracts

Full length AKTIP cDNA flanked by BamHI and NotI restriction sites, were amplified by PCR using the primers listed in following table (Table 4) and cloned in the pGEX6p1 vector (GE Healthcare) for expression in bacteria. Bacterially expressed GST fusion proteins were purified by incubating crude lysates with QIAGEN Glutathione HiCap Matrix according to the manufacturer's instructions. GST pulldown was carried out as previously described (Raffa et al., 2009). Briefly, appropriate amounts of GST-fusion proteins or GST alone were incubated in PBS at 4 °C for 2 h with whole protein extracts from 293T cells obtained as later described in Immunoblotting treating cells with lysing buffer. Complexes were collected by centrifugation and washed 3 times with PBS-1%Triton X-100 buffer before resuspension in SDS/PAGE loading buffer. Immunoblotting was performed as described later.

GST pull down for direct interaction with bacterially purified proteins

For direct interaction assays GST-AKTIP full length were obtained as described above. Purified TRF1 and TRF2 were produced from the plasmids pET 30A Tev expressed in BL21 (D3) cells and purified as previously described (Amiard et al., 2007). Briefly, supernatant from a cell lysate was bound to Ni-NTA slurry (Qiagen), after several washes with Wash Buffer (50 mM Hepes [pH8], 10 mM b-mercaptoethanol, 500 mM KCl, 20 mM Imidazole, 1 mM PMSF, 10% Glycerol), the protein was eluted with the same buffer containing 300 mM Imidazole and dialysed against Wash Buffer. Protein concentration was assessed by Bradford assay (Sigma).

Purified TRFH1, TRFH2 and PCNA were cloned into pNIC28-Bsa4 vector and expressed in BL21(DE3) Rosetta T1R cells. Supernatant from cell lysate was bound to Ni-NTA 200 SEC Superflow column, washed with Wash Buffer (20 mM Hepes [pH7.5], 300 mM NaCl, 10% v/v glycerol, 2 mM TCEP) and eluted with the same buffer containing 300 mM Imidazole. Eluted fraction containing the target protein was loaded into size exclusion HiLoad 16/60 Superdex 200 column (GE Healthcare Life Science) for further purification. Size based fractions containing the target protein were then concentrated and used for binding assay. Concentration were assessed by 280 nm absorbance quantification.

Appropriate amounts of purified full length GST-AKTIP or GST alone were individually incubated at 4 °C for 2 h or over night with purified full length TRF1, TRF2, TRFH1, TRFH2 or PCNA in NETN buffer (20 mM Tris-HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40). Complexes were collected by centrifugation and washed 3 times with NETN buffer before resuspension in SDS/PAGE loading buffer. Immunoblotting was performed as described below.

Immunoblotting and blue comassie staining

Immunoprecipitated samples or samples treated with lysis buffer [Hepes pH7.5 20mM, NaCl 150mM, MgCl₂ 5mM, EGTA 0.5mM, 0.25% NP-40, DTT 1mM, PMSF 0.5 mM, Na₃VO₄ 0.5mM in presence of the protease inhibitor cocktail (Roche)] were loaded onto pre-cast 4–12% gradient acrylamide gels (NuPAGE, Invitrogen). After electro-blotting, filters were incubated with anti-TRF2 (Novus Biologicals), anti-actin-HRP conjugated (Santa Cruz), anti-lamin A/C (Santa Cruz) anti-lamin B1 (Santa Cruz), anti-PCNA (Santa Cruz), anti-RPA70 (Santa Cruz), anti-TRF1 (Santa Cruz), anti-matrin3 (Santa Cruz) or anti-importin7 (Santa Cruz). Filters were then incubated with appropriate HRP-conjugated secondary antibodies (Santa Cruz). Immunoreactive proteins were revealed using the enhanced chemiluminescence system (ECL plus, Amersham). For direct interaction between AKTIP and TRFH2 and PCNA,

immunoprecipitated samples were loaded onto 10% acrylamide gels and stained with blue comassie (0.1% v/v comassie, 50% methanol, 10% glacial acetic acid).

Production of Ft1 kof transgenic mice

Male ES cells (HEPD0589_6_H06), derived from C57Bl/6, kof for Ft1 gene and with Agouti gene expression restored were purchase from EUCOMM (*European Conditional Mouse Mutagenesis Program*) from *International Knock-out mouse consortium* (IKMC project 46319). ES cells injections into C57Bl/6 host blastocyst were performed in EMBL Transgenic Mice Unit (Monterotondo). Chimeras were then crossed with C57Bl/6 in order to obtain germ line transmission. Heterozygote Ft1 kof animals were backcrossed with wt C57Bl/6 mice and/or intercrossed in order to amplify mice colony or generate Ft1^{ko/ko} animals.

Production of Ft1 kof and p53 ko double transgenic mice

p53 ko transgenic line was gently provided by Dr. Piaggio and Dr. Soddu (IFO, Istituto Regina Elena and San Gallicano, CRS-Centro Ricerca Sperimentale). p53 expression in these mice is disrupted by the insertion of neomycin (neo) resistance gene between exon 6 and exon 7 of p53 gene (Figure 24 A). Neo replaces approximatively 40% of the p53 coding sequences (Jacks et al., 1994).

Ft1^{+/kof} were crossed with p53^{+/ko} animals in order to obtain Ft1^{+/kof};p53^{+/ko} animals. Double Ft1^{+/kof};p53^{+/ko} animals were intercrossed to obtain fully ko animals.

Mouse manipulation

The work was conducted following European Parliament and Council Guideline 2010/63/UE of the 22nd September on the protection of animals used

for scientific purposes, after the approval of the work protocol by the Department for Veterinary Public Health and food Safety (P.le dell'Industria 20, 00144 Roma).

For Ft1 kof mouse colony establishment C57BL/6 chimeric mice were obtained from EMBL transgenic Mice Unit after injection of ES cells Kof for Ft1 gene into C57BL/6 host blastocyst. C57BL/6 outbred wt stock were obtained from Allevamenti Plaisant s.r.l.. All animals were maintained in cabin type isolators at standard environmental conditions (temperature 22–25°C, humidity 40–70%) with 12:12 dark/light photoperiod. Food and water were provided ad libitum in the conventional facility at the Plaisant s.r.l. farm animal in Castel Romano (RM). Same standard mouse maintenance conditions were applied in the mouse facility at IFO, Istituto Regina Elena and San Gallicano, CRS-Centro Ricerca Sperimentale.

All transgenic offsprings were weaned at 3 weeks of age and tail biopsies were collected for genotyping and transgene expression analysis. When needed mice were anaesthetised by intramuscular injection of Zoletil 20 (Virbac S.A., France). When required, animals were euthanised by asphyxiation with carbon dioxide or cervical dislocation.

Body weight for Ft1^{kof/kof} animals with strong phenotype and for age matched wt littermate was measured before weaning at the age of sacrifice. Growth rate was assessed in Ft1 kof colony and Ft1;p53 colony of the different genotypes starting at weaning, proper age matched controls were used.

gDNA extraction from tail biopsies and genotyping

Tail biopsies were digested overnight at 56°C with a proteinase K/SDS solution, genomic DNA was extracted using the NucleoSpin® Tissue columns kit (Macherey-Nagel, Duren, Germany) following manufacturer's instructions. Mice were genotyped by PCR amplification in a final volume of 25 µl containing 50 ng of gDNA obtained from tail, using the following primers:

Ft1 kof allele:

- Ft1 E4 forward: 5' – GTGAAGCAGAAGCTGCCAGGAGT–3';
- Ft1 E6 reverse: 5'– AGCTCACCCGAGGTGGGATCAA –3';

p53 ko allele:

- p53-X6 forward: 5' – AGCGTGGTGGTACCTTATGAGC – 3';
- p53-Neo19 forward: 5' – GCTATCAGGACATAGCGTTGGC – 3';
- p53-X7 reverse: 5' – GGATGGTGGTATACTCAGAGCC – 3'.

RNA extraction from tissue biopsies and Q-PCR assays

RNA was extracted from frozen tissues by addition of TRIzol (Invitrogen) according to manufacturer. Samples were homogenised in the presence of a grinding ball (Sartorius, Goettingen, Germany) at 2000 rpm for 60-90s (Mikro-Dismembrator U, Sartorius). RNA was extracted according to the manufacturer's instructions and after DNaseI 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 (see below) were selected using Primer Express software (Applied Biosystems). Reactions were performed as previously described (Piersanti et al., 2006).

Q-PCR primers:

- E3 forward: AACCAGTCCTCCACGAAGTGCA
- E3 reverse: TAGGGCTTCGCTATGGGTAGAGCA
- E6 forward: CCGTCTTTCACCCACTAGTTGAT
- E6 reverse: TTGCGAACGCTCTTTTCACA
- mGAPDH forward: GTGGCAAAGTGGAGATTGTTGCC
- mGAPDH reverse: TGTGCCGTTGAATTTGCCGT

X-ray analysis

X-ray images were taken using a Faxitron MX-20 Specimen Radiography System (Faxitron X-ray Corp., Wheeling, IL) at 24 kV for 6s. The images were captured with Medical Imaging Film HM Plus (Ferrania USA, Inc., Woodbury, MN).

Histology

Pathological examination on kof strong phenotype mice was performed at approximately 12 days of age. Skin, bone and heart of Ft1 kof strong phenotype mice were fixed in 10% phosphate buffered formalin. Tissues were cleared with ascendent alcohol concentration, embedded in paraffin and sectioned at 3.5 μm . Sections were hydrated with descendent alcohol concentration, stained with Hematoxylin (Carlo Erba) and Eosin (Sigma), cleared and mounted with DPX Mountant for Histology (Sigma). Representative pictures of tissue samples were taken with ZEISS-Axio Phot (Zeiss) microscope connected to Progress-C5 JENO-PTIK camera with the software Progress Mac (Capture PRO).

Statistics

Statistical analysis presented into this work were performed using GraphPad Prism software (5.0; GraphPad Software Inc.) or Microsoft Excel (Microsoft Corporation 2011-14.1.0) depending on the kind of the analysis. Statistical analyses of Kaplan-Meier survival curves were performed using the log-rank (Mantel-Cox) test. Inheritance of kof allele was analysed using the expected 1:2:1 mendelian ratio for heterozygous mating on the total number of animals obtained, expected and obtained mice were compared with χ^2 test. Quantification of the angle formed by cervical and thoracic vertebrae was measured with Photoshop CS6 analysis plugin. All results are expressed as the mean $\pm$ SEM. Two independent data sets were compared with the Student's *t* test (unpaired, 2-tailed). *p* values of 0.05 or less were considered to indicate significance.

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