The role of short telomeres as cause of natural aging in zebrafish

Madalena Carneiro

Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

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In English, with abstract in Portuguese

This thesis has been scanned for plagiarism and there was no conflict with published works.

To my family,

and my husband Francisco

Declaration/Declaração

I declare that this dissertation is a result of my own research carried out between June 2011 and May 2015 in the laboratory of Dr. Miguel Godinho Ferreira, Instituto Gulbenkian de Ciência in Oeiras, Portugal, with the co-supervision of Dr. José Pereira Leal, Instituto Gulbenkian de Ciência, Oeiras, Portugal. Chapter 2 has been published in PLOS Genetics, entitled “Telomerase is required for zebrafish lifespan”, Catarina M. Henriques, Madalena C. Carneiro, Inês M. Tenente, António Jacinto and Miguel G. Ferreira (2013). Chapter 3 is an adapted version of a manuscript under revision in PLOS Genetics, authored by Madalena C. Carneiro, Catarina M. Henriques, Tânia Carvalho and Miguel G. Ferreira.

Declaro que esta dissertação é o resultado do meu próprio trabalho desenvolvido entre Junho de 2011 e Maio de 2015 no laboratório do Dr. Miguel Godinho Ferreira, Instituto Gulbenkian de Ciência em Oeiras, Portugal, com co-orientação do Dr. José Pereira Leal, Instituto Gulbenkian de Ciência, Oeiras, Portugal. O capítulo 2 foi publicado no PLOS Genetics como “Telomerase is required for zebrafish lifespan”, Catarina M. Henriques, Madalena C. Carneiro, Inês M. Tenente, António Jacinto and Miguel G. Ferreira (2013). O capítulo 3 está integrado num manuscrito em revisão no PLOS Genetics da autoria de Madalena C. Carneiro, Catarina M. Henriques, Tânia Carvalho and Miguel G. Ferreira.

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List of abbreviations

ALT	Alternative Lengthening of Telomeres
AMPK	AMP-activated protein kinase
AT	Ataxia telangiectasia
ATP	Adenosine triphosphate
DC	Dyskeratosis Congenita
DDR	DNA damage response
DEG	Differentially expressed genes
DR	Dietary Restriction
DSB	Double strand break
FOXO	Forkhead transcription factor
GH	Growth hormone
GSEA	Gene set enrichment analysis
HGPS	Hutchinson-Gilford Progeria Syndrome
HHS	Hoyeraal-Hreidersson syndrome
HR	Homologous recombination
HSC	Hematopoietic stem cell
IIS	Insulin/IGF-1 signaling
IGF-1	Insulin-like growth factor 1
IFN-I	Type I interferon
IPF	Idiopathic Pulmonary Fibrosis
KO	Knockout
mTL	median Telomere Length

NAC	N-acetylcysteine
PGC1α	PPARg coactivator 1α
PGC1b	PPARg coactivator 1β
POT1	Protection of telomeres 1
RAP1	Repressor-activator protein
RMRP	RNA component of mitochondrial RNA processing endoribonuclease
ROS	Reactive oxygen species
SASP	Senescence-associated secretory phenotype
S6K1	Ribosomal S6 protein kinase 1
TC	Time course
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TIF	Telomere Dysfunction-Induced Foci
TIN2	TRF1 interacting protein
TOD	Time of death
TOR	Target of Rapamycin
TTI	Targeted telomere insertion
TRF1	TTAGGG repeat binding factor 1
TRF2	TTAGGG repeat binding factor 2
WS	Werner syndrome

Abstract

Why, and how, organisms age and ultimately die is a key question of modern biology. Telomeres are considered molecular timekeepers determining cellular lifespans. Because telomerase expression is restricted in human somatic cells, telomeres shorten progressively, potentially acting as central instigators for the loss of tissue regenerative capacity with age. Where – in which particular tissues – and how – by which molecular mechanisms – progressive telomere erosion promotes dysfunction in aging is still not entirely understood.

The past fifteen years have seen incredible progress in the evaluation of how telomere shortening impacts homeostasis in vertebrates, particularly with the creation of telomerase knockout mouse models. Notably, considerable differences can be found in mouse and human telomere biology, opening the stage for other telomerase deficient organisms to provide important complementary insights. More importantly, there is a growing demand to define the extent to which the artificial shortening of telomeres in these models reproduces the responses elicited by natural telomere erosion in physiological aging.

The work developed in this thesis introduces the telomerase mutant zebrafish (*tert*$^{-/-}$) as a promising model to study how short telomeres affect tissue homeostasis and lifespan. We show that first generation *tert*$^{-/-}$ die prematurely with short telomeres, developing a wide range of degenerative phenotypes that are accompanied by an acute (p53-dependent) depletion of proliferating cells. Remarkably, the majority of these degenerative phenotypes thoroughly resemble the dysfunctional events that are characteristic of natural zebrafish aging, from infertility to gastrointestinal atrophy, sarcopenia and even alterations in motor behavior. Thus, the telomerase mutant zebrafish is a powerful organism to model and study the phenomena underlying aging-associated pathologies.

How telomere erosion contributes to the dysfunction of different tissues throughout the normal lifetime of an organism remains largely unknown. It would be expected that tissue replication dictates the rate at which telomeres shorten and therefore their potential for causing tissue damage in aging. Unexpectedly, we find that this is not true for zebrafish aging. Our work shows that telomeres shorten irrespectively of tissue replication rates with age, reaching critical lengths specifically in the gut and muscle. The accumulation of short telomeres and DDRs specifically in these organs anticipates not only local dysfunction (intestinal inflammation and sarcopenia) but also the onset of aging-associated diseases in other tissues, including cancer.

The future of targeted rejuvenation therapies lies in identifying the molecular mechanisms mediating telomere-induced dysfunction in aging. This thesis offers the first global transcriptional profiling study directly identifying similar gene expression changes emerging over time in WT and *tert*$^{-/-}$ tissues. A clear transcriptional signature common to both *tert*$^{-/-}$ and WT aging involves the repression of glutathione metabolism, a key oxidative defense mechanism, in line with previous studies reporting an association between telomere shortening and elevated levels of ROS. Particularly, *tert*$^{-/-}$ zebrafish tissues also show an abnormal accumulation of ROS and diminished Sod-2 expression. We now demonstrate that two independent antioxidant treatments, with the general scavenger NAC or with the superoxide dismutase mimetic MitoTEMPO, are sufficient to rescue homeostasis and lifespan defects caused by extreme telomere dysfunction. Thus, telomere-induced disruption of homeostasis relies, at least in part, in defective oxidative stress protection. Future studies will determine the potential benefits of such therapies in natural aging contexts.

Sumário

A razão pela qual os organismos envelhecem permanece um dos grandes mistérios da biologia moderna. Os telómeros funcionam como relógios moleculares que determinam o tempo de vida de uma célula. Com a passagem do tempo, os telómeros encurtam progressivamente devido à ausência da telomerase em células somáticas humanas, atuando como causa potencial para a perda de capacidade regenerativa. Ainda não compreendemos totalmente onde – quais os órgãos – e como – através de que mecanismos –a erosão telomérica promove disfunção no envelhecimento.

O estudo do impacto do encurtamento telomérico na homeostasia de organismos vertebrados sofreu uma evolução considerável nos últimos quinze anos, particularmente com a criação de modelos de ratinho *knockout* de telomerase. No entanto, existem vários aspetos que diferenciam a biologia de telómeros em ratinhos e humanos, realçando a necessidade de utilizar modelos alternativos. Por outro lado, torna-se cada vez mais premente determinar de que forma o encurtamento artificial de telómeros nestes modelos recria os fenótipos causados por encurtamento fisiológico no envelhecimento.

Nesta tese propomos o peixe zebra mutante de telomerase (*tert*$^{-/-}$) como novo modelo promissor para determinar de que forma os telómeros curtos afetam a homeostasia de tecidos e sobrevivência. O nosso trabalho demonstra que a primeira geração de *tert*$^{-/-}$ morre prematuramente, com vários fenótipos de degeneração e defeitos de proliferação dependentes da ativação de p53. Surpreendentemente, a maioria destes fenótipos assemelha-se à disfunção característica do envelhecimento natural, desde a infertilidade à atrofia do sistema gastrointestinal, perda de tecido muscular e alterações comportamentais. Assim, o peixe zebra mutante de telomerase é um organismo que poderá ser utilizado para estudar os fenómenos que levam ao desenvolvimento de várias patologias com o envelhecimento natural.

Ainda não é claro em que medida o encurtamento de telómeros afeta diferentes tecidos no envelhecimento. Seria de esperar que as taxas de divisão celular desses tecidos determinasse a velocidade a que os telómeros encurtam e, dessa forma, o potencial desse encurtamento para causar dano. No entanto, não é isso que se verifica no envelhecimento do peixe zebra. Esta tese demonstra que os telómeros encurtam com a idade em diferentes tecidos, independentemente das suas taxas de divisão, atingindo tamanhos extremamente curtos no intestino e no músculo. A acumulação de telómeros curtos, juntamente com a ativação de respostas a dano no DNA, antecipam não só eventos de disfunção local (inflamação e perda de massa muscular) mas ainda patologias de outros tecidos, nomeadamente cancro.

A identificação dos mecanismos moleculares através dos quais os telómeros curtos promovem envelhecimento é fulcral para o desenvolvimento de novas terapias de rejuvenescimento. Parte do trabalho desenvolvido nesta tese inclui o primeiro estudo transcricional que identifica as alterações de expressão génica ao longo do tempo que são comuns a *tert*$^{-/-}$ e peixe zebra selvagens em diferentes tecidos. Uma das assinaturas mais proeminentes implica a repressão de genes envolvidos no metabolismo do glutatião, um antioxidante central do organismo. Em consonância com estes resultados, estudos anteriores reportam uma associação entre encurtamento de telómeros e elevados níveis de espécies reativas de oxigénio (ROS). Os tecidos de *tert*$^{-/-}$ também acumulam níveis anormais de ROS e mostram ainda uma diminuição na expressão de Sod-2. Tratamentos com dois antioxidantes – N-acetilcisteína (NAC) e o mimético do superóxido dismutase (MitoTEMPO) – são suficientes para reverter parcialmente os defeitos de sobrevivência em organismos com extrema disfunção telomérica. O nosso trabalho demonstra que os fenótipos causados pelo encurtamento de telómeros são em parte atribuíveis a uma proteção deficiente contra stress oxidativo. O benefício da aplicação destas terapias em contextos de envelhecimento natural deverá ser avaliado em estudos futuros.

Chapter 1 | General introduction

1.1 Why do we age?

Aging is broadly defined as the progressive loss of function in time that leads to increased susceptibility to disease and ultimately death. Such traits are clearly disadvantageous for optimum organismal fitness (1), yet various species are affected by them and show a conserved exponential rise in mortality in their last third of life (2). So why does aging occur?

One of the earliest theories on the nature of aging, advanced by August Weismann, postulated that aging and death of older organisms are altruistically programmed to limit population size and promote its turnover. This would reduce competition for resources and benefit subsequent generations in adapting to new environments (1, 3). The molecular mechanism determining programmed death of organisms would be directly connected to the number of cell divisions a somatic cell undergoes (later termed "senescence" (4)), or in Weismann's own words: "that life span is connected with the number of somatic cell generations which follow after each other in the course of an individual life" (5). Two arguments contradict this hypothesis. First, the existence of such an intrinsic programmed death mechanism implies that specific genes that "accelerate" aging are positively selected in every generation, a concept hard to reconcile given they would pose a strong disadvantage for an individual's survival. A second fact arguing against this theory is that animals reared in protected environments live much longer than in their natural wild habitats (6). This is primarily due to the absence of extrinsic hazards such as predators, starvation and harsh weather conditions (1). Thus, there is little opportunity for programmed death (and for the action of natural selection) to actually contribute to mortality in the wild.

An important principle that could be drawn from the high extrinsic mortality observed in the wild is that aging must have evolved due to a decline in the

force of selection to remove deleterious mutations acting late in life (7). This concept was key for the proposal of the “mutation accumulation” theory by Peter Medawar (8). Medawar postulated that if specific mutations have a high cost only in advanced ages (after animals have reproduced) they are not selected against and may therefore accumulate and be passed on to the next generation. The accumulation of deleterious alleles late in life versus elimination of those expressed during the active reproductive periods, would therefore predict the increase in disease observed in aging.

In 1957, an alternative theory of George Williams proposed that aging could be explained by the pleiotropic action of genes on fitness/several traits in early versus late life periods (7). William’s theory suggested that if a given gene has a beneficial impact during early reproductive years, it is favored by selection even if at the cost of becoming detrimental in advanced ages (antagonistic effect). A commonly accepted example of this ‘antagonistic pleiotropy’ hypothesis of aging is replicative senescence, a mechanism proposed to have evolved to block tumor growth in early life but that creates favorable microenvironments for cancer to develop in advanced ages (9).

A specific variant of the antagonistic pleiotropy view is the disposable soma theory (10), which postulates that energy resources must be carefully optimized between growth/reproduction and somatic maintenance/repair in species that segregate soma from germline. Given the high level of extrinsic mortality in the wild, metabolic resources (such as DNA repair processes) must invest in somatic maintenance just long enough to allow organisms to efficiently reproduce. The outcome of such strategy is post reproductive somatic disintegration. Accordingly, artificial selection experiments mostly using *Drosophila melanogaster*, show that delaying reproduction increases the force of selection in later ages and extends longevity (11, 12). Moreover, different studies show that a better investment in maintenance of somatic cell integrity,

by enhanced DNA repair and lower levels of oxidative damage, correlates directly with mammalian longevity (1, 13).

More recently, Blagosklonny proposed that aging is the consequence of hyperfunctioning of developmental processes in late life (14), possibly due to deregulation of signaling of the target of Rapamycin (TOR) pathway (15). Because early life processes fail to turn off, unintended hypertrophy or hyperplasia occur, resulting in several pathologies, such as cancer, hypertension and hypercoagulation (15). In this perspective, aging is nothing but the sum effect of these pathologies that results in death.

Despite these theories and other concepts that have emerged throughout the years, aging remains a paradoxical unsolved scientific problem. We still do not entirely understand why and how the dysfunction of different tissues and cell types occurs in time, which mechanistic processes underlie such failure (and what interconnects them) and what exactly is the role played by the microenvironment and systemic signaling in aging (16). Nevertheless, striking discoveries on the biology of aging have shed light on central molecular and cellular processes regulating lifespan and homeostasis. These discoveries are to a limited extent reviewed in the following section.

1.2 Aging is genetically regulated

Until recently, aging was considered the end product of several stochastic events leading to entropic multi-organ dysfunction. The key observations, made by Cynthia Kenyon, that single-gene mutations and environmental manipulations significantly extend the lifespan of small short-lived organisms (17-19) showed, however, that as any other biological process, aging is subject to regulation. Two additional facts support that aging is genetically regulated: 1) human lifespan is approximately 25% heritable (20) and 2) different species have extremely divergent lifespans. Average lifespan is approximately 80 years

for humans in developed countries, 3 weeks for worms and 3 years for mice, meaning it has suffered an increase of over 1,000 fold throughout the course of evolution (18). Curiously, the strongest phenotype correlating with variation in interspecies longevity is body size (21).

A portion of the mutations first found to extend lifespan in *Caenorhabditis elegans* and *Drosophila Melanogaster* turned out to have a conserved role in mouse aging (18), thus supporting the use of short-lived organisms for the study of mammalian aging and potential benefit of human health. Many of these mutations ameliorate maintenance and repair of tissues by decreasing the activity of nutrient sensing pathways and enhancing protection against various sources of cellular stress (22). Importantly, manipulating these pathways not only extends longevity, but also delays the onset of age-related disease.

Since the discovery that perturbations in nutrient sensing influences longevity, studies ranging from yeast to mammals identified a series of others hallmarks of aging, recently (23) hierarchically classified as:

- **Primary** – initiating triggers of progressive damage in time (genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis);
- **Antagonistic** – hallmarks that cause detrimental effects in response to primary triggers (deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence);
- **Integrative** – responses to accumulated damage caused by primary and antagonistic hallmarks (stem cell exhaustion, altered intercellular communication).

The next sections review the data establishing some of these hallmarks (and related molecular processes) as regulators of aging, particularly those that are more relevant to the work developed in this thesis. Furthermore, a special emphasis is given on how they are interconnected with telomere shortening. The role of short telomeres as particular initiating triggers of aging is explored in more detail in a separate chapter, as this is the core subject of this thesis.

1.2.1 – Deregulated nutrient sensing

As previously mentioned, reducing the activity of nutrient sensing pathways extends longevity across the evolutionary spectrum, from *C. elegans* to mice. This section briefly reviews how three nutrient sensors – insulin/insulin-like growth factor (IGF-1) signaling (IIS), target of Rapamycin (TOR) and AMP kinase (AMPK) – influence aging in *C. elegans, Drosophila* and mice. Curiously, all three sensors mediate to some extent the increase in lifespan obtained when organisms are subjected to Dietary Restriction (DR) (24).

Inhibition of the IIS pathway increases lifespan by 2-fold in worms (17), by ca. 26% in mice (25) and by 48%-85% in flies (26, 27). In worms and flies, lifespan extension requires the forkhead transcription factor – FOXO (17, 28, 29), which activates mechanisms of oxidative defense, xenobiotic metabolism and pathogen resistance (22). Although it remains unknown if IIS-influence on mammalian lifespan is also dependent on FOXOs transcription (there are four in mice), variants in FOXO3a have been associated with cases of extreme human longevity (30). Interestingly, IIS signaling in specific tissues is sufficient to delay aging, as fat-specific insulin receptor knockout mice and flies overexpressing dFOXO in the adipose tissue live longer (31, 32).

Similarly to inhibition of the IIS pathway, downregulation of TOR complex 1, TORC1 or of downstream target ribosomal S6 protein kinase 1, S6K1, extends longevity of worms, flies and mice, reducing the incidence of age-associated pathologies (33-36). In flies and worms, extension of lifespan by reduced TOR signaling requires activation of autophagy and a generalized shut off in translation (with the remarkable exception of mitochondrial respiration genes, whose enhanced activity is actually required for lifespan to be extended), associating with an increased resistance to thermal stress (37-39).

Finally, overexpression of AMPK, an energy and nutrient sensor that responds to high AMP/ATP ratios by activating catabolic pathways, extends lifespan in *C.*

elegans and potentially in mice via chemical stimulation with metformin (40, 41). In *C. elegans,* AMPK regulates autophagy by directly inhibiting mTORC1 (42) and improves cellular stress resistance by activating FOXO (43).

Given the lifespan-extension effect of reduced IIS and TOR signaling, it is somehow contradictory that natural aging associates with reduced levels of growth hormone GH and IGF-1 (44) and that telomere shortening in particular suppresses IGF-1, mTOR signalling, and activates AMPK (45). Perhaps, as proposed for other models of induced DNA damage, repression of the somatotrophic axis in response to telomere dysfunction is a pro-survival strategy adopted by cells to re-allocate energy resources from growth to cellular repair (46).

1.2.2 – Mitochondrial dysfunction

As we age, mitochondria become progressively dysfunctional and inefficient in synthesizing adenosine triphosphate (ATP) via the respiratory chain. Consequently, there is excessive leakage of reactive oxygen species (ROS), which accumulate in the cell and promote several types of damage (‘Free radical theory of aging’ (47)). ROS generate carbonyl derivatives that alter protein function (48), promote lipid peroxidation that decreases membrane fluidity (49), and cause DNA lesions by oxidizing bases that become prone to mispairing (50). Mitochondrial DNA is particularly sensitive to ROS-induced damage, given its proximity to superoxide generation (51), as are GC regions (including telomeres), given their high content in guanine-repeats (52, 53). ROS induced damage may therefore contribute to the increase in mtDNA mutations and telomere shortening observed in aging (54, 55). Telomere attrition in turn was shown to fuel the production of ROS, by impairing the biogenesis of mitochondria (via repression of PGC1α and PGC1β) and consequently lowering the expression of oxidative defense genes (Sod-1 and GPx) (56).

The role of oxidative stress in aging and lifespan remains an enigma. Initial studies supported the free radical theory of aging by showing that: 1) deficiencies in mouse methionine sulfoxide reductase or in flie copper/zinc-containing superoxide dismutase increase ROS levels and reduce lifespan (57, 58) and 2) overexpression of catalase and superoxide dismutase (or their supplementation via mimetics) extends lifespan in flies, worms and mice (59-62). However, recent experiments show contradictory results: reductions in superoxide dismutase activity either extends or does not affect worm and mouse lifespans (63-65) and inhibition of respiration or restriction of glucose require mild increases in ROS to extend lifespan in worms (66, 67). Because of these divergent results, ROS was recently proposed to primarily work as a "stress-elicited survival signal", having a pro-homeostatic function upon damage, which becomes compromised and decreases lifespan when the levels of damage are too high (23).

Beyond generating elevated levels of ROS, accumulation of dysfunctional mitochondria due to ineffective autophagy-mediated elimination, may compromise tissue homeostasis with aging by increasing apoptotic or pro-inflammatory signaling (68) or by inducing nuclear genome instability via inhibition of iron-sulphur cluster biogenesis (69). Conversely, increased mitochondrial activity, by overexpression of PGC1α in stem and progenitor cells of the digestive tract, delays aging and extends lifespan in flies (70).

1.2.3 – Cellular senescence

In 1961, Leonard Hayflick reported that human fibroblasts permanently arrest cell cycle after proliferating a finite number of times, reaching a state designated of replicative senescence (4). As cellular senescence prevented the uncontrolled proliferation of pro-tumorigenic cells, it was hypothesized to have evolved primarily as a tumour suppressive mechanism for early ages (71, 72). However, such mechanism would come at the high cost of restricting tissue

regeneration and repair in post-reproductive ages (73). Accordingly, indirect evidence supports a role for senescence in natural aging phenotypes. Senescent cells progressively accumulate in mammalian tissues (74) and, although unable to divide further, remain in a metabolic active state (75-78) and hyper secrete a series of pro-inflammatory cytokines, metalloproteinases and growth factors (79). Judith Campisi termed this secretory phenotype the 'senescence-associated secretory phenotype' –SASP – and showed that it alters the tissue microenvironment, promoting inflammation (which in turn is involved in different aging pathologies) (80) and attenuating tissue repair (79, 81). Curiously, the senescent response is amplified by intercellular communication, whereby senescent cells induce DDR and senescence in neighboring bystanders, via gap junction-mediated cell-cell contact or direct transfer of proteins (82, 83). Studies conducted in the progeroid BubR1 mouse background showed that elimination of senescent cells delays the premature onset of sarcopenia and cataracts, also strongly supporting a role for senescence in aging (84).

What are the mechanisms driving senescence? Telomere shortening was initially identified as the reason why replicative cells eventually senesced in Hayflick's experiments (reviewed in detail in section 1.3.4.1.2 (4, 85)). However, different studies later showed that other forms of severe DNA damage (particularly the ones that results in DSBs), including oncogene induced damage (e.g. by RAS) and changes in chromatin states (e.g. by histone deacetylase inhibitors), also induce senescence (75, 77, 78). Most of these processes converge in activation of either p53 (and, consequently p21) or p16^{INK4a}-pRB pathways (or both) (75, 77, 78). The senescence response triggered by telomere shortening and DDR in mouse cells is primarily p53-dependent (86, 87). In this context, inactivation of p53, p21 or other DNA Damage Response (DDR) proteins allows senescent cells to resume growth, if p16^{INK4a} (the second barrier to unlimited division) expression levels are low (88, 89).

While there is mounting evidence for processes that underlie senescence in cultured cells, the mechanisms driving senescence in vivo remain poorly characterized. Interestingly, mice with hyperactive p53 alleles are prematurely aged (90). In contrast, the deletion of p53 in telomere dysfunctional contexts prevents the elimination of damaged stem cells and shortens organismal lifespan (91).

As an aside, while the tumor suppressive (antagonistic pleiotropic) view on why senescence evolved has prevailed for many years, recent evidence now supports that senescence may also play instructive roles for tissue remodeling during normal mammalian development (92, 93).

1.2.4 – Stem cell exhaustion

Tissue homeostasis requires a functional somatic stem cell reservoir for continuous renewal and maintenance of cellularity over time (94). However, with aging, stem cells progressively lose replicative and differentiation properties in different mammalian organs (e.g. brain, muscle and hematopoietic tissues) (95). This loss may jeopardize the replenishment of tissues over time, and thereby contribute to their dysfunction in aging. In support of this view, progeric mouse models with deletions in different DNA repair kinases (ATM and ATR) (96, 97) show premature ROS-induced attrition of stem cells, which results in early tissue atrophy. Still, how/in which organs stem cell exhaustion contributes to loss of homeostasis in natural aging remains unknown (95).

Why does stem cell function become progressively impaired? Different studies support this is triggered by both intrinsic factors, such as DNA damage and extrinsic factors, such as alterations in stem cell niches. DNA damage accumulation occurs mainly due to replication stress, reduced operation of DNA repair systems and telomere attrition, which couple with higher expression levels of $p16^{INK4a}$ (98). Importantly, self-renewal of long-lived damaged stem cells with expansion capacities poses a high risk for malignant transformation

(99). Short telomeres in particular impair stem cell function by reducing their proliferation, differentiation and mobilization potential but also by affecting the stem cell microenvironments (100-108). In addition, parabiosis experiments have shown that the impaired function of muscle and neural stem cell compartments of 'aged microenvironments' can be restored upon exposure to 'young' systemic factors (109).

Different interventions prevent the decline on stem cell proliferation in the intestine, muscle, skin and hematopoietic tissue. Clear examples include DR and Rapamycin treatments (110), whose effects on stem cell renewal are mediated non-cell autonomously by mTORC1.

1.2.5 – Genomic instability

One of the long-standing views on aging is that it is caused by 'wear and tear', i.e. by the accumulation of DNA damage in an organism throughout its lifetime. Mutations do accumulate in the nuclear and mitochondrial genomes of human and mouse cells over time (111, 112) and aged organisms display higher mutation rates than younger counterparts (particularly genome rearrangements) (113). The causes of such mutations remain largely unknown, but seem to include various exogenous and endogenous agents, such as ROS and errors in DNA replication (23). Associated with a higher DNA damage load, DNA repair processes become inefficient with aging (114). Accordingly, mice models carrying mutations in DNA repair genes exhibit several tissue abnormalities and die prematurely. Notable examples include mice deficient in the nucleotide excision repair pathway (Xeroderma pigmentosum and Cockayne syndromes) (46), mice with mutations in RecQ helicases (Werner and Bloom syndrome), mice with genetic deletions of base excision repair genes (SIRT6) and mice with deletions in the non-homologous end-joining pathway (Ku80) (114-116). Accumulation of DNA lesions may contribute to aging by increasing apoptosis (and therefore reducing tissue cellularity and homeostasis), by affecting gene

transcription and by perturbing the structure of chromatin (114).

Interestingly, many DNA repair proteins associate with telomeres and are essential for telomere maintenance, one of them being SIRT6. In the absence of this chromatin-associated protein, mice die prematurely, develop several tissue abnormalities and show signs of telomere dysfunction (and fusions) (117, 118).

Other mechanisms are in place to ensure that the integrity of chromosome ends is maintained, but because these mechanisms function sub optimally telomeres shorten progressively, becoming themselves drivers of genome instability and tissue dysfunction. The evidence establishing telomere attrition as a primary hallmark of aging is reviewed in depth in the following sections.

1.3 Telomere shortening in aging

1.3.1 – Telomeres and telomerase: a brief historical note

1.3.1.1 Telomeres and the end replication problem

The ends of chromosomes were first proposed to have crucial roles in genome stability by Hermann Muller (119) and Barbara McClintock's pioneering findings (120). In 1939, Barbara McClintock's cytogenetic studies in maize showed that the ends of chromosomes are protected from the typical rearrangements and mitotic breakage-fusion-bridge cycles observed in other chromosome breaks (120). These cycles were known to cause genomic instability by promoting abnormal segregation of genetic information during cell division. It became clear that the ends of chromosomes required specialized structures to protect them from fusing between themselves or with other breaks and were, therefore, necessary for chromosome stability. Muller designated these structures of "telomeres", from the Greek words *telos* (end) and *meros* (part) (119). However, it was not until the discovery of DNA polymerases and their role in semi-

conservative replication (121), many years later, that the molecular nature and function of telomeres was revealed. Semi-conservative DNA replication was found to occur in the 5' to 3' direction and to rely on a template complementary molecule (122). As DNA polymerases cannot initiate synthesis of DNA on their own, they require the repeated synthesis of complementary RNA primers, which are elongated from 3' hydroxyl groups into Okazaki fragments (123). To ensure genome integrity, RNA primers are eventually removed, leaving no 3' hydroxyl group at the very 5' end to prime DNA synthesis. Consequently, a 3'-overhang is formed (124, 125). Due to this 'end replication problem', only formally demonstrated much later in *in vitro* experiments (126), continuous cell division would lead to gradual resection of DNA ends. However, the presence of a 3' G-rich overhang was later found to be a conserved feature of telomeres, required for their structural integrity, end protection, and actively maintained by resection (127). Thus, in 1995 Lingner and co-workers revised the model for the end-replication problem, proposing it was not a consequence of incomplete 5' end synthesis by DNA polymerases but rather a result of the inability to completely regenerate the G-overhang in the leading strand (128).

1.3.1.2 The discovery of telomerase

With the discovery that DNA ends gradually erode due to the end replication problem, another important question appeared: how is it that faithful chromosome replication is ensured through generations of cell division? To answer this Elizabeth Blackburn and Joseph Gall set out to determine the exact sequence of terminal ends in macronuclear ribosomal DNA molecules of the unicellular ciliate *Tetrahymena thermophila*. They found that *Tetrahymena* telomeres were composed of repetitive non-protein coding G-rich sequences (5'-T2G4-3'), with a variable number of repeats per DNA end (129). Sequencing of DNA ends of other ciliates, not long after, consistently revealed the presence of a related sequence of non-coding repeats (5'-T4G4-3') (130). Ligation of

Tetrahymena end repeats in linear plasmids introduced in budding yeast was found to maintain the plasmids in a linear stable state, allowing for replication to take place (131). This strongly suggested that the chromosomal end structure required for replication and end protection is evolutionary conserved (131). Another interesting observation in this study was that the ends of linear plasmids after transformation were not composed of *Tetrahymena* DNA sequences, but rather of yeast telomeric repeats. Together with studies showing there is extreme variation in telomere length between cells (132), these findings pointed towards the existence of one or more mechanisms capable of carrying the synthesis of telomeric DNA, without requiring a template parental strand. In search of such mechanisms, Carol Greider and Elizabeth Blackburn showed that *Tetrahymena* cell extracts possessed a specific terminal transferase enzymatic activity capable of extending *Tetrahymena*-type synthetic oligonucleotides $(TTGGGG)_4$ and yeast telomeric DNA primers (133). This enzyme, later designated of telomerase, was identified as a ribonucleoprotein complex that uses an essential RNA template, complementary to the G-rich telomeric sequence, to add new repeats to 3' ends (Figure 1.1, (134, 135)).

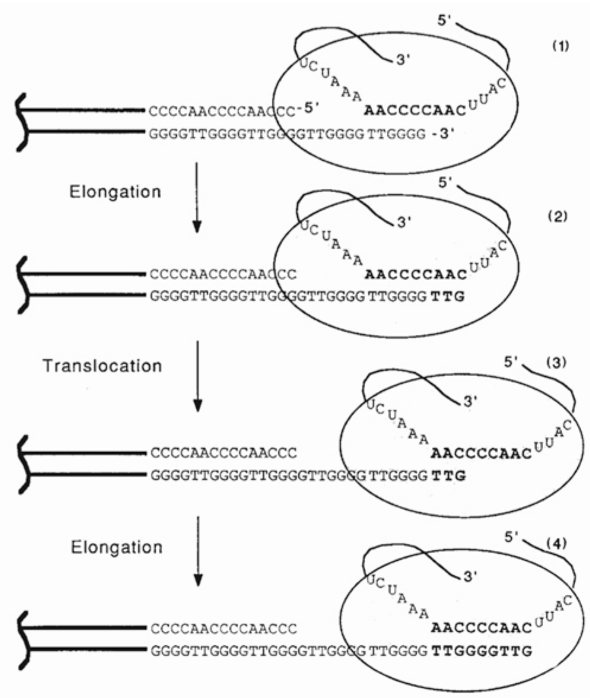

Figure 1.1 – Model for elongation of telomeres by telomerase.
'The Tetrahymena telomere is shown containing a 13-base overhanging TTGGGG strand. (1) After recognition of the TTGGGG strand by telomerase, the 3' most nucleotides are hybridized to the CAACCCCAA sequence in the RNA. (2) The sequence TTG is then added one nucleotide at a time. (3) Translocation then repositions the 3' end of the TTGGGG strand such that the 3' most TTG nucleotides are hybridized to the RNA component of telomerase. (4) Elongation occurs again, copying the template sequence to complete the TTGGGGTTGG sequence. This mechanism explains how oligonucleotides with 3' ends terminating at any nucleotide with the sequence TTGGGG are correctly elongated to yield perfect tandem repeats of (TTGGGG)n'
Figure and legend taken from (135).

Studies performed in cultured cells from several human tissues and tumors, later came to show that telomerase is repressed in somatic tissues, promoting telomere shortening with cell division, but remains active in germ and immortal cancer cells (136).

1.3.2 – Telomere structure and function

The ends of vertebrate chromosomes are composed of repetitive DNA sequences $(TTAGGG/CCCTAA)_n$, ranging from 5 to 15 kb in human cells (137), that associate with a protective protein complex termed 'shelterin' (138) (Figures 1.2 and 1.3). Telomeres have two main functions: prevent chromosome ends from being recognized as deleterious DNA breaks and ensure their efficient maintenance by recruitment of telomerase (138). These functions require both the DNA tandem array of repeats (composed of duplex structures and 3' single-stranded G-tails) and the shelterin proteins that bind them.

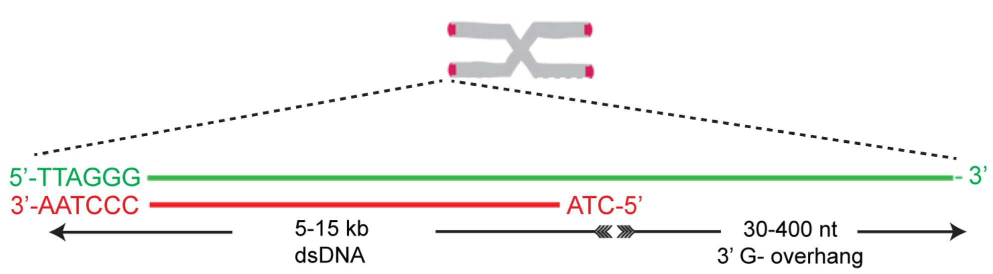

Figure 1.2 – Human telomeres.
The ends of human chromosomes consist of repetitive DNA sequences, ranging from 5 to 15 kb, with a G rich leading strand and a C rich lagging strand. The G strand forms a 3' overhang. Adapted from (139)

The generation of a 3' G-rich overhang at telomeres was initially attributed to the inability of cells to completely replicate the lagging strand (end replication problem, see section 1.3.1.1, (124, 125). However, additional regulated nucleolytic activity was found to promote resection of the 5'-end (128) (discussed in more detail in section 1.3.4.1.1). The G-rich overhang is composed of 30-400 nucleotides in mammalian chromosomes (140, 141) and is

required for stability and efficient binding of shelterin proteins, and therefore for telomere protection (138) (Figure 1.3).

A large number of proteins associate transiently with telomeric DNA but only those that fulfill certain criteria belong to shelterin: they have to be specifically abundant at telomeres, be present at telomeres in any time throughout the cell cycle and have a telomere-specific known function (138). In human cells, six proteins respect these conditions (Figure 1.3).

TRF1 and its paralog TRF2 (TTAGGG repeat binding factors 1 and 2) were the first proteins found to have telomere-binding specificity in mammalian extracts (142, 143). Both exhibit several similarities, binding double-stranded telomeric DNA as homodimers via a C-terminal Myb-type motif that recognizes specifically TAGGGTT sites and not other repetitive nucleotide sequences (138). They diverge in their N terminus domain, which is acidic in TRF1 and basic in TRF2 (142).

Protection of telomeres 1 (POT1) binds specifically the G-rich single stranded telomeric overhang and is highly conserved across eukaryotes (144). POT1 interacts with TRF1/2 (which also regulate POT1 binding at telomeres (145)) through another shelterin component, TRF1 interacting protein TIN2 (146, 147). TIN2 also bridges together TRF1 and TRF2, stabilizing the TRF complex at telomeres (148), and recruits TPP1 (149) that binds the C-terminal domain of POT1 (150, 151). Finally, repressor-activator protein RAP1, a shelterin component first described in budding yeast (152, 153), is recruited to telomeres via interaction of its C-terminal domain with TRF2 (154).

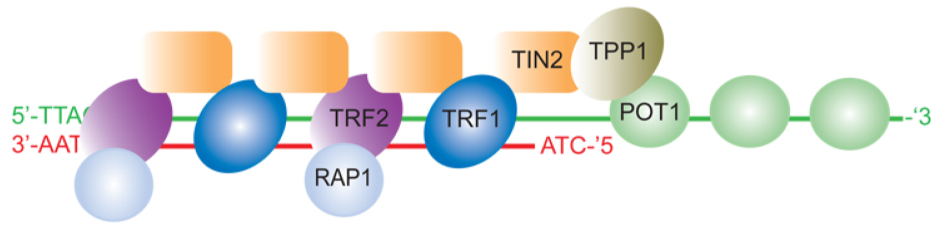

Figure 1.3 – The shelterin complex.

'Shelterin is composed of double stranded telomeric repeat binding factors TRF1 and TRF2, the TRF2 interacting factor RAP1, the bridging molecules TIN2 and TPP1 and the telomeric protection factor POT1, together covering the double and single stranded repeats'. Figure and legend taken from (139)

1.3.2.1 Shelterin regulates telomere length, 3'overhang structure and prevents DDR activation

Studies measuring the consequences of disrupting the function of different shelterin proteins have been instrumental for dissecting the functions of shelterin in telomere protection. Shelterin forms a stable complex (147) and has been found to regulate telomere length as well as the 3'overhang structure, and to prevent the activation of DNA damage responses (DDRs) at telomeres (Figure 1.4).

TRF1: this shelterin component acts as a negative regulator of telomerase, prevents activation of DDRs at telomeres and allows for proper telomeric replication (155).

Overexpression of human TRF1 (hTRF1) results in telomere shortening and inhibition of hTRF1 binding at telomeres (by expression of a dominant-negative hTRF1 mutant) leads to telomere elongation (156). TRF1 prevents telomerase from elongating telomeres via its interactions with TIN2, TPP1 and POT1 (146, 156-158). Notably, deletion of the N-terminal binding domain of POT1 abrogates TRF1 inhibitory effect on telomerase, resulting in fast telomere elongation (145). Deletion of TRF1 from mouse cells activates ATM and ATR downstream checkpoints, resulting in chromosomal instability, cellular senescence (159), and reduced telomeric association of TIN2 and TRF2 (160).

TRF2: this shelterin component has a key role in telomere capping, in stabilizing t-loops (see section 1.3.2.2) and in preventing activation of ATM-dependent DDRs at telomeres.

Inhibition or expression of a dominant-negative mutant of human TRF2 (hTRF2), results in ca. 50% loss of the G-rich telomeric overhang due to cleavage by the nucleotide excision repair nuclease ERCC1/XPF (161). Consequently, DNA ligase IV-mediated end-to-end fusions form, retaining the telomeric sequences (162, 163), an ATM-dependent DDR cascade is activated culminating in apoptosis (162) and senescence (163). It is now clear that TRF2 blocks the non-homologous end-joining (NHEJ) pathway in the G1 phase of the cell cycle (164), and together with POT1 prevents activation of homology directed repair (HDR) at telomeres in G2 (165).

Similarly to TRF1, TRF2 acts as a negative regulator of telomere length, possibly via interaction with POT1. Overexpression or targeting of hTRF2 to telomeres results in telomere shortening (166) and disruption of the TRF2-POT1 association causes telomere elongation (167). Titia de Lange and colleagues showed that overexpression of TRF2 increases the rate of telomere shortening without accelerating the onset of senescence (168).

TRF1 and TRF2/RAP1 are the main recruiters (within shelterin) of non-shelterin telomere associated proteins. These proteins are less abundant at telomeres and may perform other functions in the cell, but nevertheless are important for telomere end protection or length regulation. Examples of such proteins include tankyrase 1 and 2 – that bind to TRF1, and WRN and ORC1 – that bind the N terminus of TRF2 (169).

POT1: this shelterin component prevents the activation of ATR-dependent DDRs at telomeres, protects the G-overhang from degradation, coats the 5'end of duplex telomeric DNA and regulates telomere length via telomerase recruitment.

Reduction of human POT1 (hPOT1) with shRNA or RNAi elicits a transient DDR at telomeres, causes 30%-40% loss of the 3' overhang and disrupts the precise terminal sequence found at 5' overhangs (which normally ends in ATC-5′) (170, 171). In addition, hPOT1 knockdown significantly induces apoptosis

and cellular senescence with no overall increase in fusions (170). In contrast to humans, mice possess two isoforms of POT1 – mPOT1a and mPOT1b (172). mPOT1a mainly suppresses (ATR-dependent) DDRs at telomeres and has critical functions in overall maintenance of chromosomal stability (its absence results in sister chromatid exchanges), while mPOT1b regulates the amount of single stranded DNA at G-overhangs (165, 172). Deletion of mPOT1b promotes extension of the G-overhang, possibly via exonucleolytic degradation of the 5'-strand (172).

In addition, POT1 also regulates telomere length. Overexpression of hPOT1 (173) and deletion of the N-terminal OB-fold domain of hPOT1 both lead to rapid telomere elongation, via recruitment of telomerase (145). The interplay between POT1 and telomerase seems to be highly dynamic, as in vitro studies show that hPOT1 competes with telomerase for G-overhang binding (174).

TIN2: this shelterin component essentially tethers POT1/TPP1 to TRF1/2, ensuring overall stabilization of the shelterin complex. In addition, TIN2 prevents activation of ATR-dependent checkpoints at telomeres and acts as a regulator of telomere length.

Absence of human TIN2 (hTIN2) displaces the TRF complex and POT1/TPP1 from telomeres, promoting recruitment of RPA and activation of ATR downstream checkpoints, eliciting typical mPOT1a/b deletion phenotypes (148, 175). TIN2 may act as a negative or positive regulator of telomere length. A specific mutation in the N terminal domain of human TIN2 (hTIN2), which does not affect binding to hTRF1, elongates telomeres (146). However, other TIN2 mutations usually found in disease contexts or reduction of TIN2 levels using shRNA, actually accelerate telomere shortening, in some cases by affecting TPP1-dependent telomerase recruitment (176).

TPP1: this shelterin component enhances POT1 affinity for single stranded telomeric DNA, modulating its function in end protection and in telomere length

regulation. In addition, TPP1 plays important roles in telomerase recruitment and processivity.

Disruption of the hPOT1-TPP1 interaction or reduction of hTPP1 expression using RNAi, reduces the telomeric localization of POT1, activates DDR and promotes elongation of telomeres (151, 158, 177). At the same time, TPP1 by itself or together with POT1 recruits telomerase and increases its processivity for telomere elongation (150, 177). When telomerase is actively adding telomeric repeats, a complex formed by TPP1 and POT1 promotes translocation of telomerase and prevents primer dissociation (178). Human telomerase (hTERT) recruitment to telomeres is physically mediated by an interaction between a small patch of amino acids located in the OB-fold domain of TPP1, dubbed the 'TEL' patch, and specific amino acid residues in the TEN-domain of human telomerase (179, 180). In mice, mTPP1 also associates directly with telomerase and recruits mPOT1a/b to telomeres, forming a complex that inhibits binding of RPA (151, 172). Deletion of mouse TPP1 results in DDR checkpoint activation and release of POT1a/b from telomeres, eliciting phenotypes similar to those found in mPOT1a/b knockout cells (181).

RAP1: this shelterin component negatively regulates telomere length and aids TRF2 in prevention of telomeric end-to-end fusions.

Human RAP1 (hRAP1) increases the selectivity of TRF2 binding at telomeres (182). Artificial telomeric targeting of hRAP1 to telomeres is sufficient to prevent fusions even in the absence of TRF2 (183) and overexpression of RAP1 results in a moderate elongation of telomeres (154). hRAP1 knockdown using siRNA and expression of dominant negative deletion mutants of hRAP1 elongates telomeres (184). Deficiencies in mouse RAP1 (mRAP1) increase telomere recombination and fragility (185). In addition, mRAP1 binds to extratelomeric sites in subtelomeric regions, potentially acting as a transcriptional regulator (185).

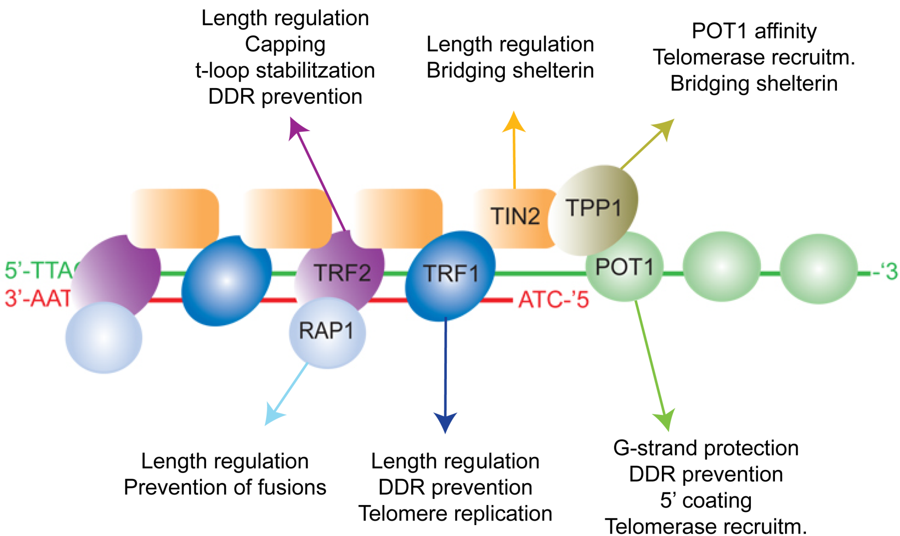

Figure 1.4 – How shelterin shapes telomeres.
Adapted from (139).

1.3.2.2 Shelterin protects telomeres from degradation by generating t-loops

One of the ways in which shelterin is proposed to shape the structure of telomeres, and also protect them from being recognized by DNA repair processes or of fusing, is by forming t-loops (138, 186-188). The protruding 3' G-rich overhang at telomeres invades the duplex telomeric repeats causing the formation of large loops by base pairing with the C-strand (187) (Figure 1.5). Telomere t-loops have been visualized in several species, including human and mouse cells, mostly by electron microscopy studies (187). The assembly of large duplex loops seems to be mediated by TRF2 (186, 187), which sits at the loop junction, and requires necessarily terminal telomeric sequences with 3' overhangs (blunt ends or 3' overhangs do not form t-loops) (188). The formation and maintenance of t-loops in vivo, possibly via TRF2 or other (shelterin) proteins, remains to be shown.

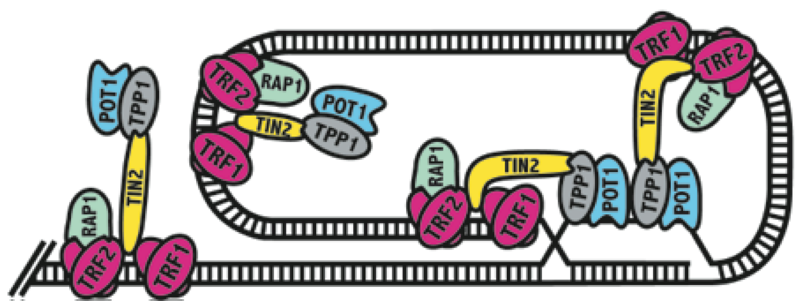

Figure 1.5 – Schematic of the t-loop structure.
The t-loop is generated by folding back of the 3' G-overhang, which invades the duplex telomeric region by forming a displacement loop, and basepairs with the complementary C-strand. Adapted from (138) and generously provided by Hugo Almeida.

1.3.3 – Mechanisms of telomere length maintenance

Cells use two mechanisms to counteract telomere attrition with cell division and ensure maintenance of telomere length and capping functions. The core mechanism involves telomerase, an enzyme that adds telomeric repeats *de novo* (133), and a secondary alternative mechanism relies on telomeric recombination.

1.3.3.1 Telomerase

Telomerase has two main components: a catalytic subunit (TERT – telomerase reverse transcriptase) (189-192), and an RNA subunit (TERC – telomerase RNA component) (135). TERC contains an RNA template (AAUCCCAAUC), which is used by TERT to catalyze the addition of new telomeric repeats to the G-rich overhang of existing telomeres (135, 193). Besides its two core components, TERC and TERT, telomerase associates with a number of other proteins that are essential for its assembly in Cajal bodies and recruitment, such as TCAB1, dyskerin, coilin, NOP10, NHP2 and GAR1

(194, 195). Mutations in TERT, TERC or in these telomerase accessory proteins result in human disease (see section 1.3.5.2 for details; (196-204)).

Telomerase recruitment and binding at telomeres is tightly regulated by shelterin components, ensuring equilibrium between telomere extension and attrition (see section 1.3.2.1). In addition, G4 DNA quartet structures formed at G-rich regions (including telomeres) limit the access of telomerase in vitro (205).

Interestingly, the expression of telomerase can be stimulated by Myc (206), NF-kB (207) and β-catenin (208, 209), and these factors are in turn regulated by telomerase in feed forward loops. Compelling evidence supports these pathways may be involved in the extratelomeric functions of telomerase (which are independent of telomere elongation). Telomerase stimulates (stem) cell proliferation by acting as a cofactor of the beta-catenin transcriptional complex (210), and promotes tumor development by potentially activating Myc and Wnt signaling (211, 212). Additional non-canonical roles of telomerase involve its interaction with the RNA component of mitochondrial RNA processing endoribonuclease (RMRP), which produces double stranded RNAs that can be processed into siRNAs (213). Finally, telomerase can be targeted to the mitochondria affecting its function, superoxide production and apoptosis (214, 215).

In humans, telomerase is found active specifically in germ and stem cells (136, 216), and can be transiently upregulated in cells of the immune system (217, 218). However, presence of telomerase activity in these cells is not sufficient to counteract telomere shortening over time (217, 219-221). Because immortality is required for continuous proliferation, 90-95% of human cancers reactivate telomerase (222). However, when telomerase is conditionally inhibited, cancer cells adapt and activate an alternative mechanism to maintain telomere length and ensure their propagation (223). This mechanism is described in the next section.

1.3.3.2 Alternative lengthening of telomeres (ALT)

In the absence of telomerase, human cancer cells maintain telomere length by activating a recombination-based mechanism, designated 'Alternative Lengthening of Telomeres' (ALT) (224). ALT can be found in approximately 10% of human cancers (225) and is more prevalent in tumors of mesenchymal origin (226). ALT tumors are characterized by very heterogeneous telomeres (with very short and very long sequences (224)), that associate with APB bodies (ALT- PML bodies) (227) and often by the presence of extra-chromosomal telomeric DNA that forms double-stranded (t-circles) (228) and single stranded telomeric circles (C or G-circles) (229). Notably, in a recent study Neumann and colleagues created a telomere-tagged transgenic mouse strain and showed that ALT activity occurs in normal somatic murine cells (230).

Several studies support that ALT is achieved by homologous recombination (HR) at telomeres. Specifically in immortalized ALT cell lines (telomerase negative) and not in telomerase positive cells, DNA tags inserted into telomeres are copied between chromosome ends (231). In addition, several recombination proteins are necessary for telomere maintenance in ALT cells, including the MRN complex (MRE11, RAD50 and NBS1) (232, 233), subunits of the SMC5/6 complex (234), FEN1 (235), MUS81 (236) and FANCD2 (237). The template used by HR for telomere synthesis may be the telomeric sequence of another chromosome, extrachromosomal telomeric DNA, or a telomere may template itself to promote recombination via t-loop formation (230, 238).

The activation of ALT may impose a higher burden of genome instability. Déjardin and colleagues recently showed that orphan nuclear receptors of the NR2C/F class bind telomeres in ALT cancer cells, inducing telomere cluster formation (required for HR) and promoting the addition of telomeric DNA to multiple chromosome internal sites. This targeted telomere insertion (TTI) process creates interstitial telomeric sequences, which generate fragile sites and promote chromosomal translocations (239).

1.3.4 – Consequences of telomere shortening – cell

As described in sections 1.3.2.1 and 1.3.2.2, different shelterin components (and associated proteins) as well as telomeric structures (t-loops), prevent the unwanted engagement of DNA repair processes at telomere ends, which would otherwise result in end-to-end fusions and have potentially catastrophic outcomes for cell viability (240). However, as telomerase activity is mainly restricted to germ and stem cell compartments in human tissues ((136, 241); see section 1.3.3.1), telomeres progressively shorten and the amount of shelterin may become limiting for maintenance of a protective capping function. What are the consequences of telomere dysfunction for a cell, a tissue and an organism?

1.3.4.1 Telomeres as internal mitotic clocks

1.3.4.1.1 Regulators of telomere attrition

Due to the absence of telomerase activity in somatic cells, human telomeres suffer programmed shortening with cell division (242). However, the relationship between these two variables is not linear. Human cells lose on average 50-300 bp per population doubling, a much faster rate than predicted by the end replication problem (243). What accounts for such loss?

One additional mechanism proposed to contribute to telomere shortening is post-replicative processing of telomere ends by various exonucleases, namely Apollo, Exo1 and MRN, which resect the telomeric 5’ end forming a protruding 3’ overhang (244, 245). Lagging-strand synthesis forms an overhang that is bigger than leading-strand synthesis (140), and thus the lagging strand overhang is proposed to act as the main determinant of telomere shortening rates (243). While some studies do confirm the existence of a robust correlation between telomere shortening and the size of 3’ overhang in cultured human cells, others

do not (243, 246). The divergent results of these studies could stem from measurements of the different overhangs generated by either lagging or leading strands.

Telomeres may also shorten due to oxidative stress, which causes base oxidation and generates abasic sites, single stranded and double stranded DNA breaks (247). Compared to the rest of the genome, G-rich sequences (including telomeres) are more susceptible to oxidative stress-induce damage given their high content in guanine triplets, which are prone to modification (52). Unrepaired persistent damage at telomeres may in turn cause stalling of replication forks or destabilize t-loops, resulting in telomere attrition (248). Accordingly, telomere shortening in human cultured fibroblasts can be either accelerated by mild oxidative stress (249) or decelerated by treatments with free radical scavengers (250). In addition, *in vitro* studies show that a single 8-oxo-guanine telomeric lesion, caused by mild oxidative damage, is sufficient to disrupt TRF1 and TRF2 binding at telomeres (251).

Another factor that may contribute to replication fork stalling at G-rich sites, including telomeres, is the formation of G4 quadruplexes (at the G-rich strand) (252).

In summary, although telomere length is in great part genetically determined (253), it may be influenced by a multitude of factors: replicative history of cells, exposure to oxidative stress and post-processing of 5’ ends. These factors contribute to the striking heterogeneity found in telomere length between chromosome ends of the same cell and between individual cells derived from clonal populations (254, 255) (Figure 1.6).

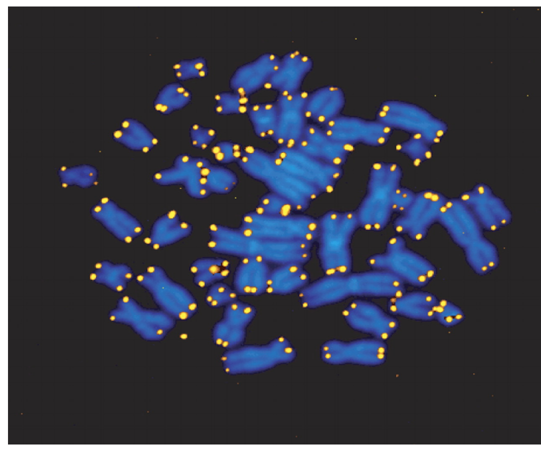

Figure 1.6 – 'The length of telomere repeats at individual chromosome ends is highly variable'.

Telomere repeats in a normal human lymphocyte are visualized using quantitative fluorescence in situ hybridization (Q-FISH) using peptide nucleic acid probes. Telomeres are shown in yellow, whereas the DNA of chromosomes, counterstained with DAPI, is shown in blue. Note that the fluorescence on sister chromatid telomeres is typically of equal intensity in line with expectations for quantitative hybridization.' Figure and legend taken from (256).

1.3.4.1.2 Short/uncapped telomeres trigger DDRs and induce senescence

When telomeres reach a critical length or lose capping function, they become indistinguishable of threatening DNA double strand breaks, triggering canonical DNA damage responses that culminate in growth arrest (89, 163). A connection between telomere shortening and replicative senescence (discovered by Hayflick in the 1960s; (4)) was first predicted by incredible foresight of Alexsei Olovnikov. Prior to existence of any profound knowledge in telomere biology, Olovnikov proposed that telomeres function as internal counting mechanisms ('mitotic clocks' or 'replicometers'), dictating the number of divisions a cell undergoes before arresting irreversibly (senescence) (124). This was one of the primary clues that telomere attrition could potentially work as a regulated biological program limiting cellular and organismal lifespan. Olovnikov's predictons were confirmed much later with studies showing that enforced

ectopic expression of telomerase in cultured somatic cells elongates telomeres and results in cellular immortalization (85, 257).

We now know that cellular senescence is induced by telomere dysfunction, which can be caused either by shortening (which may promote uncapping), or by telomere-length independent uncapping, as explained below.

What are the molecular signals triggered by short telomeres to arrest cell division?

The generalized cellular response to DNA lesions has been well characterized. Damage is recognized by PI3 kinases ATM and ATR, which become activated and phosphorylate H2AX (Ser 139). This facilitates the recruitment of checkpoints and other repair proteins, such as 53BP1, MDC1 and the Mre11 complex, which form foci important for signal amplification/repair, and phosphorylation of CHK1 and CHK2. This cascade converges in the activation of p53 and p21, which arrest cell division (169).

Several studies show that telomere uncapping or shortening activates a canonical DDR (76, 89).

Short telomeres in human fibroblasts passaged into senescence bear markers of a p53-dependent DDR (89, 258). They show accumulation of 53BP1, γ-H2AX, MRE11, CHK1, CHK2, RAD17 and BRCA1 (76, 89) foci (dubbed Telomere Dysfunction-Induced Foci – TIFs) (259). TIFs signal through ATM to p53 (and consequently activate p21), causing a G1 cell cycle arrest. Deletion of ATM in short telomere contexts is not sufficient for cells to resume proliferation, most probably due to activation of a secondary ATR-dependent pathway (76, 105, 260, 261). Interestingly, five telomeres bearing TIFs are predicted to be sufficient to induce a p53-dependent senescence response (258).

How short telomeres trigger DDRs is not entirely understood. A current model proposes that telomere erosion depletes shelterin components to a point where they can no longer prevent the activation of ATM and ATR pathways (169).

Accordingly, de-protection of telomeres via direct disruption of shelterin components induces p53 and p16-dependent senescent responses, independently of changes in telomere length. Inhibition of TRF2 binding at telomeres leads to ATM activation, upregulation of p53, $p16^{INK4a}$ and p21, and hypophosphorylation of RB, resulting in a G1/S cell cycle arrest and apoptosis (87, 162, 163). In addition, different DDR foci form at telomeres, including the proteins γ-H2AX, 53BP1, NBS1, RAD1, the Mre11 complex and RAD17 (89, 259). ATM functions as the primary mediator of TIF formation in TRF2 deficient cells, as its deletion significantly rescues the accumulation of 53BP1-TIFs, although it is not sufficient to prevent senescence (259). Cell cycle arrest can be partially abrogated if p53 is inactivated but complete bypass of senescence requires the joint inactivation of p53 and p16 (86, 87).

Mutations or RNAi-mediated inhibition of other shelterin components, such as TIN2 and POT1, also result in formation of 53BP1 and γ-H2AX TIFs, apoptosis and senescence (170, 262). In POT1 deleted cells, DDR and TIF formation are largely mediated by ATR (263).

Impressively, overexpression of TRF2 in human cells induces telomere shortening and delays the onset of replicative senescence, potentially by protecting critically short telomeres from fusions (168). Altogether these studies suggest that rather than being caused by a complete loss of telomeric sequences, senescence is instead induced by a change in the protection status of shortened telomeres.

Paradoxically, several DDR proteins are transiently recruited to telomeres during replication, such as ATM, ATR and the MRN complex, and this recruitment is essential for end protection and for effective telomerase action. However, loading of shelterin proteins somehow prevents the activation of a full checkpoint response and permanent cell cycle arrest (264).

1.3.5 – Consequences of telomere shortening – organism

1.3.5.1 Telomeres shorten with human aging

Telomerase is expressed and activated in human embryonic tissues, maintaining telomeres throughout the extensive cell replication activity that takes place in intra-uterine development (265). However, as gestation proceeds, telomerase is progressively silenced, first in the brain and bone (at 16 weeks of gestation), and later in other tissues, including the adrenal gland, muscle, lung, skin, and liver (241, 265). From extra-uterine development onwards, it becomes restricted to germ cells and tissue adult stem cells (136, 241). In addition, some cells of the immune system, such as activated T and B cells retain the capacity to activate telomerase, to support extensive cell division and clonal expansion necessary for effective immune function (217, 218). As most human somatic tissues have no detectable levels of telomerase, telomeres shorten with age (256, 266, 267). Although telomerase is present in stem cells (those of the hematopoietic compartment have been studied the most) and T cells, it is not sufficient to prevent telomere shortening over time (217, 219-221). Interestingly, Blasco and colleagues showed that the efficient reprogramming of differentiated mouse cells into induced pluripotent stem cells (iPS) requires an active telomerase that ensures telomere elongation (268). Besides cell replication, oxidative stress and post-replication end processing also contribute to telomere attrition in human cells (see section 1.3.4.1.1).

Most human studies of age-dependent telomere shortening measure variation in telomere length of peripheral blood leukocytes between different age groups, which are composed of several independent individuals (54, 269). While leukocyte telomere length is 36-84% heritable (253), these longitudinal studies typically report a large inter-individual variability. It is not uncommon to find individuals belonging to the youngest age group having equivalent telomere lengths to those in the oldest group (54, 270). This considerable scattering of

telomere length within each age group may be explained by genetic influences, but also by environmental stressors and health conditions that increase/decrease the burden of replication in stem cell pools, change inflammatory status and alter the levels of oxidative stress (271, 272). Nevertheless, there is a significant trend of telomere erosion with age, with accentuated shortening occurring mainly from birth to puberty, presumably due to expansion of the hematopoietic compartment (273). During this period, primitive HSCs divide rapidly to give rise to multipotent progenitor cells, which then expand to produce more differentiated lineages (274). In adulthood, HSC division is much lower slowing down telomere shortening to a more gradual plateau (54, 269) (Figure 1.7).

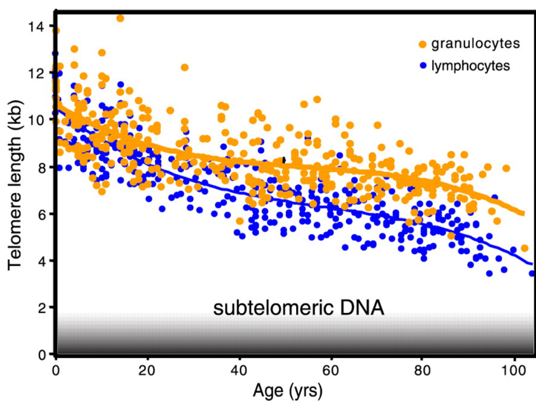

Figure 1.7 – 'The telomere length in human granulocytes and lymphocytes from human peripheral blood declines with age'.

Results of calculated median telomere fluorescence in lymphocytes (orange dots) and lymphocytes (blue dots) of 400 normal individuals over the entire age range. Note that the telomere length is highly variable at any given age and shows a biphasic decline with age. Most likely hematopoietic stem cells proliferate rapidly early in life followed by a marked decrease in turnover in infancy. The acceleration in telomere attrition over the age of 60 is as yet unexplained.' Figure and legend taken from (256).

Data on telomere length variation in other somatic tissues with age is still limited and has been reported mostly in the context of specific diseases. In this sense, different studies have found an inverse correlation between telomere length and cardiovascular disease, diabetes, inflammation, risk of infection, liver cirrhosis and pulmonary fibrosis, and a direct correlation with healthspan (275-277). Recently, a study performed by Daniali and colleagues measured age-dependent telomere length attrition in four different human somatic tissues: skeletal muscle, skin, subcutaneous fat and leukocytes. In accordance with previous observations (278), a significant synchrony in telomere length could be found between different organs, meaning that an individual with long telomeres in one tissue will have long telomeres in other tissues (270). A surprising discovery of this study is that regardless of different proliferation status, the four different tissues have equivalent telomere shortening rates of about 20-30 base pairs per year (270). In agreement, other low turnover tissues, such as heart and liver, also shorten their telomeres with human aging (266, 279). These findings imply that differences found in telomere length between high (e.g. leukocytes – shorter telomeres) and low turnover tissues (e.g. muscle – longer telomeres) are established with replication during early life (270).

1.3.5.2 Short telomeres associate with disease

A prominent role for telomeres in aging is supported by degenerative disorders that exhibit premature aging-phenotypes (progerias dubbed 'telomeropathies') due to loss of function mutations in genes necessary for telomerase maintenance. Typical adult symptoms of telomeropathies include Idiopathic Pulmonary Fibrosis (IPF), characterized by lung failure and inflammation (197), and liver cirrhosis, a common cause of death (280).

The first telomeropathy to be identified was Dyskeratosis Congenita (DC), a syndrome where patients carry mutations in TERT, TERC or in other genes involved in telomere maintenance, including DKC1 (dyskerin) and TIN2 (202,

281, 282). DC patients have much shorter telomeres than their relatives and die prematurely (Figure 1.8 A). In addition, they suffer from severe bone marrow failure, exhibit abnormal skin pigmentation, nail dystrophy, pancytopenia, several mucocutaneous alterations and show higher cancer rates (281). The Hoyeraal-Hreidersson syndrome (HHS) is a more severe variant of DC, where patients additionally suffer from cerebellar hypoplasia and microcephaly (283). Other exceptionally rare variations of DC include the Revesz syndrome and the Coats Plus syndrome (284, 285). The high degree of overlapping symptoms and molecular mechanisms between these telomeropathies indicates they resemble more of a spectrum disorder, rather than distinct diseases (286). Interestingly, these disorders exhibit a pattern of genetic anticipation, in which later generations of carriers have shorter telomeres and suffer from an earlier onset of disease with aggravated symptoms (286) (Figure 1.8 B). Even wild type children of TERT heterozygote carriers may express some form of DC, given they also inherit short telomeres (287).

Finally, telomere maintenance defects can also be found associated with other progerias such as Werner syndrome (WS) and ataxia telangiectasia (AT) (95, 197, 280, 288). Mice develop WS and AT only when provided with a telomerase deficient background (105, 289). Conversely, overexpression of telomerase prevents the proliferative defects, DDR activation and premature senescence induced by progerin, a mutated form of lamin A that causes the Hutchinson-Gilford Progeria Syndrome (HGPS) (290).

Altogether, these studies have contributed to dissect the role of short telomeres in maintenance of tissue homeostasis and lifespan. However, care needs to be taken when extending findings in these progerias to natural aging scenarios, as many of these 'accelerated aging' phenotypes are often segmental and an exacerbated version of what one may find in natural aging (95).

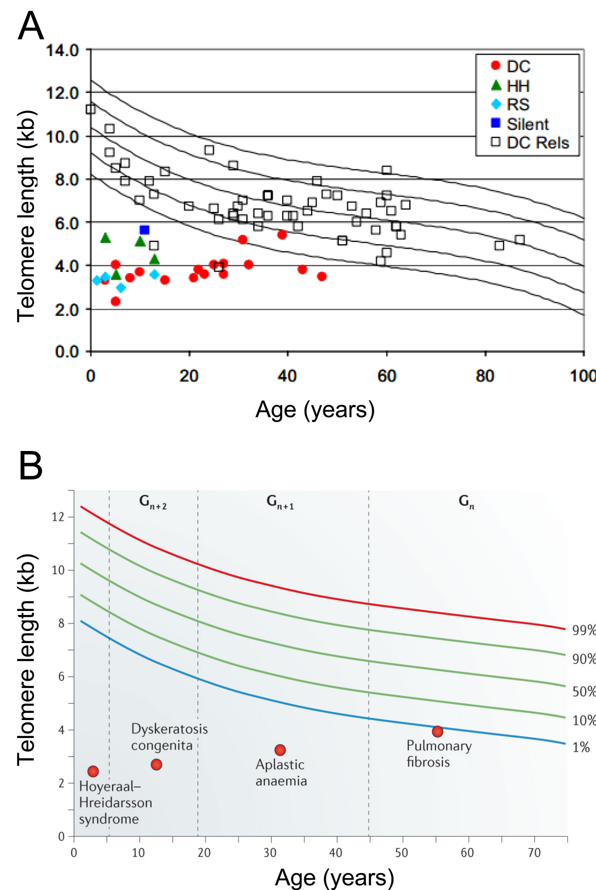

Figure 1.8 – 'Lymphocyte telomere length according to age in patients with dyskeratosis congenita and their relatives'.

A) *'Lines in the figures indicate the first, tenth, 50th, 90th, and 99th percentiles of results from 400 normal control subjects. Symbols represent subjects: 17 patients with dyskeratosis congenita (red solid circle), 4 Hoyeraal-Hreidarsson variant (green solid triangle), 4 Revesz syndrome (turquoise solid diamond), one silent carrier (blue solid square), and 54 relatives (open square)'.* Figure and legend taken from (291). B) *'A schematic drawing that illustrates the typical range of telomere lengths by age in, for example, peripheral blood lymphocytes. At every age, telomere length displays a normal distribution that is defined by the percentile lines labelled on the right. Telomere length in individuals with four different clinical presentations across the age range is indicated. The dashed lines represent a typical age range in which these disorders may first manifest, and 'G $_n$ ', 'G n_{+1}' and 'G n_{+2}' designate three successive generations manifesting with earlier-onset and evolving disease type owing to progressive telomere shortening.'* Figure and legend taken from (292).

1.3.5.3 A model of vertebrate accelerated aging: the telomerase knockout mouse

Genetically modified mouse models have been instrumental to explore the consequences of telomere shortening in tissue homeostasis, tumourigenesis and lifespan. It should be noted that the common lab mouse has invariably long telomeres (20-150 kb) (293) in comparison to human telomeres that span the range of 5-15 kb (137). Nevertheless, it was recently found that mouse telomeres do shorten and accumulate persistent damage with aging, particularly after the second year of age, in stem cell compartments and differentiated populations of several tissues (294-296). Importantly, the increase in percentage of short telomeres with age is a strong predictor of mouse lifespan (297).

Blasco and colleagues generated the first mouse model completely lacking telomerase activity in 1997, by deleting the germline copy of telomerase RNA (*mTerc*$^{-/-}$) in a mixed C57BL6/129Sv strain (298). Surprisingly, they found that the first generation (G1) of *mTerc*$^{-/-}$ mice 1) is born normally and develops without apparent degenerative phenotypes, 2) is fertile and can be bred up to 6 generations and 3) derived cells can be immortalized in culture and form tumors when introduced in nude recipients (298). Thus, these results strongly indicated that telomerase RNA and activity were not limiting for tissue homeostasis, germ cell function or tumourigenesis in the mouse.

However, it was found that continuous inbreeding of *mTerc*$^{-/-}$ for six generations results in progressive telomere length decline (4–5 kb/generation), cellular growth defects (299), telomere uncapping, aneuploidy and end-to-end fusions in lymphocytes and embryonic fibroblasts (298, 300). Phenotypes of telomere dysfunction were found predominantly in chromosomes with very short telomeres (potentially the ones limiting cell survival) (301).

Following detailed studies of these later generations of *mTerc*$^{-/-}$ mice showed that they suffer obvious reductions in lifespan particularly after G4, with average survivals ranging 18 months for G6 *mTert*$^{-/-}$ vs. 24 months for WT (302). However, it should be noted that in a more recent study medium and maximum survival reductions could already be found in the first generation (G1) of *Terc*$^{-/-}$ mice (303). Reduced survival of *mTerc*$^{-/-}$ mice is accompanied by widespread tissue dysfunction and atrophy (summarized below, Figure 1.9).

Testis: mTerc$^{-/-}$ (G3 onwards) have increased rates of germ cell apoptosis (and less proliferation), which culminate in defective spermatogenesis and infertility (304, 305). Consequently, these mice produce smaller litters and their offspring often dies from defects in neural tube closure (306). Because of these reproductive issues, *mTerc*$^{-/-}$ continuous matings can only be performed up to the sixth generation.

Hematopoietic tissue: G6 *Terc*$^{-/-}$ mice are anemic and develop progressive lymphopenia, with splenic lymphocytes undergoing apoptosis when stimulated to divide (304). *mTerc*$^{-/-}$ hematopoietic cells have reduced repopulation ability and show a proliferative disadvantage in competitive transplantations with WT cells (307).

Heart: G3 *mTerc*$^{-/-}$ cardiac myocytes proliferate less and show increased rates of apoptosis, resulting in myocyte hypertrophy and cardiac failure (308).

Skin: a progressive increase in hair graying becomes obvious in G6 *mTerc*$^{-/-}$ after 15 months, accompanied by alopecia, impaired hair growth, ulcerative skin lesions (102, 300, 302) and delayed wound healing capacity (302).

Subcutaneous adipose tissue: in aged G6 *mTerc*$^{-/-}$, the subcutaneous fat layer disappears and is replaced by fibrous tissue. This couples with body weight loss after 10 months (which in turn correlates directly with survival) (302).

Intestine: the proximal intestine of G6 *mTerc*$^{-/-}$ shows severe villi atrophy/blunting (302).

Brain: neural stem cells of G4 *mTerc*$^{-/-}$ have shorter telomeres and show decreased self-renewal activity and impaired differentiation in the subventricular zone of the brain (105, 309).

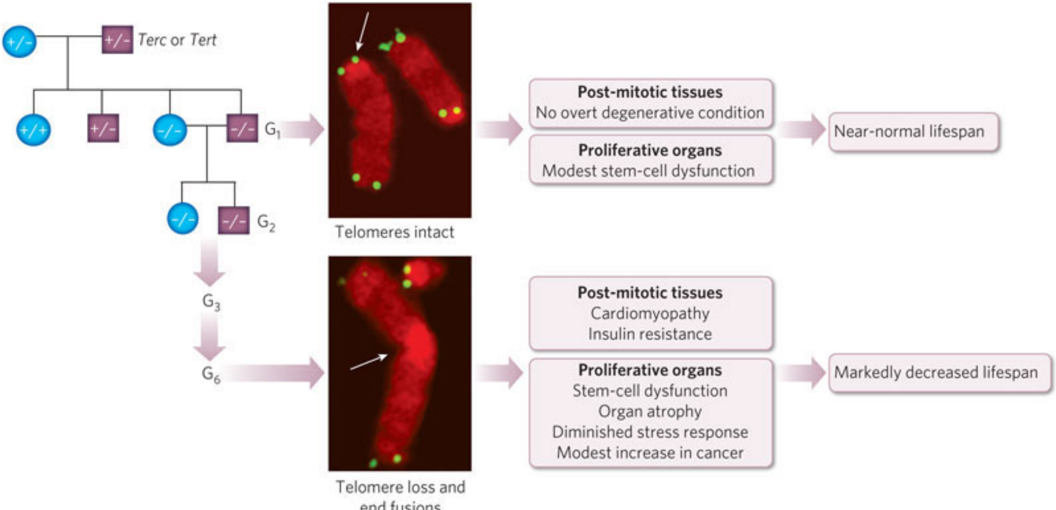

Figure 1.9 – 'Telomerase knockout mice with dysfunctional telomeres develop premature aging'.

Telomerase knockout mice (G1) are viable, with intact chromosomes, and have minor physiological abnormalities in the case of long telomeres (top image; arrow); however, with advanced age, they develop degenerative symptoms sooner than do age-matched mice with wild-type Terc. Continuous interbreeding of telomerase knockout mice leads to subsequent generations of mice (G2, G3 and so on) with telomeres of decreasing length. Mice with dysfunctional telomeres have chromosomal abnormalities (bottom image; arrows point to loss of telomere signal, resulting in fused chromosomes), and they develop multiple ageing-associated degenerative disorders in highly proliferative organs, as well as in post-mitotic tissues. (…) Such mice have a shortened lifespan and a modest increase in cancer, in line with the role of telomeres in preventing illegitimate recombination events.' Figure and legend taken from (95).

The timing of onset and severity of many of these phenotypes correlates inversely with telomere length and directly with the presence of critically short telomeres (301, 302). This point is clearly demonstrated when comparing the effects of telomerase deletion across different mouse genetic backgrounds, which have extremely different telomere lengths. The consequences of *mTerc*

deletion (described above) were originally assessed in a mixed genetic mouse C57BL6/129Sv background (MB), where telomere size normally varies between 30 and 120 kb (298, 302, 310). As described above, these *Terc*$^{-/-}$ mice develop most degenerative phenotypes when allowed to age in late generations (G3 onwards), and cannot be inbred for more than six generations (298, 302). In comparison, absence of telomerase in the C57BL6 (B6) mouse strain, which still has heterogeneous telomere length distributions but shorter telomeres on average (306), restricts successful mating to only four generations (311). G3/4 B6 *mTerc*$^{-/-}$ mice show higher levels of chromosomal instability, much lower survival (50% of G4 *Terc*$^{-/-}$ mice die by 5 months) and comparable tissue degeneration phenotypes to those found in later generations (G6) of MB *Terc*$^{-/-}$ mice (311).

A much more striking example is found when telomerase is deleted in the wild-derived inbred mouse strain (Cast/EiJ). This strain has short telomeres (human-like), which are homogeneously distributed in length, ranging the sizes of 18–20 kb (310). Upon *mTerc* deletion, already the first generation of mice (G1) suffers a drastic lifespan reduction, accompanied by a series of pathologies that are more severe than those found in G5 MB *Terc*$^{-/-}$ mice (298). These pathologies include severe depletion of the intestinal epithelium coupled with villi atrophy, ineffective bone marrow hematopoiesis, formation of intestinal microadenomas and germ cell apoptosis that limits mating beyond the 3rd incross (G3) (312).

As in *Terc*$^{-/-}$ mice, disruption of the catalytic subunit of telomerase (*mTert*) also results in progressive telomere shortening, high levels of genomic instability, aneuploidy and telomeric fusions in embryonic stem cells (313). Consequently, late generation *Tert*$^{-/-}$ mice (G4 onwards) develop fertility defects that are equivalent to those found in *mTerc*$^{-/-}$, and can be successfully mated for eight generations (314).

Interestingly, successive crossings of heterozygous mice also result in progressive telomere shortening and in an increase of signal free ends, implying

that *Terc*$^{+/-}$ and *Tert*$^{+/-}$ mice are haploinsufficient for telomere length maintenance (312, 314). Telomere shortening in these mice is accompanied by several defects in tissue renewal in the bone marrow, intestine and testis (312). Strikingly, the WT progeny of late-generation telomerase heterozygote incrosses also inherits shorter telomeres, germ cell hypoplasia/degeneration, hematopoietic and immune function defects (312, 315). Altogether, these findings indicate that in telomerase null or happloinsufficient backgrounds, short telomeres are sufficient to induce tissue degenerative phenotypes (312, 315).

In summary, loss of telomerase activity by deletions of either *mTerc* or *mTert* compromises the maintenance of telomere length in vivo, disrupting tissue homeostasis and survival. What are the mechanisms regulating telomere dysfunction-induced phenotypes? Is telomerase reactivation sufficient to rescue these phenotypes and delay aging?

1.3.5.4 Mechanisms by which short telomeres drive tissue dysfunction

One of the essential functions of telomeres is to ensure chromosome ends are not recognized as DNA double strand breaks (138). However, with continuous cell division in the absence of telomerase, telomeres progressively shorten and trigger DDRs that converge in the activation of p53 (see section 1.3.4.1.2). This short telomere-DDR signaling cascade promotes tissue atrophy and dysfunction in several high and low turnover organs of inbred telomerase knockout mice (see previous section) (56, 302, 304). p53 (316) (but not p16^{Ink4a} and p19^{Arf} (317)) is one of the major downstream executers of short telomere-induced defects. Accordingly, the high levels of germ cell apoptosis and fertility defects found in late generation *Terc*$^{-/-}$ mice are significantly rescued by deletion of p53. However, absence of p53 also results in a rampant increase in genomic instability and cancer (316, 318, 319). Of note, in surprising contrast to p53, the absence of ATM exacerbates the phenotypes of *Terc*$^{-/-}$ mice, increasing the already high levels of apoptosis and chromosome fusions, overall resulting in

lower survival (105). This suggests there are ATM-independent pathways activating p53 in response to short telomeres *in vivo*. Interestingly, abrogation of other components of the DDR pathway (involved in signaling DNA damage through the p53 pathway), rescues the survival and tissue degenerative phenotypes of late generation *Terc*$^{-/-}$ mice. Deletion of exonuclease 1 (EXO1) improves lymphopoiesis, decreases gut apoptosis and reduces chromosomal fusions of G3 *Terc*$^{-/-}$ mice, without increasing cancer formation (261). Deficiencies in CHK2 or mismatch repair gene PMS2 improve the intestinal atrophy of *Terc*$^{-/-}$ mice (320, 321). All three deletions (*Exo1*$^{-/-}$, *Chk2*$^{-/-}$ and *PMS2*$^{-/-}$) rescue independently the survival of *Terc*$^{-/-}$ mice, and only *Terc*$^{-/-}$*Chk2*$^{-/-}$ mice show higher cancer incidences (261, 320, 321).

Several studies have now characterized in more depth two main types of mechanisms acting downstream of the short telomere-DDR-p53 axis to compromise tissue homeostasis. These mechanisms are explored in the next sections (Figure 1.10).

1.3.5.4.1 The short telomere-p53 axis limits homeostasis of highly proliferative tissues by inducing cell cycle arrest and apoptosis

The classic and most studied mechanism acting downstream of p53 in short telomere contexts involves restriction of proliferation, via induction of a permanent cell cycle arrest (which evolves to senescence), associated with depletion of genetically unstable cells by apoptosis. This mechanism has been causally linked to defective maintenance of high turnover organs (101-103, 105, 107).

Studies performed in late generation telomerase knockout mice (G3/G4 *Terc*$^{-/-}$), show that short telomeres restrain division and increase apoptosis particularly in stem and progenitor cells, compromising their function in the intestine, skin, brain, muscle and hematopoietic tissue (100-107). In the epidermis, hematopoietic tissue and brain, blunted proliferation responses associate with

defective mobilization/differentiation of telomere dysfunctional stem cells. Because of decreased mobilization capacity, dysfunctional epidermis stem cells accumulate in their niche located at the bulge of the hair follicle, limiting the wound healing capacity of this tissue (102). The defective proliferation of short-telomere HSCs limits their repopulation capacities in serial transplantations (101), and alters their differentiation patterns, resulting in defective lymphopoiesies (322, 323). Finally, impaired self-renewal of neural stem cell compartments in G4 *Terc*$^{-/-}$ brains also couples with defective differentiation of individual neurospheres into neurons (105, 106).

Some of the molecular players regulating stem cell dysfunction in response to short telomeres have been identified, particularly those acting in the intestine and in HSCs. Insofar, different studies reveal a prominent role for p53 dowstream targets, including cell cycle inhibitor p21, and p53 upregulated modulator of apoptosis PUMA (100, 103).

In G3 *Terc*$^{-/-}$ mice, deletion of p21 rescues the defective proliferation of stem and progenitor cells, particularly in intestinal crypts, improves regeneration and repopulation capacities of HSCs and restores lymphopoiesis, without affecting apoptotic responses (100). PUMA deletion also rescues the decreased proliferation of intestinal stem cell niches, without significantly increasing the levels of chromosome instability (103). Furthermore, when both PUMA and p21 are inhibited, the intestinal stem cell self-renewal capacity increases even further, but results in high levels of chromosomal instability (91, 103). Interestingly, deletion of p53 function specifically in the intestine of telomere dysfunctional mice rescues proliferation in colonic crypts but also impairs the depletion of chromosomal unstable stem cells, leading to premature degeneration of the organ (91). In the skin, p53 deletion also partly rescues epidermal stem cell numbers and their defective mobilization upon telomere shortening (324).

1.3.5.4.2 The short telomere-p53 axis limits homeostasis of low turnover organs by impairing mitochondrial biogenesis and dampening the somatotrophic axis

Recent evidence proposes a second short-telomere induced mechanistic response for dysfunction of quiescent organs (e.g. heart and liver). Accumulation of short telomeres represses mitochondrial biogenesis, resulting in lowered activity of oxidative defense mechanisms and decreased gluconeogenesis (56). Impaired mitochondrial biogenesis relies on p53 activation and potentially changes in energy homeostasis that lead to suppression of IGF-1 and mTOR signaling, as well as activation of AMPK (45, 56). The consequent accumulation of ROS may raise further genotoxic damage at telomeres, altogether creating a potential feedback loop that results in increased mitochondrial dysfunction and tissue degeneration, although experiments demonstrating this are still missing (56).

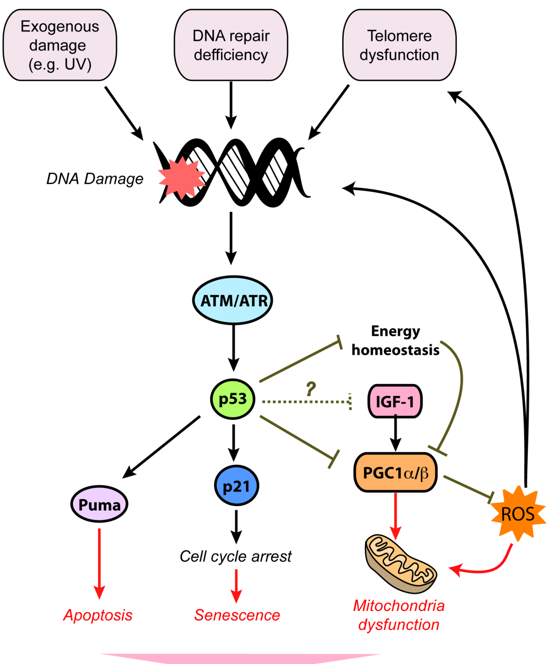

Figure 1.10 – Pathways modulated by the short telomere-p53 axis.

Telomere dysfunction activates p53 (316) causing Puma-mediated apoptosis (103) and p21-cell cycle arrest (and consequently senescence) (100). Furthermore, p53 upregulation leads to impairments in energy homeostasis and suppression of IGF-1 signaling (59), which result in repression of master regulators of mitochondrial biogenesis (56) and consequently mitochondrial dysfunction.

1.3.5.5 Alternative (emerging) telomerase deficient vertebrate models

As described in previous sections (1.3.5.3), the laboratory mouse model has been crucial for our understanding of the impact of telomere shortening in vertebrate tissue homeostasis. However, considerable differences can be found in mouse and human cells, not only in telomere length, but also in cell immortalization and entry into senescence (325). Furthermore, telomerase-deficient lab mice are viable up to several generations and severe disease phenotypes manifest primarily in later generations (298, 304) (section 1.3.5.3). This has instigated the development and characterization of complementary vertebrate models that have telomeres with human like lengths. While these models are still far from being as established as the mouse, they can substantiate even further our knowledge on vertebrate telomere biology.

In this regard, two fish species have emerged as promising vertebrate models: zebrafish and killifish.

African turquoise killifish have telomeres of lengths close to those found in humans (6–8 kb) (326) and develop a wide range of phenotypes and pathologies in natural aging, including decreased fertility, sarcopenia and cancer (327). A particular advantage of these fish is that their lifespan is relatively short (compared to other vertebrates), varying between 4 to 6 months, making them the naturally shortest lived vertebrate model in the lab (328). Recently, Brunet and colleagues generated the first TERT-deficient killifish model using the CRISPR/Cas-based genome editing technique (328). They showed that the in the absence of telomerase, killifish develop premature defects including low

fertility, gut villi atrophy, loss of blood cellularity and epithelial adenomatous changes. However, telomerase absence does not significantly affect the lifespan or telomere length of first generation TERT-deficient killifish (328).

Zebrafish have human-like length telomeres (of about 5-15 kb). Telomerase activity (and expression), although detected in most zebrafish tissues, declines with age (329). In addition, only a few studies have reported that zebrafish develop aging-associated phenotypes including increased spinal curvature (330), accumulation of oxidized proteins in the muscle (331), defective regenerative potential (329) and reduced cognitive function (332).

Strong evidence supports a vital role for telomerase and telomere-associated proteins in maintenance of zebrafish tissue homeostasis and regeneration. *terf2* mutants are embryonic lethal and exhibit signs of premature senescence (333). Adult *terf2* heterozygotes (which are viable) exhibit signs of premature retina degeneration and suffer a significant reduction in lifespan (333). Supporting a role for telomerase in tissue regeneration, Flores and colleagues recently showed that telomerase hyperactivation (and consequent telomere elongation) are required for cardiomyocyte proliferation and heart regeneration after zebrafish cardiac injury (334).

Several morpholino-based zebrafish knockdown models of telomerase and telomere-associated proteins have been further proposed as promising models for the study of dyskeratosis congenita (DC, details on this telomere-syndrome in section 1.3.5.2). Knockdown of *tert, terc* or *dkc1* triggers larval phenotypes that are reminiscent of human DC, such as pancytopenia, impaired hematopoietic differentiation or p53-dependent apoptosis (335-337). In agreement, the same disease symptoms are recapitulated by zebrafish carrying a mutation in *nop10*, a member of the H/AXA RNP complex, essential for telomerase trafficking to Cajal bodies, which has been linked to DC (338). Importantly, the premature degenerative phenotypes of these DC models develop independently of changes in telomere length (335-338).

Finally, the impacts of telomerase absence in zebrafish lifespan and adult tissue homeostasis have been directly addressed by part of the work developed in this thesis (the core subject of Chapter 2).

1.3.6 – Telomerase reactivation extends lifespan

The effects of telomerase reactivation were first tested in cell culture studies during the 1990s. Enforced expression of Tert was found to provide retinal pigment epithelial cells and foreskin fibroblasts with unlimited replicative potential (85) and to delay the onset of senescence in fibroblasts derived from patients with premature aging syndromes (339, 340).

Hints that telomerase gene therapies could have an effect *in vivo* came soon after the generation of the telomerase knockout mouse models. Different studies tested if the re-introduction of a WT telomerase allele in the germline of late generation *Terc*$^{-/-}$ or *Tert*$^{-/-}$ mice was sufficient to prevent the tissue dysfunction caused by telomere shortening (314, 341, 342). This was accomplished by studying the progeny of G3 *Terc*$^{-/-}$ x *Terc*$^{+/-}$ or G3 *mTert*$^{-/-}$ x *mTert*$^{+/-}$ crosses. It was found that the G4 progeny specifically receiving the telomerase wild-type allele re-sets telomere length to detectable levels in all chromosomes, extending the lifespan and significantly rescuing the degenerative phenotypes of G3 *Terc*$^{-/-}$ or *Tert*$^{-/-}$ mice (314, 341, 342). Recently, DePinho and colleagues created a new mouse model to test the effects of telomerase reactivation in progeroid adult mice with already established short telomere-induced tissue degenerative phenotypes (343). This mouse has an engineered knock-in allele encoding a 4-OH inducible telomerase-oestrogen receptor (TERT-ER) under the control of the endogenous *Tert* promoter (343). Strikingly, tamoxifen induced *Tert* reactivation for a four-week period alone is sufficient to restore telomere length, quell DDRs, and rescue tissue degenerative phenotypes in the testis, intestine, spleen and brain (343).

Accordingly, different studies also substantiate that telomerase overexpression in mouse adult tissues may have a beneficial impact in lifespan and in prevention of age-related pathologies. Tert overexpression targeted to basal keratinocytes (bovine keratin 5 promoter; K5-*Tert* mice) ameliorates the renal and testicular dysfunction observed in aging but increases mortality due to a higher incidence of spontaneous tumors (344). However, if these K5-Tert transgenic mice are crossed with a cancer-resistant background (with increased expression of p53, p16 and p19ARF), *Tert* overexpression decreases the level of telomeric damage and confers a ca. 40% extension in median survival (345). In addition, these mice have improved fitness of skin and intestinal epithelial barriers, better neuromuscular coordination and insulin sensitivity (345).

Telomerase activation late in life can lead to a beneficial extension of lifespan and delay aging, without increasing cancer incidence. This was recently shown using gene therapy strategies with recombinant non-integrative adeno-associated viruses (AAV) (346). Injection of AAV-mTERT in a single treatment extends median lifespan and reduces the incidence of age-related pathologies, including osteoporosis, insulin resistance, while improving neuromuscular coordination and cognition. Importantly, tissue rejuvenation and lifespan extension require a catalytically active telomerase and elongation of telomeres (346).

Finally, preliminary data shows that chemical activators of telomerase, such as TA-65 and AGS-499, have neuroprotective effects and improve mouse metabolic function (347) and potentially endow humans with improved immune response (348).

Altogether these findings highlight the role of telomere dysfunction as a regulator of aging and raise the potential of using telomerase reactivation therapies to delay or prevent aging associated pathologies.

1.3.7 – Telomeres, telomerase and cancer

Because telomere shortening limits the uncontrolled proliferation of cells, by committing them to a permanent growth arrest (senescence) after a certain number of divisions, it is considered a robust tumor suppressor mechanism (4, 242). Interestingly, cells undergo senescence before telomeres reach critically short lengths (85, 168, 242). If these cells bypass senescence, for instance by acquiring a mutation in p53, they may resume proliferation (316). Continuous proliferation drives telomere dysfunction and high levels of chromosomal instability (‘crisis’), resulting in massive death mediated by mitotic telomere deprotection and fusions (316, 349). This genetic instability enhances the probability for selection of cells that acquired mutations that allow them to sustain indefinite proliferation (through telomere length maintenance) (Figure 1.11). Thus, while telomere shortening may have initially evolved to keep cancer at bay, it also favors the selection of unstable cells that find a way to bypass tumor suppressor checkpoint signaling (318). Nevertheless, even if tumors arise from short telomere cells, their growth and progression require the activation of a telomere length maintenance mechanism. Most human cancer cells accomplish this by reactivating telomerase (136, 191, 225). Accordingly, telomerase overexpression in murine models typically results in increased cancer rates (350, 351).

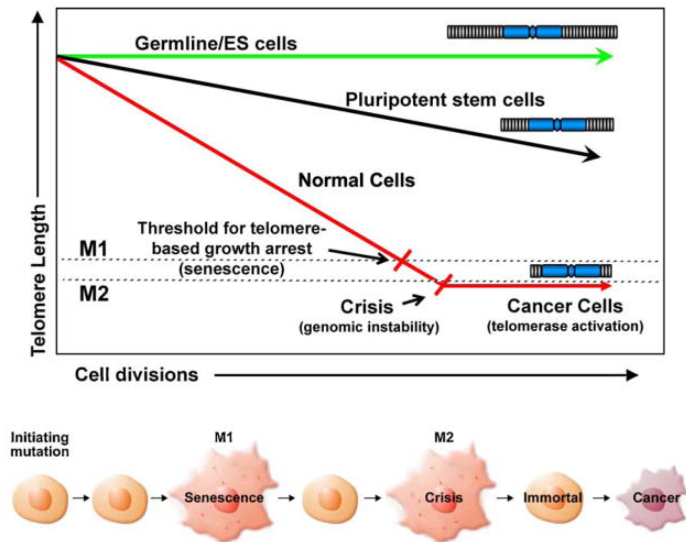

Figure 1.11 – How telomere dysfunctional cells may progress to cancer.
'Most somatic cell do not express telomerase activity and thus lose telomere length with each division at a faster rate until the cells uncap a few of their telomeres and undergo a growth arrest called replicative senescence. In the absence of cell cycle checkpoints (e.g. p53/pRB pathway), cells bypass senescence until they reach crisis. In crisis telomeres are so short that chromosome end fusions occur and there is increased genomic instability (probably due to chromosomal, breakage, fusion, bridge cycles). A rare cell that escapes crisis almost universally does so by reactivating telomerase and this cell can now become a cancer cell with limitless potential to divide.' Figure and legend taken from (222).

Recently, several mutations in the TERT promoter were identified in human melanomas. These mutations generate *de novo* consensus binding motifs for ETS transcription factors, increasing telomerase transcription by up to four fold (352, 353). TERT mutations have since been found to associate with a wide range of other human cancers, particularly glioblastomas, liposarcomas and bladder cancers (354).

A recent report by Hockemeyer and colleagues elucidates how these TERT promoter mutations may confer a selective advantage and contribute to cancer development. Contrary to what happens in normal cells, when embryonic stem cells engineered to carry telomerase-expressing mutations in the TERT

promoter are differentiated, telomerase is not silenced (355). Differentiated cells carrying the TERT promoter mutation have much longer telomeres, and present telomerase activity levels similar to other cancer cell lines (355).

Aging associates independently with telomere shortening and with an increase in cancer incidence (54, 269, 356). However, the role of telomere length in cancer remains somewhat controversial. In mouse models deficient for telomerase, telomeres shorten and eventually become dysfunctional, leading to different outcomes in terms of tumourigenesis. In this context, cancer rates may increase (302, 318, 357), decrease (but in some cases with higher number of initiation events) (223, 358-361), or remain grossly unaffected (362). The outcome seems to be largely dependent on the genetic bcakground and on p53 status (318, 363, 364). In addition, patients who suffer from Dyskeratosis Congenita (DC, see section 1.3.5.2), have much shorter telomeres and an increased probability of developing cancer (365). Notably, cells from DC patients show extreme chromosomal instability, telomere fusions and rearrangements (366).

A recent study suggests that the effect of telomere dysfunction (in telomerase deficient contexts) in tumourigenesis may rely on how efficiently an organism adapts to activate ALT mechanisms (see section 1.3.3.2; (223)). In a controlled experiment, Hu and colleagues showed directly that the activation of telomere lengthening mechanisms (that may or may not depend on telomerase), determines cancer progression and malignancy (223). Furthermore, if telomerase is specifically activated following a period of telomere dysfunction (and consequently, high chromosomal instability), cancers emerge with much more aggressive and invasive properties (319, 367, 368). Ultimately, telomere length and telomerase activity may have to be maintained in a certain 'safe range', to simultaneously ensure tissue/organism homeostasis and prevent malignancy (286) (Figure 1.12) In this sense, two key studies show that if telomerase is reactivated only during a transient period in late generation

telomerase knockout mice or in late life of normal wild type mice, it significantly delays aging and extends lifespan without increasing cancer incidence (343, 346).

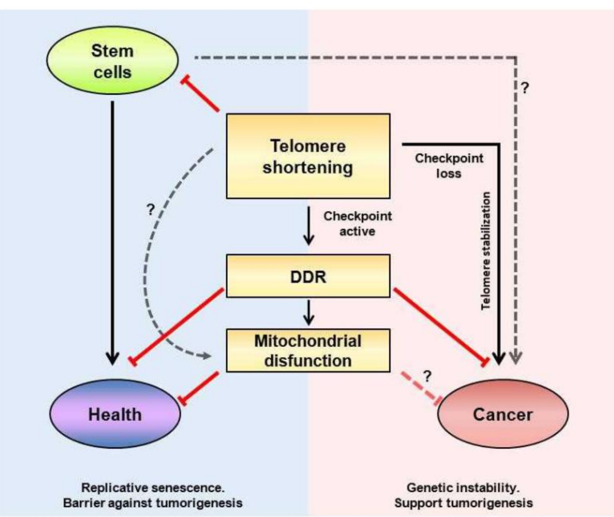

Figure 1.12 – 'Short telomeres in aging and cancer'.

Major pathways affected by short telomeres and their impact on aging or cancer. DNA damage and tumor suppressor activity have been shown to impact tissue decline and aging. When DNA damage checkpoints are bypassed, cells with short telomeres could progress to cancer. Both the role of stem cells with short telomeres in cancer and whether short telomeres modulate other pathways independently of p53 (such as mitochondrial dysfunction) remains unknown. Figure and legend taken from (369).

1.4 Aims and thesis scope

Even though progressive telomere loss is considered a primary culprit of aging, many questions remain unresolved. How are different individual organs affected by telomere shortening with age? Is telomere shortening in one key tissue sufficient to promote systemic aging of the whole organism? Which precise molecular events mediate the loss of homeostasis caused by dysfunctional telomeres in aging?

Telomerase deficient mice have for long been the main characters of studies assessing how vertebrates respond to telomere shortening (298, 302). Curiously, in normal settings, these mice have much longer telomeres than humans (325). In this thesis we propose that the telomerase mutant zebrafish (*tert*$^{-/-}$) can be used as an exciting complementary model (to the current mouse models) for studying how telomere dysfunction impacts tissue homeostasis and lifespan in vertebrates. In chapter 2 we focus on characterizing this new model. We establish the choreography of premature dysfunctional phenotypes emerging over time in *tert*$^{-/-}$ tissues, determining how it is accompanied by changes in telomere length, proliferation, apoptosis and senescence. Finally, we test directly whether these phenotypes are mediated by the activation of p53.

Establishing *tert*$^{-/-}$ zebrafish as a promising model for aging research requires understanding the extent to which its premature degenerative phenotypes are an acceleration of natural tissue dysfunction in aging. Chapter 3 addresses carefully this point, introducing a detailed comparative prospective study of aging-related dysfunction and disease in several WT and *tert*$^{-/-}$ tissues.

A primary goal of this thesis is to determine in which specific organs progressive telomere erosion with age is likely to become a limiting factor for maintenance of homeostasis. In chapter 3 we take two first steps to address this issue: 1) we establish the dynamics of telomere shortening in different zebrafish

tissues with age; 2) we determine which specific aging phenotypes are anticipated by local accumulation of critically short telomeres and DDRs.

The identification of key molecular mechanisms mediating telomere-induced tissue dysfunction is a key step for the development of targeted rejuvenation therapies. In chapter 4 we introduce a global transcriptional profiling study that aims to find new molecular mediators of short telomere-induced defects, by comparing changes in gene expression of WT and *tert*$^{-/-}$ tissues over time. We follow one specific leading candidate (oxidative stress de-protection) and test whether it has functional relevance in mediating the loss of homeostasis and lifespan defects caused by extreme telomere dysfunction.

Finally in chapter 5, we discuss the main contributions of this thesis work and propose future avenues of research that may 1) help clarify whether telomere shortening in specific tissues contributes to systemic phenotypes of aging; 2) bring novel mechanistic insight into how short telomeres disrupt homeostasis and limit survival.

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Chapter 2 | Telomerase is required for zebrafish lifespan

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Authors' contributions

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Analyzed the data: MGF CMH MCC IMT

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Abstract

Telomerase activity is restricted in humans. Consequentially, telomeres shorten in most cells throughout our lives. Telomere dysfunction in vertebrates has been primarily studied in inbred mice strains with very long telomeres that fail to deplete telomeric repeats during their lifetime. It is, therefore, unclear how telomere shortening regulates tissue homeostasis in vertebrates with naturally short telomeres.

Zebrafish have restricted telomerase expression and human-like telomere length. Here we show that first generation *tert*$^{-/-}$ zebrafish die prematurely with shorter telomeres. *tert*$^{-/-}$ fish develop degenerative phenotypes, including premature infertility, gastrointestinal atrophy and sarcopenia. *tert*$^{-/-}$ mutants have impaired cell proliferation, accumulation of DNA damage markers and a p53 response leading to early apoptosis, followed by accumulation of senescent cells. Apoptosis is primarily observed in the proliferative niche and germ cells. Cell proliferation, but not apoptosis, is rescued in *tp53*$^{-/-}$*tert*$^{-/-}$ mutants, underscoring p53 as mediator of telomerase deficiency and consequent telomere instability. Thus, telomerase is limiting for zebrafish lifespan, enabling the study of telomere shortening in naturally aging individuals.

2.1 Introduction

Telomeres constitute the ends of linear chromosomes, comprising DNA (TTAGGG)n repeats and its associated proteins, known as the shelterin complex (1). Telomeres provide protection against erosion of chromosome-ends that occurs with each cell division as a result of the “end-replication problem” (2). Additionally, they prevent the recognition of chromosome termini as deleterious DNA double strand breaks (DSBs). If this function fails, chromosome-ends induce DNA damage responses (DDRs) that comprise the

activation of p53 (3). Telomerase, a reverse transcriptase, counteracts chromosome-end depletion by elongating telomeres through the action of its catalytic unit (Tert) and RNA template (Terc) (4, 5). Because telomerase expression is restricted in human somatic cells, telomeres shorten during our lifespan (6). Human somatic cells lose around 100 base pairs of telomeres per population doubling (7), leading to a limit of about 50-80 cell divisions in culture, known as the Hayflick limit (8).

Impaired tissue homeostasis is at the core of several human diseases, including ageing-associated degeneration (9). Premature aging syndromes such as Werner, Hutchinson-Gilford and Dyskeratosis Congenita (DC) share the common trait of shorter telomeres, accelerated aging and reduced lifespan (10). DC, in particular, can be caused by mutations in the telomerase *tert* or *terc* genes, and there is a direct correlation between telomere length and disease severity (11).

Telomeres become dysfunctional due to critical shortening, oxidative damage or uncapping (12). Dysfunctional telomeres induce DDRs characteristic of damage-induced DSBs (13). Depending on the cell type, level of DNA damage and p53/p63/p73 status, dysfunctional telomeres initiate an apoptotic response or a G1 cell cycle arrest, leading to senescence (14). While high levels of DNA damage are thought to trigger apoptosis via *puma* (p53 upregulated modulator of apoptosis) activation, low levels are most likely to cause cell cycle arrest via *p21* activation (14). Telomere maintenance, therefore, dictates survival and replicative potential of cells, directing tissue homeostasis.

Most of our knowledge of vertebrate telomeres comes from inbred mice strains with long telomeres (15). Several generations of intercrossing between telomerase deficient mice are needed before telomere shortening has a noticeable impact at the organism level (16-18). Data from late generation telomerase knockout mice suggest that cell senescence (19) and/or apoptosis (20) play a critical role in the observed degenerative phenotypes. Either *puma*

(21) or *p21* (19) deletion separately ameliorate degenerative phenotypes observed in late generation telomerase knockout (KO) mice. Stem cell exhaustion via *puma*-mediated apoptosis is crucial in limiting the life span of late generation *terc* knockout mice (21). Whether artificially shortening telomeres in the long telomere mouse strains or the use of other genetic backgrounds with shorter telomeres reproduces the way in which human tissues respond to telomere lifetime erosion remains an open question.

Recently, a wild-derived inbred mouse strain (Cast/EiJ) has been proposed as a better model for understanding telomere dysfunction in humans, given its shorter telomeres (22). Telomerase deficiency in this strain gives rise to first generation defects similar to the ones observed in human DC syndromes (22). Thus, telomere length may be limiting for Cast/EiJ longevity, making it a promising alternative to the current mouse models. However, molecular responses to dysfunctional telomeres in this model remain to be elucidated.

It is critical to investigate complementary vertebrate models to understand what is the most likely impact of telomere exhaustion in a biological system that, like humans, has evolved to have telomere length as an internal cell division "clock". Zebrafish, a teleost fish that exhibits gradual senescence, is a promising vertebrate model for telomere biology. Contrary to the inbred laboratory mouse, zebrafish have heterogeneous telomeres of human-like length (23). Despite detection of telomerase activity in various tissues, zebrafish telomeres shorten with age (24). Like humans (6), telomerase expression in zebrafish somatic cells is not sufficient to prevent telomere shortening (24). Telomere shortening was associated with impaired regenerative responses in the aged fish, denoting a role for telomere in homeostasis of adult tissues. Accordingly, a zebrafish mutant for the telomere repeat binding factor 2 (Terf2) accumulates senescence markers and this is accompanied by central nervous system necrosis and decreased survival (25).

Here we show that first generation telomerase deficient zebrafish have shorter telomeres than wild-type siblings and die prematurely. *tert*$^{-/-}$ fish are born and develop normally until adulthood, but progressively develop accelerated degenerative phenotypes characteristic of disrupted tissue homeostasis. These include premature infertility, gastrointestinal atrophy, loss of body mass, increased inflammation and sarcopenia at terminal stages. Underlying these phenomena is a sustained decrease in cell proliferation, an acute apoptotic response and accumulation of DDR foci. Removal of p53 function rescues cell proliferation, but not apoptosis, in high turnover tissues, such as testis and gut. This implicates p53 as a critical mediator of telomerase-dependent proliferative defects observed in *tert*$^{-/-}$. Thus telomerase and consequent telomere shortening play a key and limiting role in tissue maintenance during zebrafish lifespan.

2.2 Results

2.2.1 – Telomerase mutant zebrafish have shorter telomeres

In order to examine the consequences of telomerase depletion in zebrafish, we used the currently available but yet uncharacterized, tert^{hu3430} line produced by ENU-tilling screen at Utrecht University, Netherlands (26). This telomerase mutant line carries a T→A transition in the second exon of the *tert* gene giving rise to an early stop codon. For simplicity, we will refer to the tert^{hu3430} homozygous mutant strain as *tert*$^{-/-}$.

To test whether *tert*$^{-/-}$ mutants had a functional telomerase, we performed the commonly used Telomere Repeat Amplification (TRAP) assay (27). We observed no amplification bands corresponding to telomere elongation in the TRAP assay as compared to *tert*$^{+/+}$ controls (Figure 2.1A), indicating that *tert*$^{-/-}$ mutants lack active telomerase. The consequence of absence of telomerase is continuous telomere shortening. Accordingly, Telomere Restriction Fragment

(TRF) analysis by Southern blot revealed a significant reduction in average telomere size (Figure 2.1B and D). This attrition was highlighted by the significant reduction in intensity of the higher molecular weight TRFs (~16 kb), as compared to $tert^{+/+}$ siblings (Figure 2.1B), in all tissues tested (Supplementary Figure S2.1). Additionally, a lower molecular weight TRF population of approximately 6 kb is present in both $tert^{+/+}$ and $tert^{-/-}$ (arrows in Figure 2.1B and D and Supplementary Figure S2.1). Zebrafish telomere sequences are exclusively terminal, as all TRF signal disappears after BAL-31 (5'- and 3'-exonuclease) digestion (Supplementary Figure S2.1).

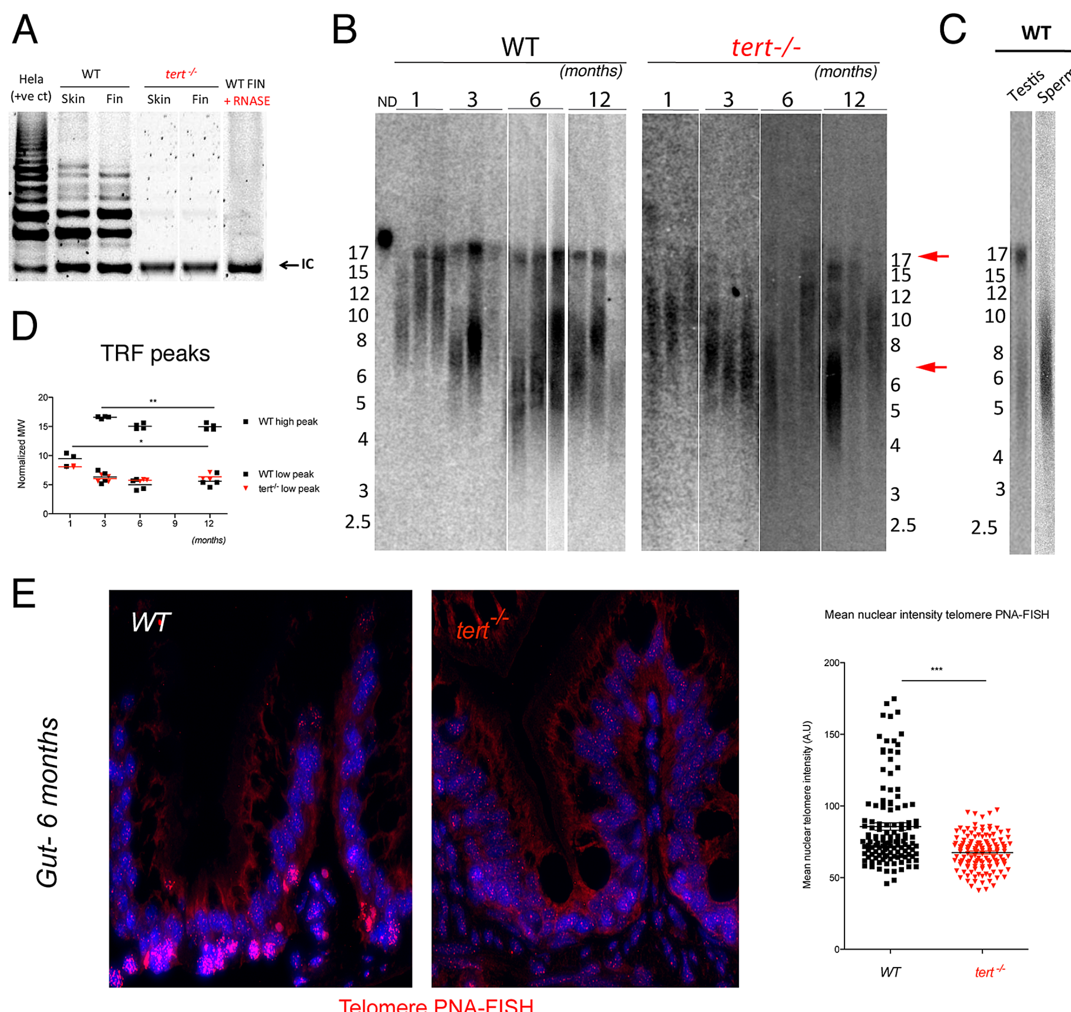


Figure 2.1 – Telomerase mutant zebrafish have shorter telomeres than WT siblings.

A) Representative image of TRAP assay showing that telomerase is not active in the *tert*$^{-/-}$ zebrafish, as compared to *tert*$^{+/+}$ siblings. Here shown are caudal fin and skin protein extracts. Hela cell extract is shown as positive control. N = 4. B) Representative image of restriction fragment analysis of caudal fin genomic DNA of 3 different individuals at different ages, by southern blot (random primer-labelled telomeric probe $(CCCTAA)_{12}$ ^{32}P-dCTP). *tert*$^{+/+}$ Zebrafish have heterogeneous telomeres, with two distinct peaks of different lengths. In *tert*$^{+/+}$ the highest peak (~16 Kb, top red arrow) becomes more distinct after 1 months of age and decreases in length over-time (B and D). The lowest peak of telomere intensity also decreases in length (bottom red arrow, B and D). *tert*$^{-/-}$ zebrafish have shorter telomeres than *tert*$^{+/+}$ siblings in different tissues (see also Supplementary Figures S2.1A-B), observed by the decrease in length

of the higher TRF peak. The shortest TRF peaks accompany those of *tert*$^{+/+}$ siblings, and decrease over-time at similar rates. C) Testes fractionation in *tert*$^{+/+}$ reveals the two-telomere length populations in whole testis, whereas mature sperm only shows the shorter TRF smear of about 6 kb, suggesting different telomere lengths in different cells within a tissue. D) TRF mean sizes were calculated as described in (50). E) Telomere PNA-FISH in 6-month-old gut tissue shows cells with different telomere intensities in the wild type, mainly localizing to the proliferative niche. In contrast *tert*$^{-/-}$ mutants display cells with less bright and more homogeneous telomere intensity.

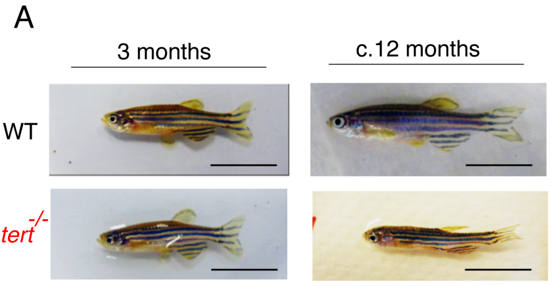
The presence of two telomere populations in *tert*$^{+/+}$ zebrafish is suggestive of restricted telomerase activity. Accordingly, the lower population of TRFs shortens over-time both in *tert*$^{+/+}$ and *tert*$^{-/-}$ (Figure 2.1B, D, E). We also observe discrete but significant shortening of the higher TRF population in *tert*$^{+/+}$, suggesting that telomerase activity is not sufficient to prevent telomere loss. This bimodal TRF pattern was observed in all *tert*$^{+/+}$ tissues except for blood, where we detect a single TRF of higher molecular weight (~16 kb; Supplementary Figure S2.1A-B) and in sperm, where we observed a single TRF signal of approximately 6 kb (Figure 2.1C).

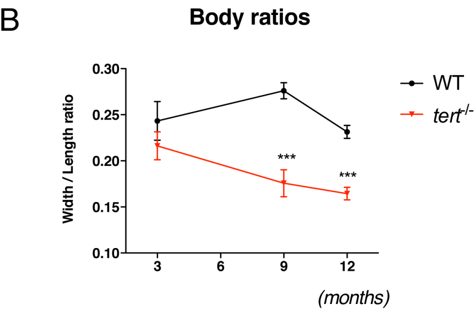
Consistent with our TRF analysis, we observe the presence of cell populations with different telomere intensities by telomere-PNA FISH (Figure 2.1E). This technique clearly shows that *tert*$^{+/+}$ tissues, such as the gut, are composed of cells with different telomere intensities. Cells with high intensity in telomere signal localize primarily to the base of the villus, suggestive of being early precursor cells. In contrast, *tert*$^{-/-}$ tissue shows more homogeneous, low intensity, telomere-PNA FISH. Such pattern mirrors our TRF results of high and low telomere lengths and suggests that telomerase expression is restricted to certain cells types as observed in humans (6).

2.2.2 – Telomerase zebrafish mutants die prematurely of body wasting

First generation *tert*$^{-/-}$ mutants, resulting from a heterozygous incross, are born healthy and develop past sexual maturity without any obvious defects (Figure 2.2A). From 4-6 months onwards, we observed a consistent gradual decrease in body mass reflected in declining width/length body ratios (Figure 2.2B). This wasting phenotype was observed in all *tert*$^{-/-}$ individuals at their time of death (N=24). In contrast, we were unable to detect wasting in any of the *tert*$^{+/+}$ siblings (N=45) until the end of the experiment (22 months). Wasting (also known as cachexia) is a common phenotypic alteration in aged organisms, including humans, and is usually associated with muscle sarcopenia and frailty syndromes (28).

Progressive wasting in *tert*$^{-/-}$ fish was accompanied by an increase in mortality (Figure 2.2C). *tert*$^{-/-}$ mutants die significantly earlier than their *tert*$^{+/+}$ siblings (average lifespan of 9 versus >22 months, $p<0.005$). Due to a male sex bias in our crosses that affected all *tert*$^{+/+}$, *tert*$^{+/-}$ and *tert*$^{-/-}$ progeny, we were unable to obtain significant numbers for female analysis and so the data hereafter presented reflects the study of males alone. Sex determination in zebrafish is still largely unknown, but it is thought to be highly influenced by environmental factors (29).





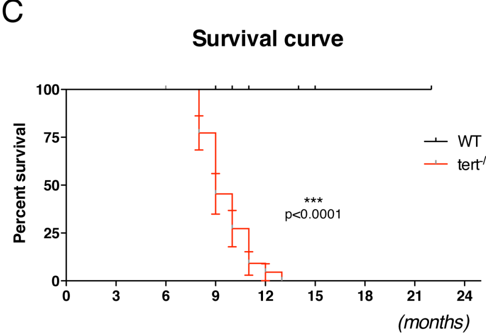


Figure 2.2 – First-generation telomerase mutant zebrafish show progressive body wasting and die prematurely.

A) Representative images of *tert*$^{+/+}$ and *tert*$^{-/-}$ zebrafish show that *tert*$^{-/-}$ fish are born and develop normally until reproductive maturity at ~3 months of age, but progressively lose body mass since then, B) represented as an overall reduction in width/length ratios as compared to wild-type siblings N≥6 $p<0.001$. This progressive wasting phenotype is accompanied by increase in mortality. C) Kaplan-Meier curve showing that *tert*$^{-/-}$ zebrafish have significantly reduced survival when compared to *tert*$^{+/+}$ siblings (AVG lifespan 9 *versus* >22 months ($p<0.005$)). N = 24 *tert*$^{-/-}$; N = 45 *tert*$^{+/+}$. Data are represented as mean +/− SEM. Scale bar = 1 cm.

2.2.3 – Telomerase depletion produces a time- and tissue-specific degeneration

Histopathological analysis of different tissues revealed important phenotypic alterations in *tert*$^{-/-}$ mutants. Telomere shortening has been shown to affect primarily high turnover tissues in both humans (10) and late generation *terc* KO mice (30). Accordingly, we noticed an order of events during tissue atrophy of *tert*$^{-/-}$ zebrafish. *tert*$^{-/-}$ zebrafish testis were the first to depict histopathological abnormalities; second were the liver and intestine; the third to be affected was the kidney and remaining organs, including the muscle (Figures 2.3, 2.4 and summarized in Supplementary Table S2.2).

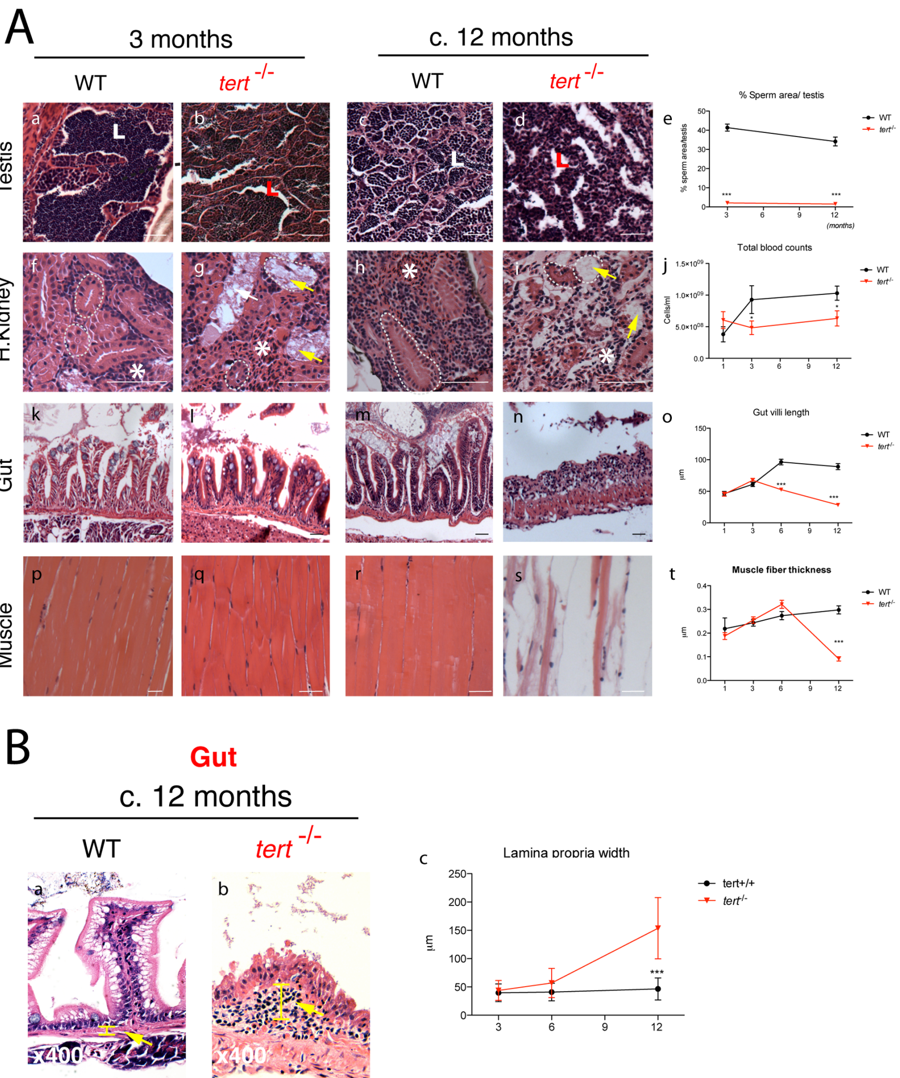


Figure 2.3 – Telomerase depletion leads to a time- and tissue-dependent degeneration. A) Representative images of *tert*$^{-/-}$ and *tert*$^{+/+}$ zebrafish sections stained with hematoxilin-eosin. *tert*$^{-/-}$ show reduced sperm in testis lumen (L) (Ab and d; p<0.001), defects in the marrow area (white asterisks) (Ag and i) that correlate with a decrease in total blood cells (p = 0.0228, N≥5, Aj) and degeneration of mesonephric tubules in the head kidney (Ag and i dashed outlines). Gut atrophy in *tert*$^{-/-}$, reflected as decreased villi length (Al, n, o), becomes significant after 6 months (p<0.001) and muscle fibers are thinner (p<0.001) at c.12 months (Aq, s, t, dashed outline); N≥5. B) *tert*$^{-/-}$ display progressive thickening of the gut lamina propria, indicative of inflammation (Bb, c, yellow bar and arrow, N≥4). Data represented as mean +/− SEM. Scale bar = 50 µm.

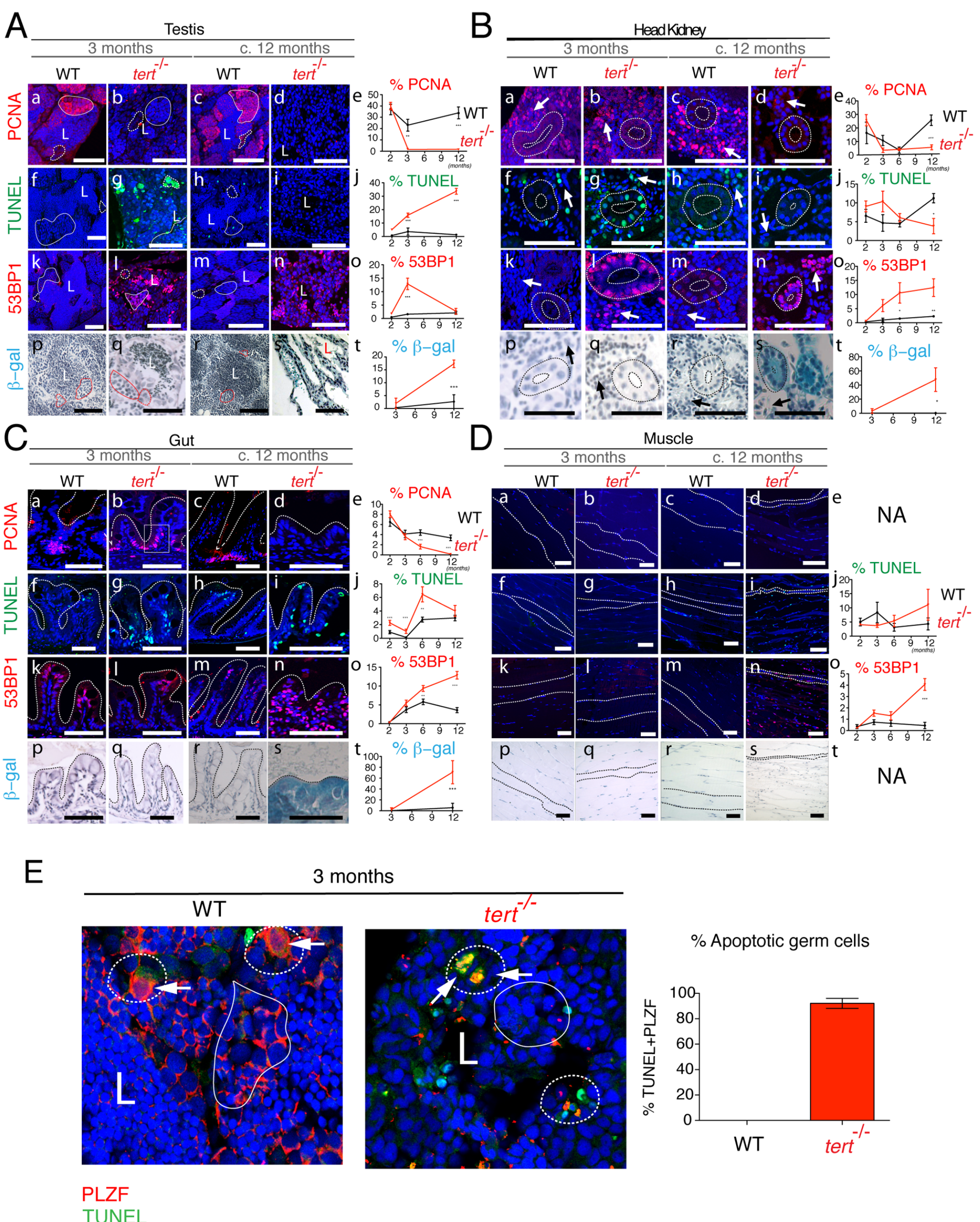


Figure 2.4 – Proliferative tissue degeneration is accompanied by a sustained decrease in proliferation, acute apoptotic responses, and progressive accumulation of DDR foci.

A) Representative IF images of tissue sections in F1 *tert*$^{-/-}$ and *tert*$^{+/+}$ zebrafish show levels of proliferation (PCNA), apoptosis (TUNEL), DNA damage (53BP1) and senescence-associated β–

galactosidase (SA-β-GAL) at the ages of 3 to c.12 months. A) Testis, B) head kidney and C) gut sections show sustained decrease in proliferation in *tert*$^{-/-}$ as compared to *tert*$^{+/+}$ siblings (panels b, d and e) (p<0.001) and an acute apoptotic response at 3 months (p<0.001) (panels g, i and j). This is accompanied by a progressive increase in 53BP1 foci (panels l, n and o; p<0.001) and SA-β-GAL (panels s and t). In the testis, most apoptosis seems to localize to the spermatogenic zone (Ag, dashed outline) and panel E, where we see an increase in TUNEL-labeled germ cells, labeled with the marker PLZF. Most of 53BP1 signal locates to the proliferative zone of maturing spermatocytes (Al, uniform outline). Note in the head kidney B), both *tert*$^{-/-}$ proliferative hematopoietic tissue (Ba–d, arrows) and the non-proliferative mesonephric tubule epithelium (Ba–d, dashed outline) show increased apoptosis (Bg, I, j), DNA damage (Bl, n and o) and senescence (Bs and t). D) Muscle, a largely non-proliferative tissue (Da–d) shows significant accumulation of 53BP1 foci in *tert*$^{-/-}$ by c.12 months (Dn and o; p<0.001). Quantifications performed in at least 3 different fields of view of at least 3 different individuals (each genotype/time-point). Gut IF quantifications calculated as number of positive cells per “crypt” zone (C) uniform square outline exemplified. Other tissues' IF quantified as overall % positive cells. SA-β-GAL quantified as % area stained blue, per field of view. Data are represented as mean +/− SEM. Scale bar = 50 μm.

Testis of *tert*$^{-/-}$ zebrafish shows a severe imbalance, as early as 3 months of age, in size and ratio of the main spermatogenic classes: spermatogonia, spermatocytes and spermatids (31). There was an atrophy of the differentiating and maturing spermatogenic stages (Figure 2.3Ab and Ad). This atrophy is consistent with what was observed in spermatogonia in the late generation *terc* KO mice (30) and *tert* KO mice (18, 32). A consequence of these alterations is the significant decrease in mature sperm volume observed in the lumen of seminiferous tubules of *tert*$^{-/-}$ male fish (L in Figure 2.3Ab and Ad). Consistently, we observed that *tert*$^{-/-}$ zebrafish males are prematurely infertile (Supplementary Figure S2.2). The scarce progeny originating from a *tert*$^{-/-}$ incross was not viable and displayed embryonic deficiencies consistent with lack of cell proliferation, such as failure to close the neural tube and body truncations (Supplementary Figure S2.2). Female *tert*$^{-/-}$ mutants were initially fertile (Supplementary Figure S2.2) but became infertile later in life, when body wasting became apparent (data not shown).

Similar to telomerase deficiencies in humans and mice with critically short telomeres, *tert*$^{-/-}$ zebrafish display blood defects, translated into a mild but significant decrease in total blood cells (Figure 2.3Aj). Accordingly, histopathological analysis of the head kidney marrow (the major hematopoietic organ in fish) revealed a trend towards depletion of the hematopoietic compartment, particularly at late time points (asterisks in Figure 2.3Ai). Other proliferative tissues, such as the gut, show a progressive decrease in microvilli length (Figure 2.3Al,n,o) and increased inflammation of the lamina propria, significant after the age of 6 months (Figure 2.3Bc). These changes progress into severe gut degeneration (necrotizing enteritis), most visible at terminal stages (Figure 2.3An). During this period, we observed a pervasive mucosal thickening (sloughing; bar in Figure 2.3Bb), denuded villous tips, and inflammatory cell infiltration (arrows in Figure 2.3Bb). Atypical intestinal epithelium compatible with severe dysplasia was also observed (data not shown).

Low-proliferative tissues such as the muscle (Figure 2.3Ap-t) and liver (data not shown) only exhibit obvious degeneration at the latest time points of the *tert*$^{-/-}$ zebrafish lifespan. We observed acute and significant muscle degeneration (sarcopenia) at terminal stages (Figure 2.3As) consistent with the wasting phenotype (Figure 2.2A and B). These observations suggest that low-proliferative tissues are also targets of telomere dysfunction, as has been suggested before in mice models (33-35). Whether this happens in a cell autonomous or non-autonomous manner remains to be clarified.

Severe gut degeneration (necrotizing enteritis) is a prime candidate for cause of death of *tert*$^{-/-}$ zebrafish. This could account for mal-nutrition, consequent loss of muscle and wasting.

2.2.4 – Tissue atrophy is preceded by lack of cell proliferation, apoptosis and senescence

Telomere depletion was shown to affect primarily proliferative tissues with a high cell turnover. Quantification of cell division in *tert*$^{-/-}$ zebrafish using the S-phase marker PCNA revealed an overall decrease in cell division in proliferative tissues (Figure 2.4A-C, panels a-e). This was particularly clear in the spermatogenic zone of the testis (filled-line outline in Figure 2.4Ab,d). Spermatogenesis is initiated by the proliferation of stem cells (Spermatogonia A and progenitor stem cells - Spermatogonia B). These are responsible for the continuous renewing of this highly proliferative tissue. Decrease in cell proliferation in the testis was accompanied by an initial burst in apoptosis, as detected by TdT-mediated dUTP nick end labelling (TUNEL; dashed line outline in Figure 2.4Ag). This burst of apoptosis in *tert*$^{-/-}$ is restricted to the germinal centers, as shown by co-localization with the specific germ cell marker PLZF (36) (Figure 2.4E), explaining why tissue homeostasis is compromised in these animals, as shown in mice (37). Initial increase of apoptosis was followed by a progressive decline, losing statistical significance at later time points (Figure 2.4Ag,i,j).

A second player in disrupting tissue integrity is the accumulation of senescent cells (9, 20, 38). We observed a progressive increase in cells presenting strong 53BP1 *foci* in *tert*$^{-/-}$ testes, indicative of persistent or irreparable DNA damage (Figure 2.4Al,n,o). Persistent 53BP1 has been described as a hallmark of cell senescence, accumulating preferentially at telomeres (39, 40). Consistently, we observed an increase in senescence-associated β-galactosidase (SA-β-gal) staining at later time points (Figure 2.4As,t).

Similar to testis, both kidney marrow and gut show an initial up-regulation of apoptosis, statistically significant at 3 months old (Figure 2.4Bg,j and Cg,j). The head kidney in zebrafish has a dual function of excretion and haematopoiesis

(41). Apoptosis is up regulated both in the kidney mesonephric tubules and haematopoietic tissue (dashed outline and arrow, respectively, in Figure 2.4Bg), consistent with the decreased blood cell levels observed (Figure 2.3Aj). In the gut, this increase in apoptosis is accompanied by decreased proliferation at the base of the villi, statistically significant from 6 months onwards (Figure 2.4Ca-e). Decreased cell proliferation in the gut is accompanied by progressive increase in DNA damage *foci*, as detected by 53BP1 staining (Figure 2.4Ck-o). In all proliferative tissues, 53BP1 staining reached its peak at terminal stages, where no apoptosis is detected (Figure 2.4A-C, panels n,o). Senescent cells have been described to be resistant to apoptosis (42). Consistently, in proliferative tissues, we noticed that 53BP1 foci containing cells corresponded to regions where SA-β-gal staining was evident (panels n and s in Figure 2.4A-C).

Low-proliferative tissues, such as the muscle, only show significant defects at later time points. Muscle sarcopaenia is accompanied by an acute increase in cells stained with 53BP1 (Figure 2.4Dn,o). Contrary to proliferative tissues, 53BP1 staining in the muscle (Figure 2.4Dn) did not correlate with an increase in cell senescence, as we were unable to detect significant SA-β-gal staining (Figure 2.4Ds,t).

2.2.5 – p53 expression leads to *puma*-apoptosis and *ccng1*-senescence.

In late generation telomerase KO mice, telomere shortening activates a p53 dependent DDR that culminates in apoptosis and cell-cycle arrest (21). Similarly, we observed a p53 response in proliferative tissues of *tert*$^{-/-}$ zebrafish, such as the testis and gut (Figure 2.5A). This response is accompanied by an acute up-regulation of *puma* (Figure 2.5B a, c). Consistent with our apoptosis data, *puma* expression is reduced at later stages (c.12 months of age; Figure 2.5B a, c). In the gut, *puma* expression is replaced by sustained up-regulation of the cell cycle arrest targets *cdkn1a* and *cyclin G1* (Figure 2.5Cc and Dc).

Recently, *cyclin G1* has been depicted as a p53-dependent target in zebrafish (43). Up-regulation of cell cycle inhibitors through p53 activation culminated in cell cycle arrest and senescence. This has been described as recurrent phenomena in telomerase knockout murine tissues with dysfunctional telomeres (19). Finally, we do not detect a significant p53 response in either the head kidney (an heterogeneous tissue), or in the muscle, a low-proliferative tissue.

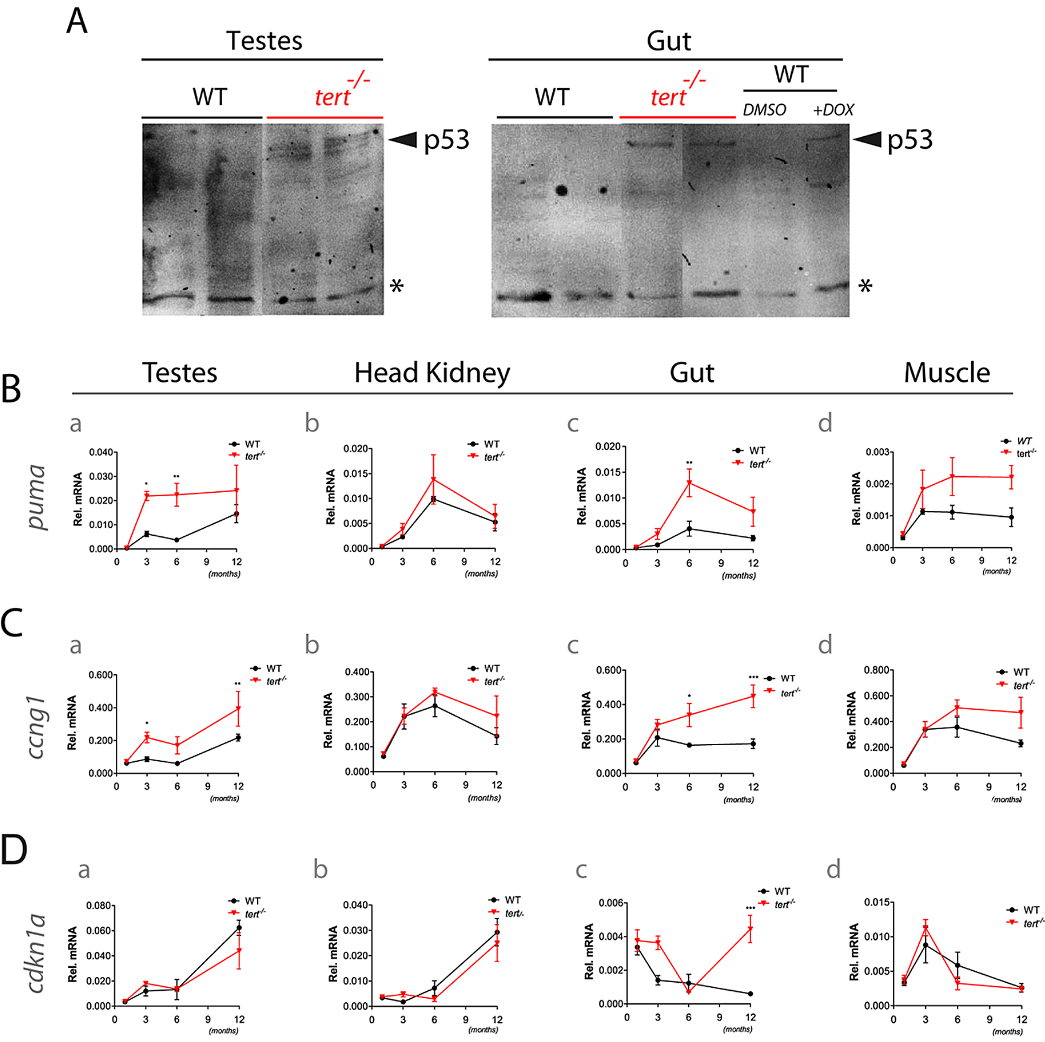


Figure 2.5 – Tissue degeneration is accompanied by p53 induction with a *puma* acute response and sustained increase of *cyclin G1* and *cdkn1a* expression.

A) Immunoblot analysis of p53 in 10-month old WT and *tert*$^{-/-}$ testis and gut lysates. 6 month-old WT zebrafish were injected with the DNA damaging agent doxorubicin to serve as positive control for p53 activation. Asterisk depicts a non-specific cross-reactive band that serves as loading control. RT-qPCR analysis showing expression of B) pro-apoptotic (*puma*) and cell cycle arrest targets (C) *cyclin G1* and D) *cdkn1a*) in testis, head kidney, gut and muscle of 1, 3, 6 and c.12 months old WT and *tert*$^{-/-}$ zebrafish (N = 3 to 8 fish per genotype). Data are represented as mean +/− SEM. *Rel. mRNA* refers to relative mRNA levels of each gene normalized to *beta-actin*.

2.2.6 – Elimination of *tp53* function partially rescues *tert*$^{-/-}$ phenotypes

Our data show that telomerase deficiency in zebrafish gives rise to a p53 response, which ultimately culminates in cell cycle arrest (Figure 2.5), as observed in mice models (19). Accordingly, tp53 deletion in mice was shown to ameliorate late generation *tert* KO phenotypes such as infertility (44). To test whether p53 could be mediating the phenotypes observed in the *tert*$^{-/-}$ zebrafish, we crossed *tert*$^{+/-}$ with *tp53*$^{-/-}$ mutants (*tp53*$^{zdf1/zdf1}$ (45)) to produce double mutant *tert*$^{-/-}$*tp53*$^{-/-}$ zebrafish. Mortality was significantly rescued, since at c.12 months, approximately 87% of *tert*$^{-/-}$ *tp53*$^{-/-}$ (N= 31) were alive, compared to 0% of *tert*$^{-/-}$ (N=24). Immunofluorescence analysis in *tert*$^{-/-}$*tp53*$^{-/-}$ proliferative tissues, revealed a dramatic rescue of cell proliferation in tissues that were most affected in *tert*$^{-/-}$ (testis and gut at c.12 months; Figures 2.6 Ac, d and Bc, d).

This rescue in cell proliferation, however, was not sufficient to prevent testis atrophy and decreased number of mature sperm in *tert*$^{-/-}$ *tp53*$^{-/-}$ (Figure 2.6Aa,b). In contrast, rescue of the proliferative capacity in the gut of the double mutant clearly suppressed *tert*$^{-/-}$ gut villi length defects (Figure 2.6Ba,b). Despite an increased cell proliferation, this partial phenotypic rescue was accompanied by a significant increase in TUNEL-labelled cells in the *tert*$^{-/-}$ *tp53*$^{-/-}$ testis (Figure 2.6Ae, f) and gut (Figure 2.6Be, f) and maintenance or slight decrease in 53BP1 staining (Figures 2.6Ag, h and Bg, h). Thus, similar to the mouse model (44),

p53 appears to mediate *tert*$^{-/-}$ phenotypes, as *tp53* function depletion partially rescues or delays proliferative tissue degeneration observed in *tert*$^{-/-}$ zebrafish.

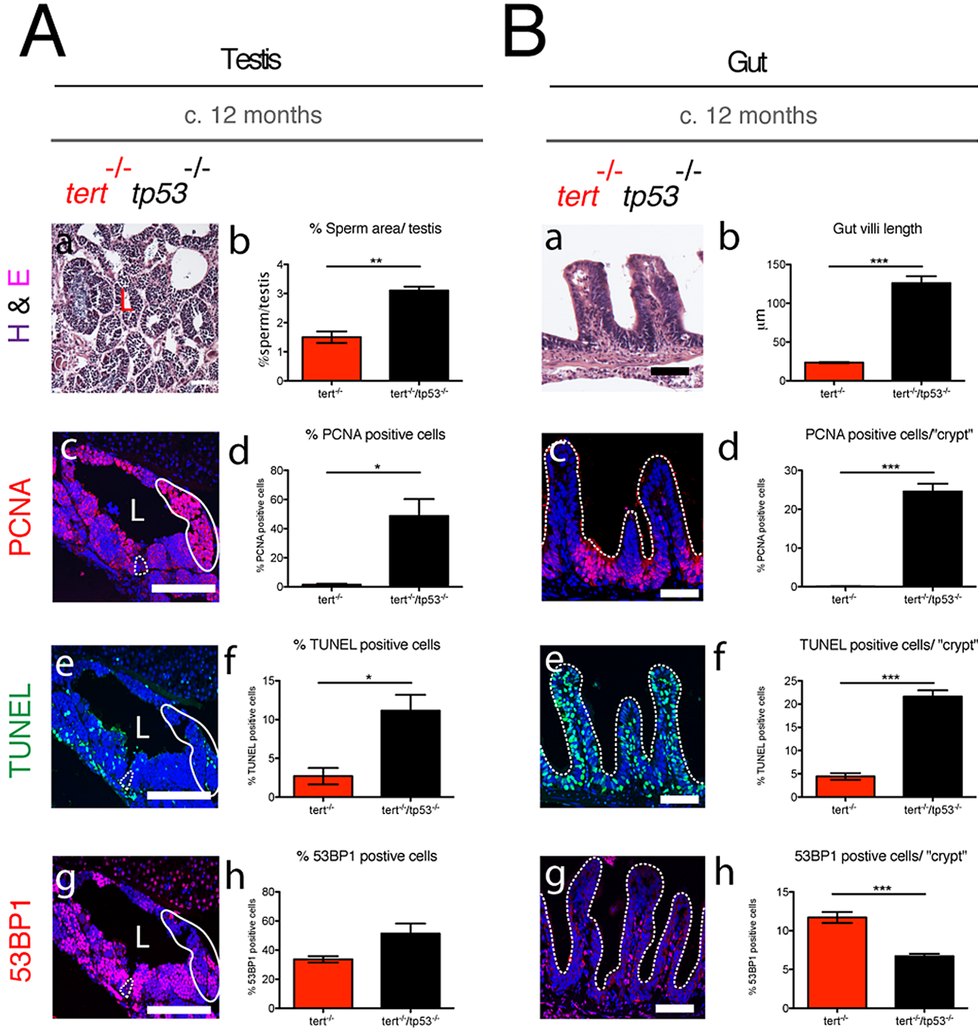


Figure 2.6 – Elimination of *tp53* function partially rescues *tert*$^{-/-}$ degeneration in proliferative tissues.

A) *tert*$^{-/-}$*tp53*$^{-/-}$ show increased proliferation and apoptosis in both testis (Ac, d and e, f) and gut (Bc, d and e, f), as compared to *tert*$^{-/-}$ alone. In the testis, dashed outline represents spermatogenic zone, uniform outline proliferative zone of maturing spermatocytes and L the lumen where mature sperm is located. Elimination of *tp53* function partially rescues mature sperm numbers (Aa, b) but completely rescues gut villi length (Ba, b). DNA damage as assessed by

53BP1 is maintained in *tert*$^{-/-}$*tp53*$^{-/-}$ testes (Ag and h) and decreased in the gut (Bg and h), as compared to *tert*$^{-/-}$. N≥3. Data are represented as mean +/− SEM. Scale bar = 50 μm.

2.3 – Discussion

Most of our knowledge on how vertebrates respond to short telomeres derives from laboratory mice, which are particularly different from humans not only in what respects telomere length, but also in cell immortalization and entry into senescence (46). Zebrafish has recently emerged as an attractive complementary vertebrate model for studying telomere biology. Zebrafish possess short telomeres that decline with age and correlate with impaired tissue repair (24). These observations motivated us to investigate the impact of telomere shortening in a biological system that, like humans, may have evolved to use telomere length as an internal cell division "clock".

In our current work, we show that zebrafish telomerase mutants have premature degenerative phenotypes and decreased lifespan in the F1 generation. This contrasts with results obtained from telomerase KO mice, where degenerative phenotypes are only observed upon several generations of null crosses (16-18, 32). The phenotypes we observe correlate well with the accumulation of short telomeres in *tert*$^{-/-}$ zebrafish, along with the disappearance of long telomeres. The two telomere populations may reflect different phenomena: 1) Presence of undigested genomic DNA; 2) Distinct populations of sub-telomeric sequences in the same cells or 3) Telomeres of different lengths present in distinct cells. We excluded the first two possibilities. Regarding the first point, undigested genomic DNA samples used as controls run at different sizes to the long TRF population (ND in Figure 2.1B). As for the second, different DNA methylation patterns in *tert*$^{+/+}$ and mutants could account for the presence of unequal TRF populations due to restriction enzyme sensitivity. To exclude this possibility, we used a restriction enzyme insensitive to DNA methylation, Tru9I, and observed an equivalent TRF pattern (data not shown).

We favour the third hypothesis in which high molecular weight TRFs are derived from cells with longer telomeres present in the same tissue. This is probably the case for testes. Mature sperm from the same individual exhibited only the shorter TRF population, whereas whole testes tissue possesses both. Thus, cells that comprise the testes, other than sperm, must have longer telomeres and are maintained through time by telomerase. In contrast to mature sperm, blood cells isolated from WT individuals exhibited exclusively long telomeres. Consistent with other tissues, *tert*$^{-/-}$ zebrafish also have shorter telomeres in blood.

The hypothesis that tissues harbour cells with telomeres of different lengths is corroborated by our telomere PNA-FISH data. *tert*$^{+/+}$ tissues, such as the gut, have cells with very different telomere intensities suggestive of shorter and longer telomeres. In contrast, *tert*$^{-/-}$ tissues show more homogeneous, low intensity, telomere-FISH. Notably, the majority of high-telomere-intensity cells in *tert*$^{+/+}$ are located to the villus proliferative zone. These cells are generally absent in the *tert*$^{-/-}$ tissues, suggesting that cells with longer telomeres are strictly telomerase dependent. This suggests that telomerase is activated during embryogenesis and elongates telomeres in stem and early precursor cells. In *tert*$^{-/-}$ mutants this cannot happen and, even though cells proliferate initially, their telomeres shorten with each cell division giving rise to populations with shorter telomeres.

Together, our data support the idea that telomerase is limiting for zebrafish telomere maintenance, since we observe long and short telomeres in WT. Accordingly, shorter telomeres in *tert*$^{+/+}$ are equivalent in size to the ones present in *tert*$^{-/-}$ zebrafish. Shorter telomeres decline rapidly in the first 3 months of life both in *tert*$^{+/+}$ and *tert*$^{-/-}$ fish, steadily decreasing for the next 3-6 months. This period of fast telomere shortening correlates with intense body growth preceding sexual maturity, also observed in humans (47).

Our work describes a choreography of defects caused by telomere shortening in different tissues over-time. Consistent with the late generation telomerase KO mice, *tert*$^{-/-}$ zebrafish die prematurely due to an acute depletion of proliferating cells (30). The choice between senescence and apoptosis in different cells may dictate the homeostatic threshold of individual tissues. This regulation will determine how the whole organism responds to telomere dysfunction. Proliferative tissues, such as testis, gut and kidney marrow are affected first. Progressive gut degeneration, with severe necrotizing enteritis, is the most likely cause for body wasting and premature death. We observed different apoptotic and senescent responses in different tissues. Immunofluorescence data identified an acute apoptotic response in proliferative tissues, namely the testis and gut and, to a lesser extent, in head kidney. This correlated with increased p53 levels and *puma* expression measured by RT-qPCR, particularly evident in proliferative tissues, such as the gut and testis. This increase in apoptosis is largely cleared at later time points, where DNA damage and senescence became the dominant phenomena in degenerated *tert*$^{-/-}$ tissues.

Interfering with *tp53* function in *tert*$^{-/-}$*tp53*$^{-/-}$ double mutants significantly increased proliferation in testes and gut, partially rescuing degenerative phenotypes. This indicates that p53 is a crucial mediator of the *tert*$^{-/-}$ degenerative phenotypes in proliferative tissues. Rescue of cell proliferation is not without consequence, since cells continue to accumulate 53BP1 DDR markers and enter apoptosis. However, the apoptotic response in *tert*$^{-/-}$*tp53*$^{-/-}$ zebrafish differs from the *tert/tp53* KO mice (44, 48), since apoptosis increases in comparison to *tert*$^{-/-}$ single mutants, whereas it decreases in mice. Increase in apoptosis in *tert*$^{-/-}$*tp53*$^{-/-}$ zebrafish is mediated via p53-independent mechanisms, such as those under the control of p63 or p73 (14). This suggests different mechanistic responses in zebrafish to telomere dysfunction to those in present in the mouse. It also reinforces the idea of separate homeostatic thresholds in

different tissues, since p53 removal can only partially rescue testes integrity whereas gut villi length is completely restored in *tert*$^{-/-}$*tp53*$^{-/-}$.

In conclusion, zebrafish is a suitable model system to understand the effects of telomere shortening during lifespan of an organism. Telomerase is also required for low-proliferative tissue homeostasis. Whether this occurs in an autonomous or non cell-autonomous manner remains to be clarified. Nevertheless, *tert*$^{-/-}$ also have shorter telomeres in low-proliferative tissues, suggesting that both scenarios are possible. Reduced or absent telomerase may trigger whole body degeneration by blocking cell proliferation causing an unbalanced tissue homeostasis, and this is largely p53-mediated in high proliferating tissues.

2.4 – Materials and Methods

2.4.1 – Ethics Statement

All Zebrafish work was conducted according to Institutional and National Guidelines and approved by the Ethical Committee of the DGV (Portuguese Veterinary Authority) and the European Union Regulatory Agency.

2.4.2 – Zebrafish lines and maintenance

The telomerase mutant line *tert* $^{AB/\ hu3430}$ possesses a T→A point-mutation in the tert gene. Zebrafish mutant lines were generated by N-Ethyl-N-nitrosourea (ENU) mutagenesis (Utrecht University, Netherlands) (49). Briefly, adult male zebrafish were randomly mutagenized with ENU and outcrossed against wild-type females. A library of F1 animals was then constructed. Genomic DNA of these F1 animals was isolated and arrayed in PCR plates. The DNA was screened for mutations in target genes by re-sequencing or TILLING. Animals with interesting mutations were recovered from the library (either re-identified from a pool of living F1 fish or recovered by in vitro fertilization with frozen

sperm) and outcrossed against wild-type fish. *tert* $^{AB/hu3430}$ line is available at the ZFIN repository (ZFIN ID: ZDB-GENO-100412-50) from the Zebrafish International Resource Center (ZIRC) and was generously provided to us by Dr. L. Bally-Cuif at the Zebrafish Neurogenetics Department, German Research Center for Environmental Health. The *tert*$^{AB/hu3430}$ used in this paper was subsequently outcrossed 5 times with WT AB for clearing of potential background mutations derived from the random ENU mutagenesis from which this line was originated. The *tert*$^{hu3430/hu3430}$ homozygous mutant is referred in the paper as *tert*$^{-/-}$ and was obtained by incrossing our *tert*$^{AB/hu3430}$ strain. Genotyping was performed by PCR of the *tert* gene (Table S1) followed by sequencing. Overall characterization of *tert*$^{-/-}$ zebrafish was performed in F1 and F2 animals produced by *tert*$^{+/-}$ incross. All 1, 3 and 6 months analysis refers to F1 animals and 12 months analysis refers to F2. The premature death phenotype depicted in Figure 2.2C refers to F1 animals only.

2.4.3 – Telomerase activity assay (TRAP)

Telomerase activity was measured using the TRAPEZE® Telomerase Detection Kit (S7700, Millipore, MA, USA) as described by manufacturers. Briefly, fish were sacrificed in 200 mg/L of MS-222 (Sigma, MO, USA) and a small portion of skin and fin were extracted from at least three different individuals of the different genotypes. Protein extracts were prepared by mashing tissue sections on ice with a micro-pestle in a 1.5ml eppendorf tube, in 100µl of CHAPS buffer with proteinase inhibitors cocktail (Sigma, MO, USA) and RNase inhibitor (200U/ml, Invitrogen, UK). Cell extracts were incubated on ice for 30 minutes and centrifuged at 12,000xg for 20 minutes at 4°C. The supernatant was quantified using Bradford protein quantification reagent (Pierce, IL, USA) and the TRAP assay was performed using 2µg of protein/sample. The positive control was provided by the TRAP kit and used as described (S7700, Millipore, MA, USA).

2.4.4 – Telomere restriction fragment (TRF) analysis by Southern blot

TRF analysis was performed as previously described (50). Briefly, genomic DNA extraction from freshly isolated tissue was performed using extraction buffer (50mM Tris-HCl pH8; 50mM EDTA; 10mM NaCl; 1% SDS) supplemented with 1mg/ml Proteinase K (Sigma, MO, USA) and RNase A (1:100 dilution, Sigma, MO, USA) prior to use. Samples were incubated at 50ºC for 18h in a thermomixer and genomic DNA was extracted by equilibrated phenol-chloroform (Sigma, MO, USA) and chloroform-isoamyl alcohol extraction (Sigma, MO, USA). Blood genomic DNA was extracted with TNES buffer (10mM Tris pH7.4; 100mM NaCl; 10mM EDTA; 0.5% SDS), supplemented with RNase A (1:100 dilution, Sigma, MO, USA) prior to use. Samples were incubated 10 minutes at RT and extracted as for other tissues. Genomic DNA was quantified and normalized so the same amount of DNA was digested with RSAI and HINFI enzymes (NEB, MA, USA) as described previously (50) for 12h at 37°C. BAL31 (NEB, MA, USA) digestion was performed at 30°C for different time points. Samples were ran on a 20cm 0.6% agarose gel, in 0.5% TBE buffer, at 4°C for 17h at 110 constant voltage. Southern blotting was performed as previously described (51).

2.4.5 – Histological preparation and phenotypic analysis

Fish were sacrificed as described above, fixed overnight in 4% paraformaldehyde and decalcified in 0.5M EDTA for 24-48h at 4ºc. Whole fish were then paraffin-embedded and 5 micrometer sections were stained with Hematoxylin-Eosin for histopathological analysis. Embryos were fixed overnight in 4% PFA at 4ºC as above and then placed in 100% methanol at 4ºC before processing. After several washes in PBS (phosphate buffer saline) the embryos were cryoprotected on a sucrose 15%/PBS solution and then embedded in

7,5% pork skin gelatine (Sigma)/15% sucrose/PBS for one hour at 37ºC. The $1cm^2$ blocks were frozen in isopenthane/liquid nitrogen and stored at -80ºC until sectioning. The embedded samples were cut in 12um sections with a cryostat (Leica CM 3050S). Hematoxilin-Eosin staining was performed in serial cuts.

2.4.6 – Immunofluorescence (IF) and confocal analysis

Whole fish slides were sub-boiled for 10 minutes at 800W in a microwave in citrate buffer (10mM Sodium Citrate, pH6) for antigen retrieval. Slides were washed 3 times in dH_20 for 5 minutes each, followed by TBST (Tween 0.1%) for 5 minutes. After washes, slides were blocked for 1 hour at RT in 0.25% BSA in PBST (Triton 0.3%). The following primary antibodies were used: rabbit monoclonal antibodies against Proliferation Cell Nuclear Antigen (PCNA, Santa Cruz, CA, USA, 1:50 dilution), 53BP1 (Life-span Biosciences, WA, USA, 1:100) and anti-PLZF (Life-span Biosciences, WA, USA, 1:100). Incubation with primary antibodies was performed overnight in the dark, at 4ºC, followed by 5 minutes, 1 hour and two 10 minutes PBS washes. Secondary antibody Alexa Fluor 568 goat anti-rabbit (Invitrogen, UK, 1:500 dilution) was then applied overnight at 4°C, followed by final washes in PBS, as described for the primary antibody washes. Apoptosis was detected using the In Situ Cell Death Detection Kit (Roche, SW) according to manufacturer's instructions. Briefly, after de-paraffinization, slides were incubated with 40mg/ml Proteinase K in 10mM Tris-HCl pH7.4, 30 minutes at 37°C. Slides were washed in 2x 5 minutes in PBS and then incubated with TUNEL labelling mix (protocol indicated by the supplier). Washes were performed as previously described. Slides were incubated with TO-PRO3 (Molecular Probes, Invitrogen, UK, 1:5000 dilution) and DAPI (Sigma, MO, USA, 1:2000 dilution) nuclear staining for 30 minutes at room temperature in the dark, followed by two 5 minutes' washes with PBS. Coverslips were then mounted with DAKO Fluorescence Mounting Medium (Sigma, MO, USA). Confocal images were acquired on Leica TCS SP5 II (Leica Microsystems,

GER) equipped with Leica Las AF Lite software and with appropriate configurations for multiple colour acquisition. For quantitative and comparative imaging, equivalent image acquisition parameters were used.

2.4.7 – Telomere PNA-FISH

Immuno-FISH was performed as in (39). Briefly, zebrafish paraffin sections, processed as for IF were hydrated by incubation in 100% Histoclear, 100, 95 and 70% methanol for 5 min and in distilled water for 5 min. Whole fish slides were sub-boiled for 10 minutes at 800W in a microwave in citrate buffer (10mM Sodium Citrate, pH6) for antigen retrieval. After cooling the slides were washed with distilled water for 5 min (2x). Slides were washed three times in PBS and dehydrated with 70, 90 and 100% ethanol for 3 min each. Sections were denatured for 5 min at 80°C in hybridization buffer (70% formamide (Sigma), 25 mM MgCl2, 1 M Tris pH 7.2, 5% blocking reagent (Roche)) containing 2.5μg/ ml Cy-3-labelled telomere specific (CCCTAA) peptide nuclei acid probe (Panagene), followed by hybridization for 2h at room temperature in the dark. The slides were washed twice with 70% formamide in 2×SSC for 15 minutes, followed by 10 minutes wash with 2×SSC and PBS. Sections were incubated with DAPI (SIGMA), mounted and imaged. Z stacking was performed (a minimum of 40 optical slices with ×100 objective) followed by Image-J deconvolution.

2.4.8 – Senescence-Associated β-galactosidase assay

β-galactosidase assay was performed as previously described (25). Briefly, sacrificed zebrafish adults were fixed as before and then washed 3 times for 1h in PBS-pH7.4 and for a further 1 h in PBS-pH6.0 at 4°C. β-galactosidase staining was performed for 24h at 37°C in 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 2mM MgCl2 and 1 mg/ml X-gal, in PBS adjusted to

pH6.0. After staining, fish were washed 3x for 5 minutes in PBS pH7 and processed for de-calcification and paraffin embedding as before. Sections were stained with hematoxilin for nuclear detection and images were acquired in a bright field microscope (Leica DMLB2, GER).

2.4.9 – Real-time quantitative PCR

Age- and sex-matched fish were sacrificed in 200 mg/L of MS-222 (Sigma, MO, USA) and portions of each tissue (gonads, gut, liver, head kidney and muscle) were retrieved and immediately snap-frozen in liquid nitrogen. RNA extraction was performed in TRIzol (Invitrogen, UK) by mashing each individual tissue with a pestle in a 1.5ml eppendorff tube. After incubation at RT for 10 minutes in TRIzol, chlorophorm extractions were performed. Quality of RNA samples was assessed through BioAnalyzer (Agilent 2100, CA, USA). Transcription into cDNA was performed using random primers (20 mg) (Promega C1181, WI, USA). Quantitative PCR (qPCR) was performed using PerfeCTa SYBR Green FastMix, ROX (Quanta, MD, USA) and an ABI 7900HT Sequence Detection System (Applied Biosystems, CA, USA). qPCRs were carried out in triplicate for each cDNA sample. Relative mRNA expression was normalized to beta-actin and rpl13 α (data not shown) mRNA expression using the ΔCT method (derived from the Livak & Schmittgen method, 2001). Primer sequences are listed in Table S1.

2.4.10 – Immunoblot analysis

Each tissue was dissected and homogenized in HEPES buffer (HEPES 10mM, KCl 300mM, MgCl2 3mM, CaCl2 100mM, Triton X-100 0.45%, Tween-20 0.05%, pH 7.6) including complete protease inhibitor cocktail (Roche Diagnostics). Cell extracts were incubated on ice and centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was collected and quantified using

Bradford protein quantification reagent (Pierce). Loading mix was added to protein extracts, heated at 95ºC for 5 minutes and loaded onto a 12.5% SDS-PAGE gel (80µg of protein/ sample). After electroblotting the gel onto a PVDF membrane, incubation was performed overnight at 4ºC with anti-Tp53 (AnaSpec, 55342) specific for zebrafish, in TBS-T with 5% milk powder (using a 1:300 dilution). Chemiluminescence detection was performed with an ECL KIT (Amersham). Western blots were performed on WT and *tert*$^{-/-}$ 10 month-old tissue samples (N= 3-4).

2.4.11 – Doxorubicin assay

To induce a DNA-damage p53-dependent response, adult fish were anesthetized in MS-222 (Sigma) and doxorubicin (Sigma) was injected intraperitoneally. A dosage of 15mg/kg body weight was used. Fish were allowed to recover for 24h, after which they were sacrificed in 200mg/L of MS-222 (Sigma) and the organs collected for analysis of p53 protein levels by Western Blot.

2.4.12 – Statistical analysis

Immunofluorescence (IF): Image edition was performed in Adobe Photoshop CS5.1. All statistical analysis was performed in GraphPad Prism5, using Mann Whitney's unpaired t-test when only two points were compared. More than one point comparison over-time was performed by two-way ANOVA test with Bonferroni post-correction. A critical value for significance of $p<0.05$ was used throughout the study. Real-time quantitative PCR: Statistical analysis was performed in GraphPad Prism5, two-way ANOVA with Bonferroni post-correction. A critical value for significance of $p<0.05$ was used throughout the study.

2.5 – Supplementary Figures

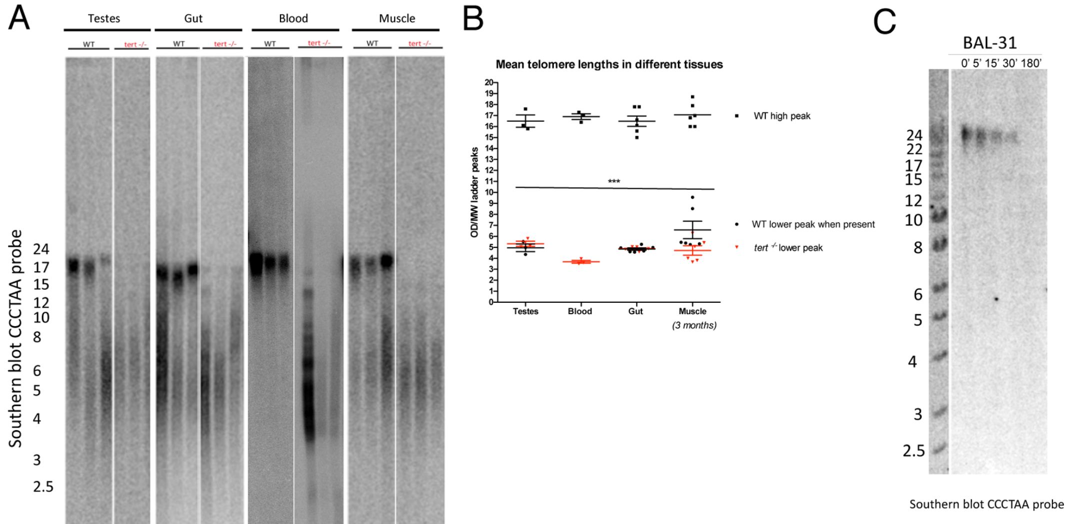


Figure S2.1 – *tert*$^{-/-}$ zebrafish have shorter telomeres than *tert*$^{+/+}$ in all tissues tested.

A) Representative Southern blots and TRF analysis of different 3-month old tissues, show decreased telomere sizes in *tert*$^{-/-}$ as compared to *tert*$^{+/+}$. All tissues have long and short TRF populations in *tert*$^{+/+}$, except the blood, where only a long TRF of approximately 15 Kb is detected. *tert*$^{-/-}$ show a severe decrease of these long telomeres, and mainly show the short TRF smear of approximately 6 Kb. B) Mean TRF peak quantifications of A). C) Representative Southern blot of Bal31 (a 5′ and 3′ terminal exonuclease) restriction of fin genomic DNA shows that all telomeric signals correspond to terminal sequences. N≥3. Data represented as mean +/− SEM.

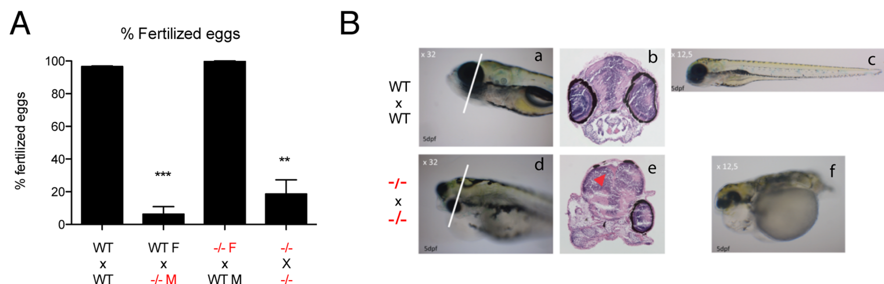


Figure S2.2 – First generation *tert*$^{-/-}$ show premature male infertility.

A) *tert*$^{-/-}$ mutant males are infertile by 6 months, represented here as percentage of fertilized eggs per cross (Fertilized/total eggs). Number of crosses = 3. B). F1 maternal zygotic progeny is not viable due to gross developmental abnormalities. N≥3. Data are represented as mean +/− SEM.

Table S2.1 – List of primers used in RT-qPCR expression analysis and *tert* genotyping.

Gene name	Primer sequences	Reference
beta-actin	forward – 5’ TTCACCACCACAGCCGAAAGA 3’ reverse – 5’ TACCGCAAGATTCCATACCCA 3’	(52)
cdkn1a	forward – 5’ ATGCAGCTCCAGACAGATGA 3’ reverse – 5’ CGCAAACAGACCAACATCAC 3’	Duan et al., 2011
cyclin G1	forward – 5’ GTGATGAAGATTCAGCCCAAGC 3’ reverse – 5’ CACTGGCCAGAGGGACATTTTTCT 3’	Our study
puma	forward – 5’ CCTCACATGATGCCTTCAGC 3’ reverse – 5’ CATTGATGGTGTCCGAGACC 3’	(43)
tp53	forward – 5’ GGTGCTGAATGGACAACTGTGCT 3’ reverse – 5’ GCAACTGACCTTCCTGAGTCTCC 3’	Our study
tert	forward – 5’ GACGACCAGTTCGGATCCCTTC 3’ reverse – 5’ CTTTACCCTCCGCCGCTTTACC 3’	Our study

Table S2.2 – Time-dependent histopathological changes in *tert*$^{-/-}$ zebrafish.

Semi-quantitative histopathological analysis was performed using a score ranging from (−) to (+++), depending on the severity and extent of the lesions: (−) none, (+) minimal to mild, (++) moderate, (+++) severe.

	3 month-old	**6 month-old**	**c. 12 month-old**
Testis	+++	n.a.	+++
Liver	++	++	++
Gut	-	+	+++
Kidney	-	++	+++
Bone marrow	-	-	++

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Chapter 3 | Short telomeres in key tissues initiate local and systemic aging in zebrafish

This chapter is an adapted version of a manuscript that is under review: Carneiro et al. *Short telomeres in key tissues initiate local and systemic aging in zebrafish.* PLOS Genetics. (2015).

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Authors' contributions

Conceived and designed the experiments: MGF MCC

Performed the experiments: MCC performed most of the experiments; CMH performed Southern Blots on Figures 3.1 and 3.3 and flow cytometry experiments; Tânia Carvalho performed the histopathological analysis; Ana Faustino collaborated in the behavioral assays.

Analyzed the data: MGF MCC CMH (RO collaborated in the behavioral assays)

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Abstract

Telomeres shorten with each cell division and telomere dysfunction is a hallmark of aging. Tissue proliferation is expected to dictate the rate at which telomeres shorten. We therefore set out to test whether proliferative tissues age faster than non-proliferative due to telomere shortening in zebrafish aging. We performed a prospective study linking telomere length to tissue pathology and disease. Contrary to expectations, we show that telomeres shorten to critical lengths only in specific tissues and independently of their proliferation rate. Short telomeres accumulate in the gut but not in other highly proliferative tissues such as the blood and gonads. Notably, the muscle, a low proliferative tissue, accumulates short telomeres and DNA damage at the same rate as the gut. Together, our work shows that telomere shortening and DNA damage in key tissues triggers not only local dysfunction but also anticipates the onset of age-associated diseases in other tissues, including cancer.

3.1 Introduction

Telomeres are the structures, composed of DNA repeats and a specific set of protective proteins (the shelterin complex), that cap eukaryotic chromosomes. Telomeres have two main functions: 1) preventing recognition of the chromosome ends as deleterious DNA double strand breaks (DSBs) and 2) ensuring their complete replication by recruitment of telomerase, a reverse transcriptase that carries a RNA subunit serving as template for the synthesis of new telomeric DNA repeats (1, 2). Due to the “end-replication problem”, telomeres shorten with each round of cell division in cells that do not express telomerase (3, 4). On average, human cells lose 50-100 bp from their extremities in every round of division (5-7).

Telomerase expression is restricted in most human somatic cells (8). Consequently, as observed in population studies, telomeres shorten significantly during human aging (9). The rate of telomere decline is most pronounced in early life, from birth to puberty, slowing down in adulthood (9, 10). Telomere erosion is a strong barrier to cell proliferation and thus a robust tumor suppression mechanism (11). However, by limiting the function of stem cell reserves necessary for tissue renewal, telomere shortening may also act as a strong promoter of aging-associated degenerative phenotypes. Indeed, short telomeres are a frequent feature of diseases that anticipate facets of aging, such as Dyskeratosis Congenita, DC (12-14). DC harbors mutations in several components involved in telomere maintenance such as DKC1 (dyskerin), a component of the active telomerase enzyme complex (15), hTERC (telomerase RNA), hTERT (telomerase reverse transcriptase) and in shelterin component TINF2 (16-19). Mutations in DKC1 associate specifically with an X-linked form of DC (20). Short telomeres as cause of age-related disease is further supported by other syndromes, such as Idiopathic Pulmonary Fibrosis (IPF) and Hoyeraal-Hreiderasson (16, 21). These so-called “telomeropathies” exhibit a pattern of genetic anticipation, in which later generations suffer from increased disease severity (12, 22, 23). These diseases are characterized by dysfunction of proliferative tissues, namely bone marrow, lung and liver, ultimately leading to multi-organ failure. Dysfunction and disease severity can be predicted by the extent of short telomere accumulation in different tissues (24, 25). Apart from these symptoms, DC patients show severe mucocutaneous alterations, such as oral leukoplakia and skin hyperpigmentation, and an increased susceptibility for developing cancer (12).

A direct association between telomere shortening and tissue specific dysfunction in vertebrates has been clearly demonstrated in late-generation telomerase knockout mice (both catalytic enzyme, *mTert*$^{-/-}$ and RNA subunit, *mTerc*$^{-/-}$) and, more recently, by us and others in zebrafish (26-30). Telomerase

plays a pivotal role in high-turnover mouse and zebrafish tissues (26, 27, 29). In G6 *Terc*$^{-/-}$ mice, intestinal epithelial cells and male germ cells have reduced proliferative capacity and increased apoptosis, resulting in tissue atrophy and infertility (29). In low proliferative tissues, such as heart and liver, G4 *Tert*$^{-/-}$ mice incur in metabolic failure by repressing master regulators of mitochondrial biogenesis, via upregulation of p53 (31). In zebrafish, first generation telomerase mutants show a tissue and time dependent dysfunction that associates with short telomeres and diminished proliferative rates (27).

Telomere shortening may also disrupt tissue integrity in a non-cell autonomous manner, by inducing cell senescence. Senescent cells accumulate with age in a variety of mammalian tissues, such as skin and eye (32). These cells secrete a specific set of molecules, including inflammatory cytokines and chemokines, growth and survival factors, which are referred to as the "Senescence-Associated Secretory Phenotype" (SASP) (33). SASP creates a microenvironment which compromises homeostasis and promotes features of tumor malignancy, including invasiveness (33).

While short telomeres may underlie part of the cascade of tissue homeostasis decline with aging, mammalian species are affected by many other factors with time, such as mitochondrial dysfunction, epigenetic alterations and impaired proteostasis (34, 35). It remains to be defined in which tissues telomeres shorten to critical levels with natural aging, triggering DNA Damage Responses (DDR) and, ultimately, organ dysfunction. Identifying the tissues where short telomeres dictate phenotypes of aging and how these tissues then influence the organism as a whole is crucial for understanding the impact of telomere length on aging.

Zebrafish has emerged as an important vertebrate model to study both telomere biology and aging (26, 27, 36-39). First generation telomerase mutant zebrafish age and die prematurely (26, 27), reminiscent of human telomere shortening syndromes. Yet, a detailed analysis of organ dysfunction and

respective telomere shortening dynamics throughout lifetime in wild type animals remains largely unexplored. We conducted for the first time a comparative prospective study of telomere dynamics, DNA Damage Response (DDR) and aging-related dysfunction and disease in different tissues in both WT (from 3 to 42 months) and *tert*$^{-/-}$ zebrafish (from 3 to 12 months, when at least 50% of *tert*$^{-/-}$ mutants are dead). This allowed us to define the choreography (timings and kinetics) of age-related lesions and how these are conserved between WT and *tert*$^{-/-}$. We discovered that *tert*$^{-/-}$ mutants anticipate the tissue-specific dysfunctional events observed during normal zebrafish aging, including tumorigenesis.

3.2 – Results

3.2.1 – Zebrafish telomere length varies between different tissues

Human population studies revealed variation in telomere length between different tissues, irrespective of their proliferation rates (40). In birds, variation in length was also found between different somatic tissues (41). Zebrafish has telomeres of human like length, ca. 5-16 kb (27, 36, 42) and absence of telomerase limits its lifespan (26, 27). To test if different zebrafish tissues have different telomere lengths within an individual, we dissected juvenile zebrafish and performed Telomere Restriction Fragment, TRF (43) analysis of high proliferation (gut, testis) and low proliferation tissues (muscle). We observed that, whereas at larval stages telomeres are more homogeneous and longer (larvae, “L”, Figure 3.1A), different tissues have acquired different telomere lengths by the time of sexual maturity, at 3 months (shown for two independent WT zebrafish “1” and “2” in Figure 3.1A; densitometry shown only for WT individual “1”, Figure 3.1B). We observed most WT tissues have a mixture of long and short populations of TRFs and *tert*$^{-/-}$ mutants predominantly have the

latter (shown for two independent *tert*$^{-/-}$ zebrafish "1" and "2" in Figure 3.1A; densitometry shown only for *tert*$^{-/-}$ individual "1", Figure 3.1B). The longer telomere population is of the same size observed in blood and thus likely to reflect circulating cells in all tissue samples. We therefore decided to use the median Telomere Length (mTL) as a conservative measurement of telomeres in different cell populations/tissues.

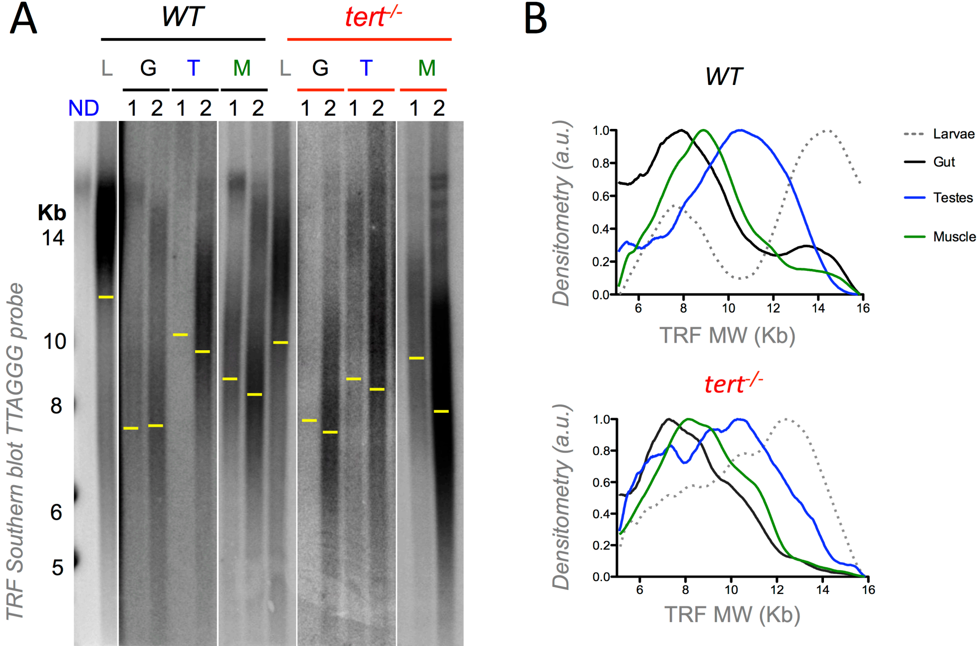

Figure 3.1 – Different zebrafish tissues have different telomere lengths.

A) Representative images of restriction fragment analysis of genomic DNA by Southern Blot (random primer-labelled telomeric probe (TTAGGG)n ^{32}P-dCTP). WT telomeres are longer in whole larvae ("L", ca. 12 kb) and shorter in 3 month-old zebrafish gut (G), testis (T) and muscle (M), but show significant variation in length between tissues – shown for two independent WT zebrafish "1" and "2"; densitometry shown for WT "1" in B). Differences in telomere length between tissues are independent of telomerase as they are globally maintained in *tert*$^{-/-}$ tissues – shown for two 3-month mutants "1" and "2" in Figure 3.1A; densitometry shown for *tert*$^{-/-}$ "1" in B). Yellow line indicates median telomere length, mTL, for each tissue sample/lane. TRF mean sizes were calculated as previously described (43). mTL data is represented as mean +/- SEM.

Three-week-old WT zebrafish larvae had long telomeres (ca.12 Kb) that declined with growth and development (Figure 3.1A). Sexually mature males had long mTL in the testis (ca. 9.9 Kb) followed by the muscle (ca. 8.7 Kb) while the shortest telomeres measured were in the gut (ca. 7.9 Kb, Figure 3.1A). Strikingly, these relative telomere length differences proved to be telomerase independent as they were globally maintained in *tert*$^{-/-}$ tissues (larvae had telomeres of ca. 10.2 Kb which declined to ca. 8.9 Kb in the testis, followed by muscle ca. 8.4 Kb and gut ca. 7.8 Kb; Figure 3.1A). This suggests that, like in humans, where telomerase activity is restricted in most tissues, telomere length and set points are established during zebrafish development.

3.2.2 – Zebrafish telomeres decline with age and the rate of shortening varies between tissues

In order to determine the dynamics of telomere shortening with aging in different tissues, we performed TRF analysis at different ages in the fin, a less proliferative tissue that we previously reported to have shorter telomeres, and in the kidney marrow (the hematopoietic organ in zebrafish), a proliferative tissue with long telomeres (27). We measured telomeres in four independent individuals and observed significant shortening of WT fin telomere length with age, at the population level (quantified in graph in Figure 3.2A, shown for only two individuals per age group in the blot). We detected a linear decline on mTL from 3 to 24 months of age, ranging ca. 8.6 Kb to 7.3 Kb (Figure 3.2A). From 24 months of age onwards, we did not detect further telomere shortening (Figure 3.2A), suggestive of an exhaustion of proliferation potential in later life stages. In humans, the rate of telomere decline is also more pronounced during the first periods of life, up to 18 years of age (9). Additionally, we were able to calculate the telomere shortening rate for two zebrafish for their first 24 months of life, by taking advantage of the well-studied regenerative capacity of zebrafish fin. We estimated a decline of 45 bp/month and 90 bp/month respectively. Since

telomerase expression was reported to increase during fin regeneration (36), this "non-invasive" assay was performed by collecting different fins at different ages in each individual.

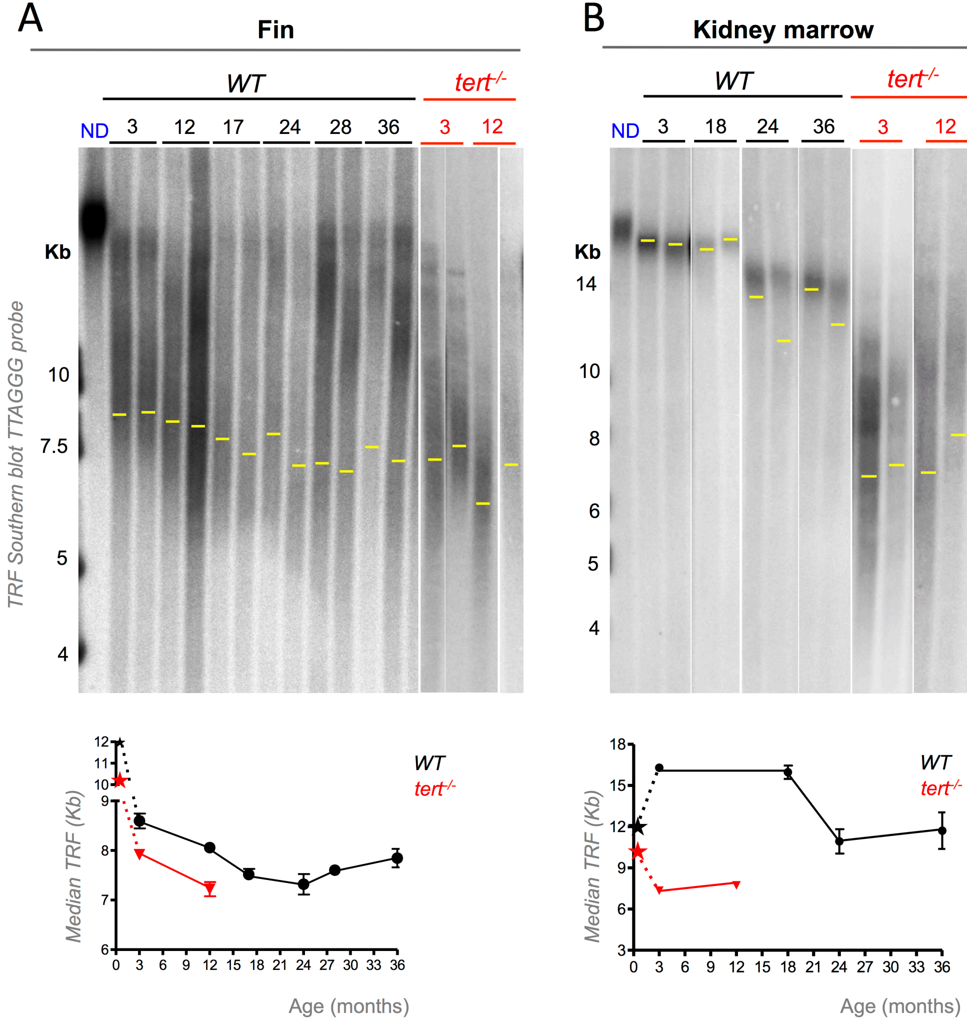

Figure 3.2 – Zebrafish has tissue-specific dynamics of shortening with aging.
A) WT telomeres significantly shorten in the fin and B) kidney marrow with age, but follow different shortening kinetics with time (black star represents WT larvae telomeres; two zebrafish shown for each age after sexual maturation – 3, 12, 17, 24, 28, 36 months for the fin and 3, 18, 24, 36 months for the kidney marrow). In A) lanes 1 and 2 of each time point are the same individual over time between the ages of 3 and 24 months. WT telomeres in the fin match the shorter length of 12-month old *tert*$^{-/-}$ mutants by 18-24 months (quantifications based on N=4 for WT and N=3 for

tert$^{-/-}$ fin; red star represents *tert*$^{-/-}$ larvae telomeres), but B) WT kidney marrow telomeres never reach *tert*$^{-/-}$ levels (quantified for N=3-6 per time point for WT and N=3 for *tert*$^{-/-}$ mutants).

Contrary to any other tissue analyzed, WT kidney marrow telomeres dramatically elongated from larvae to fully developed adult stages and shortened with age after 18 months (Figure 3.2B). At the population level, by averaging different individuals' telomere length (quantified N=3-6 for each time point from 3 months onwards), no appreciable shortening could be detected in the first 18 months of life, after which telomeres declined (Figure 3.2B). This is suggestive of higher telomerase activity and, consequently, lesser telomere shortening in kidney marrow than in the fin.

Telomeres shortened significantly with age in the fin of WT, matching the length of 12-month old *tert*$^{-/-}$ mutants (quantifications for N=3-4 for each time point/genotype) by 18-24 months (Figure 3.2A). In contrast, WT telomeres of the kidney marrow never reached the length of *tert*$^{-/-}$ mutants (quantifications for N=3-6 for each time point/genotype) during their lifetimes (Figure 3.2B). This data shows that telomere length, as measured by TRF, correlates directly with zebrafish age up to 18-24 months albeit in a tissue specific manner. In addition, mTL shortening reaches a limit bellow which we are unable to detect further decrease. As previously seen by others (9, 10), this is likely due to a selection process that eliminates cells with extremely short telomeres thus creating a virtual barrier to telomere decrease.

3.2.3 – WT gut and muscle telomere lengths reach *tert*$^{-/-}$ mutants' levels with aging

We have previously shown that *tert*$^{-/-}$ mutants have shorter telomeres than WT siblings already at larval stages (Figure 3.1A and (27)). As consequence, *tert*$^{-/-}$ mutants develop premature cell proliferation defects and DDR which culminate in early tissue degeneration (27). Thus, in our study, we used *tert*$^{-/-}$ mTL as

proxy for the presence of critically short telomeres sufficient to cause tissue dysfunction in WT.

To probe for accumulation of short telomeres, we determined the variation in telomere length in WT proliferative tissues gut and testis and less proliferative tissue muscle, over time (quantifications based on N=3-4 per time point for gut and muscle; N=5-6 per time point for testis; Figure 3.3A-C). While WT kidney marrow maintained longer telomeres of ca. 16Kb (Figure 3.2B), mTL shortened to ranges of 6 to 12Kb in other tissues (Figure 3.3A-C, densitometries shown for one individual per age in Figure 3.4). For the time points tested after sexual maturity, mTL ranged 6.5-8 Kb in the gut, 7.8-10.2 Kb in the muscle and 9.3-11.1 Kb in the testis (Figure 3.3A-C). We further detected significant mTL shortening in the gut and muscle of WT with aging, at 24 months (Figure 3.3A-B). Similarly to the fin, gut and muscle telomeres significantly declined in a linear fashion between 3 and 24 months in WT, stabilizing in the last time point of 36 months (Figure 3.3A-B). *tert*$^{-/-}$ mutants had shorter mTL already by 3 months of age in all tissues and we could not detect further significant shortening by the age of 12 months (N=3-4 per time point for gut and muscle, N=4-6 per time point for testis; Figure 3.3A-C). When comparing WT and *tert*$^{-/-}$, we found that telomeres in both gut and muscle of old WT reached the length of 12-month-old *tert*$^{-/-}$ (6.8 Kb for gut and 8.5 Kb for muscle; Figure 3.3A-B), around the age of 20 months (indicated by blue arrow in Figure 3.3A-B). In contrast, WT telomeres did not shorten appreciably with age in the testis (indicated by blue arrow in Figure 3.3C).

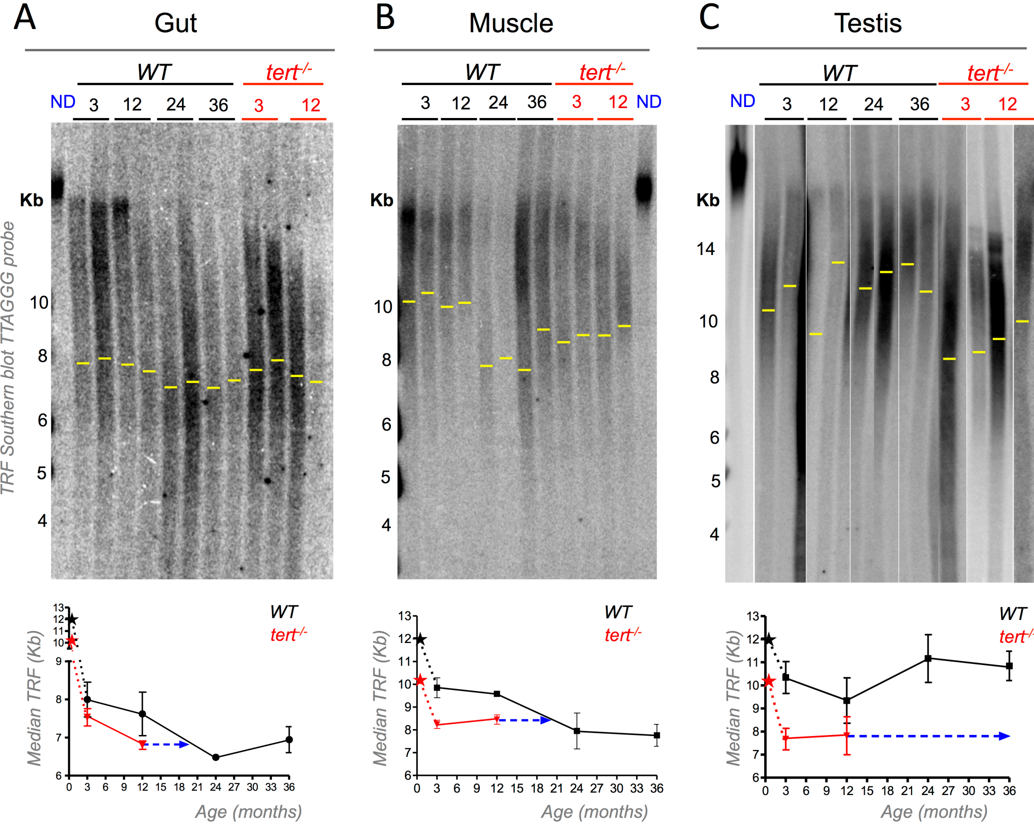

Figure 3.3 – Telomeres shorten in WT gut and muscle reaching the length of *tert*$^{-/-}$ tissues. A-C) Representative images of restriction fragment analysis of genomic DNA by Southern Blot (random primer-labelled telomeric probe (TTAGGG)n ^{32}P-dCTP) and quantifications of median telomere length. Black and red stars represent WT and *tert*$^{-/-}$ larvae telomeres, respectively, in quantifications of median TRF over time. WT telomeres shorten linearly with aging, from 3 to 24 months, in A) gut (N=3-4 per time point) and B) muscle (N=3-4 per time point), stabilizing in later ages. C) No significant telomere shortening is detected in the testis (N=5-6 per time point). Around 20 months of age WT telomeres reach the shorter length of 12 month-old *tert*$^{-/-}$ in the gut (graph in figure A – ca. 6.8 Kb) and muscle (graph in figure B – ca. 8.5 Kb) but not in testis (graph in figure C), indicated by blue arrow. N=3-4 per time point for *tert*$^{-/-}$ gut and muscle, N=4-6 per time point for *tert*$^{-/-}$ testis. TRF mean sizes were calculated as previously described (43). Data are represented as mean +/- SEM.

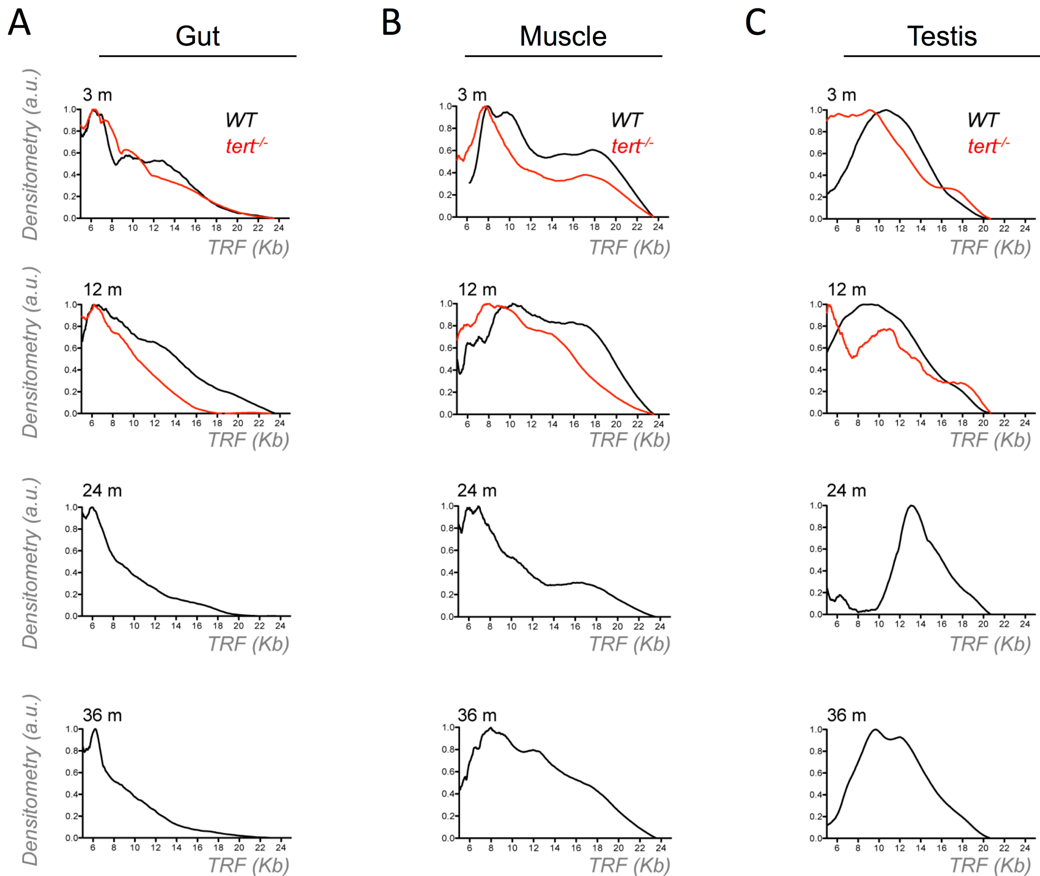

Figure 3.4 – Telomeres shorten in WT gut and muscle, but not testis (densitometries for Fig. 3.3)

A-C) Representative densitometries of TRFs in Figure 3.3, for one zebrafish of each age (WT at 3, 12, 24 and 36 months and *tert*$^{-/-}$ mutants at 3 and 12 months). *a.u.* represents arbitrary telomere length units.

In summary, telomeres shorten in the fin, gut and muscle (but not in testis and kidney marrow) of old WT zebrafish, to the level of *tert*$^{-/-}$, regardless of the distinct proliferation rates of these tissues.

3.2.4 – Short telomeres precede activation of DDRs in aging

One of the hallmarks of aging is the general accumulation of DNA damage (44). This leads to robust activation of DDR pathways, which accumulate preferentially at short or damaged telomeres (45, 46). In order to determine if short telomeres precede activation of persistent DDRs in tissues, we measured the appearance of nuclear foci of phosphorylated H2AX at Ser139 (γ-H2AX) over time. Presence of short telomeres in *tert*$^{-/-}$ tissues resulted in the accumulation of DDR markers. From 3 to 12 months, DDR markers increased significantly from 0.5 to 11.4% in the gut (p=0.0077, Figure 3.5A, 3.5E), 1.4 to 10.5% in the kidney marrow (p=0.0096, Figure 3.5B, 3.5E) and 0.5 to 5.0% in the testis (p=0.0065, Figure 3.5C, 3.5E) and showed a slight tendency to increase in the muscle (Figure 3.5D, 3.5 E). During this period, telomere length was always shorter in *tert*$^{-/-}$ tissues when compared with WT lengths (Figures 3.2 and 3.3).

In order to investigate whether short telomeres dictated the onset of tissue dysfunction in WT tissues, we asked if changes in molecular markers related to DDR, cell proliferation and apoptosis were correlated with mTL decline (Figure 3.5E). In the gut and muscle of old WT, shorter mTL directly correlated with the increase in DDR markers, which reached *tert*$^{-/-}$ levels (p=0.021 and p=0.044, Figure 3.5E). More specifically, the percentage of WT cells bearing five or more γH2AX foci increased from 2% to 18% in the gut (p<0.0001) and from 0 to 9% in muscle (p=0.0007), between the ages of 3 and 36 months, as mTL declined from 8 to 6.9Kb and from 10.2 to 7.8Kb respectively (Figure 3.5A, 3.5D-E). However, an increase in DDR with age, from 3 to 36 months, was also detected in the testis and kidney marrow (of 12%, p=0.0027, and 28%, p=0.0003, respectively, Figure 3.5B-C, 3.5E) of WT zebrafish, even though these tissues failed to significantly shorten their telomeres to *tert*$^{-/-}$ levels. Thus, DDR activation in cells of naturally aged zebrafish strongly correlates with telomere

shortening. However, some highly proliferative tissues, such as testis, exhibit marks of DDR independently of critically short telomeres, as set by *tert*$^{-/-}$ mTL.

We next asked whether the observed decline in mTL could anticipate other cellular phenotypes of aging. We looked for changes in rates of cell proliferation and apoptosis emerging with time and correlated them with mTL decline. Indeed, using PCNA immunostaining as readout for cell division, we observed decreased proliferation in the gut and testis of *tert*$^{-/-}$ mutants already by 3 months of age and by 12 months in the kidney marrow (Figure 3.5A-C). Accordingly, the percentage of proliferating cells dropped in the gut of older WT individuals (3 month vs. 36 months) from 38% to 10% ($p<0.0001$, Figure 3.5A) and this strongly correlated with telomere shortening ($p=0.015$, Figure 3.5E). Testis and kidney marrow also showed a marked decrease in cell division with aging. We observed a decline of 19% ($p=0.0005$) and 12% ($p< 0.0001$) of PCNA stained cells between 3 months and 36 months, respectively (Figure 3.5B-C). Overall alterations in proliferation were particularly evident in areas where stem cells and progenitors reside, such as the basal compartment of the seminiferous tubules in the testis and the base of intestinal villi (Figure 3.5A and C). However, similarly to DDR, testis and kidney marrow showed decreased proliferation with aging regardless of appreciable telomere length variation (Figure 3.5E). In a similar way, decrease of mTL with age did not correlate with apoptotic index in any studied tissue ($p=0.31$ for gut, $p=0.13$ for muscle, $p=0.40$ for testis, $p=0.32$ for kidney marrow, Figure 3.5E). Nevertheless, a significant increase of apoptosis with age was observed by the higher TUNEL staining, detected in all 36 month old WT tissues when compared with 3-month old groups ($p=0.031$ for gut; $p=0.0008$ for kidney marrow; $p=0.0091$ for testis; $p=0.0279$ for muscle, Figure 3.5A-D).

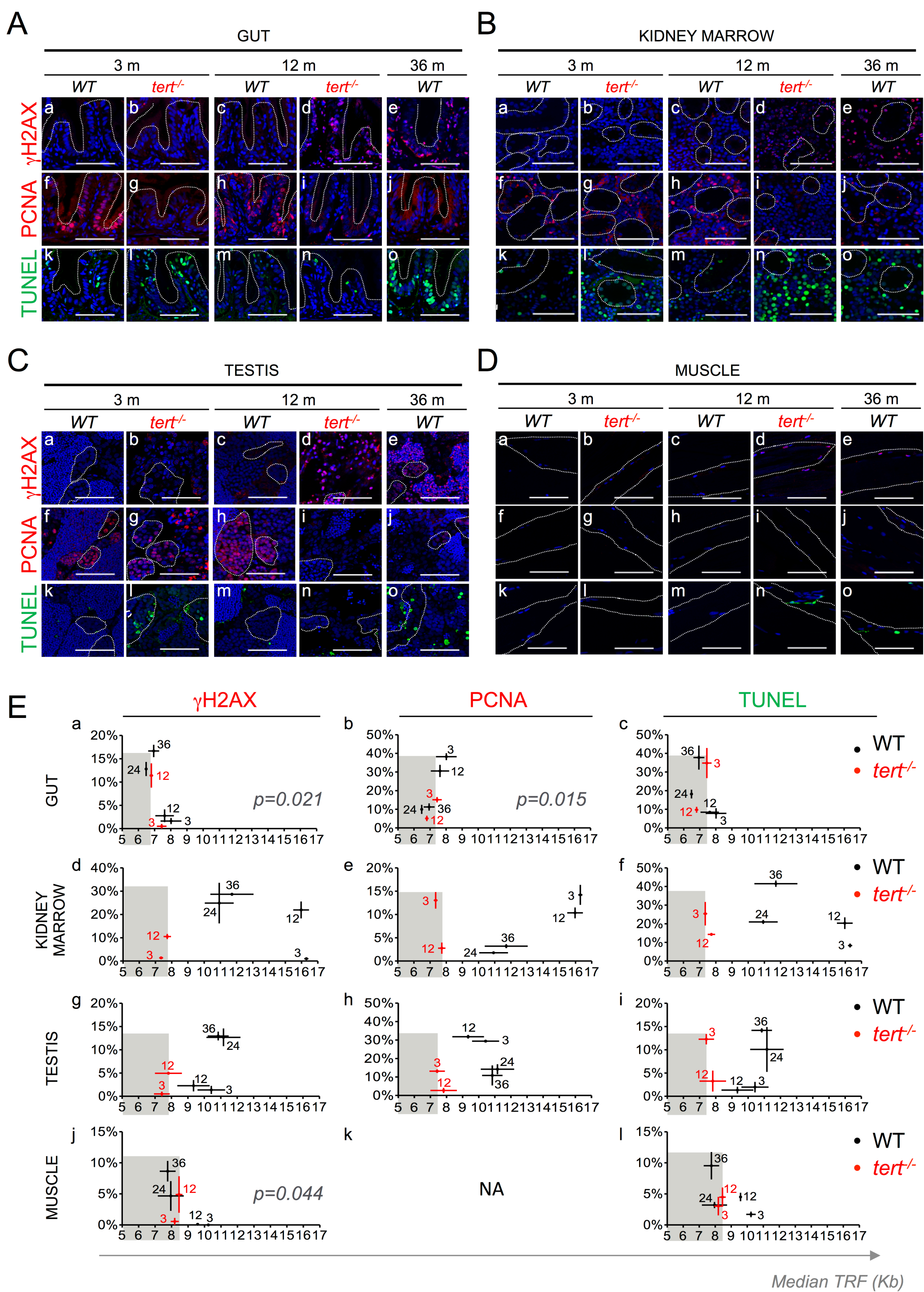

Figure 3.5 – Shortening of mTL anticipates the accumulation of DDR in the gut and muscle.

A-D) Representative IF images of DNA damage (γH2AX), proliferation (PCNA) and apoptosis (TUNEL) for gut (A), kidney marrow (B), testis (C) and muscle (D) of WT and *tert*$^{-/-}$ zebrafish. All tissues show a significant increase in number of γH2AX foci by 12 months in *tert*$^{-/-}$ zebrafish (panels b, d) and by 36 months in WT (panels a, c and e). Increased DDR correlates with shorter mTL in Ea) Gut (p=0.021) and Ej) Muscle (p=0.044) but not Ed) Kidney Marrow or Eg) Testis. Grey shaded area identifies the telomere length at which significant DDR activation is observed in *tert*$^{-/-}$ (Ea, Ed, Eg, Ej). A) Gut B) kidney marrow and C) testis, show a sustained decrease in cell proliferation by 12 months in *tert*$^{-/-}$ (panels g, i) and by 36 months in WT (panels f, h and j). Eb) Decrease in proliferation correlates with shorter mTL in the gut (p=0.015). Grey shaded area identifies the telomere length at which significant proliferation defects are observed in *tert*$^{-/-}$ (Eb, Ee, Eh). A) Gut B) kidney marrow and C) testis show increased apoptotic responses in 3 month-old *tert*$^{-/-}$ (panel l), while WT show a continuous accumulation of apoptotic signals with age (panels k, m, o). Apoptotic responses are not anticipated by shorter mTL (Ec, Ef, Ei, El). Grey shaded area identifies the telomere length at which significant apoptotic responses are observed in *tert*$^{-/-}$ (Ec, Ef, Ei, El). WT and *tert*$^{-/-}$ age groups are indicated in each graph by black and red colored numbers. IF quantifications were performed in at least 3 different fields of view for 3-5 different individuals per time point per genotype. Gut IF quantifications were calculated as number of positive cells per "crypt" zone. Other tissues' IF was quantified as overall % positive cells. Scale bar = 50 mM. N=3-6 for tissue mTL telomere length quantifications per genotype per time point (x-axis in graphs of Figure 3.5E). IF and mTL quantifications are represented as mean +/- SEM.

Senescent cells accumulate gradually in aging tissues (32) and critically short telomeres determine the onset of replicative senescence in human cells (47). We assessed whether mTL decline would also correlate with the increase in senescence found in aging zebrafish tissues. Our results showed that senescence levels increased significantly with age in the gut, measured by senescence-associated β-galactosidase (SA-β-gal), but this increase did not correlate significantly with telomere length (p=0.10, Figure 3.6). Senescence was visible in testis and kidney marrow, but was undetectable in the muscle, and also did not correlate with mTL decline with age in these tissues (Figure 3.6).

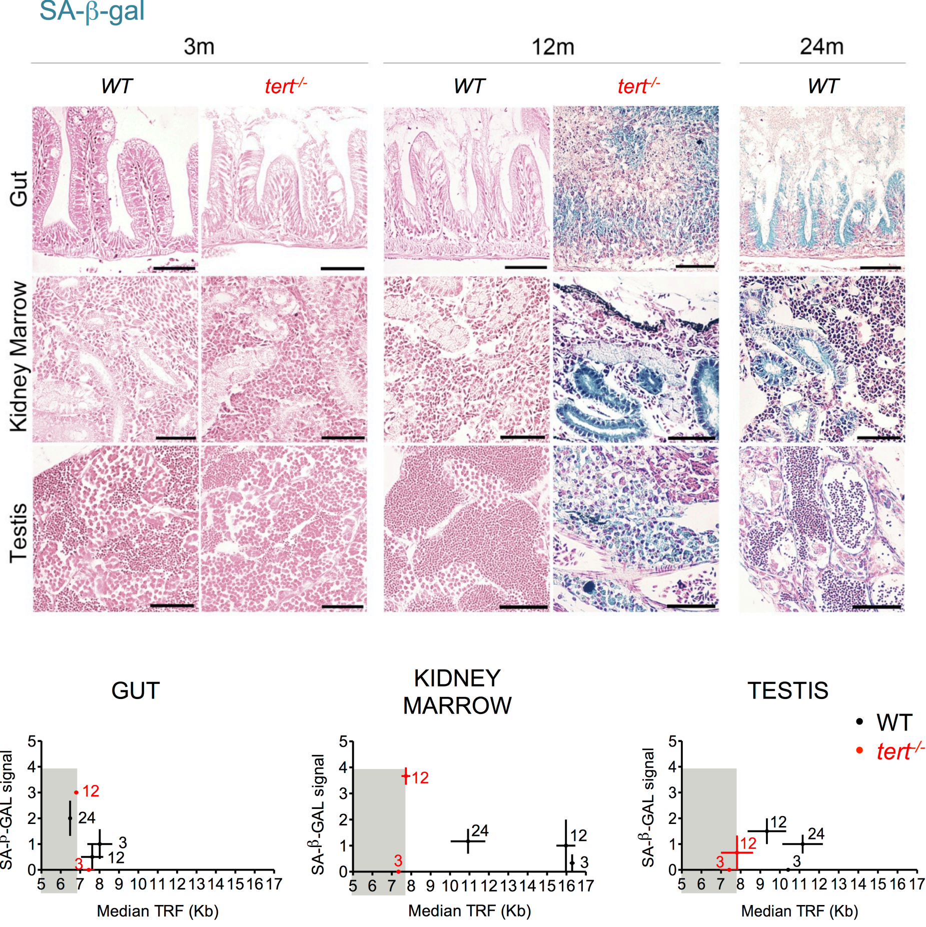

Figure 3.6 – Increase in senescence with age is not anticipated by shorter mTL.

A) Representative haematoxylin-eosin and senescence-associated β-galactosidase (SA-β-gal) staining in gut, kidney marrow and testis sections of WT (at 3, 12 and 24 months) and *tert*$^{-/-}$ mutant siblings (at 3 and 12 months). A significant increase in SA-β-gal positive cells is seen by 12 months of age in *tert*$^{-/-}$ tissues and by 24 months in WT tissues (when comparing both with 3 month-old controls). No staining is observed in the muscle. B) Increase in SA-β-gal staining does not significantly correlate with mTL decline in any of the tissues tested. Grey shaded area identifies the telomeric length at which significant SA-β-gal staining is observed in *tert*$^{-/-}$ mutants' tissues. WT and *tert*$^{-/-}$ age groups are indicated in each graph by black and red colored numbers, respectively. Quantifications of SA-β-GAL signal were performed using an arbitrary scale of signal intensity (where weaker signal is represented by 1 and stronger signal by 4). N=3-6 per genotype per time point. Scale bar = 50 mM. . N=3-6 for tissue mTL telomere length quantifications per genotype per time point (x-axis in graphs). Data represented as mean +/- SEM.

Thus, using *tert*$^{-/-}$ mutant zebrafish mTL as reference, the above analysis establishes the gut and muscle as primary tissues where short telomeres anticipate increased DDR in aging.

3.2.5 – Accumulation of short telomeres and DDRs culminate in aging-associated tissue dysfunction

Short telomeres, as defined by *tert*$^{-/-}$ length, accumulate with zebrafish aging and correlate with activation of persistent DDRs in specific tissues: gut and muscle. We therefore tested whether these cellular phenotypic alterations preceded a decline in tissue integrity. We first looked for changes in tissue inflammation. Aging is characterized by a systemic chronic pro-inflammatory condition with rising levels of TNF, IL1 and IL6 (48, 49). This "proinflammatory state" has been postulated to lead to several chronic degenerative disorders (48, 49). We found that *tert*$^{-/-}$ zebrafish developed early (by 6 months) enteritis and epithelial erosion that was further aggravated in older individuals (18% incidence by 12 months, N=12 in a total of 66 *tert*$^{-/-}$ mutants analyzed; Supplementary Figure S3.1A). As short telomeres accumulated in the gut, the incidence of enteritis increased from 0% to 13% in aged WT zebrafish (3 month vs. 36 months, N=30 in a total of 238 zebrafish analyzed, Supplementary Figure S3.1A).

We also looked for general alterations in intestinal structure. The gastrointestinal tract is known to suffer several alterations in microbiota composition, mucosal immune system and regeneration capacities with aging (50, 51). These changes compromise normal organ function and nutrient absorption. We detected a progressive tendency for thickening of the lamina propria and submucosa in the anterior part of the intestine with *tert*$^{-/-}$ mutants' aging, from 3 to 12 months, associated with inflammatory cell infiltration (Figure 3.7B). WT zebrafish showed significant thickening of the lamina propria and submucosal layers at 36 months (Figure 3.7Ae, indicated by asterisk, quantified

in Figure 3.7B). This morphological feature correlated well with mTL decline in the gut (p=0.002, N=3 per time point, Figure 3.7B). However, we were unable to find significant changes in general gut villi length in older WT individuals that would correlate with shorter mTL (Supplementary Figure S3.1B).

Parallel to the gut, decline in telomere length and activation of DDRs in the muscle prompted us to assess whether these could underlie alterations occurring in myocytes with age. Humans are known to lose muscle mass (also known as sarcopenia) progressively from adulthood to older ages primarily due to nutrient absorption defects (1-2% decrease per year after the age of 50 (52)). Atrophy of type IIa muscle fibers leads to a decay in muscle strength and motor capacity (52). 12 month-old *tert*$^{-/-}$ mutants showed significant muscle fiber thinning (when compared with 12 month WT siblings, Figure 3.7A-B). We observed a 44% reduction in myocyte width in WT zebrafish by the age of 36 months, when compared with 3 months (p=0.0001, Figure 3.7A), compatible with progressive sarcopenia. Severe myocyte degeneration and endomysisal edema directly correlated with the presence of short telomeres (<8.5Kb, p=0.028, Figure 3.7B). These results show that short telomeres directly correlate with the degenerative changes associated with aging, particularly intestinal inflammation and sarcopenia.

3.2.6 – Telomere shortening does not predict infertility or anemia associated with aging

Even though mTL does not appreciably decline to levels that compromise tissue integrity in testis and kidney marrow (when taking *tert*$^{-/-}$ mTL as reference), we investigated how aging affected tissue homeostasis and function in these organs. Adult *tert*$^{-/-}$ mutants showed reduced germ cell compartment by 12 months of age (Figure 3.7A). Here, we show that WT male zebrafish display a progressive inability to produce mature spermatozoa with age (Figure 3.7A, L-mature spermatozoa in the lumen of seminiferous tubules). In addition, 3% of

WT zebrafish after 24 months of age (N=7 in a total of 238 zebrafish analyzed, Supplementary Figure S3.1C) displayed hyperplasia of seminiferous epithelium, mostly spermatogonia. Hyperplasia of spermatogonia in *tert*$^{-/-}$ populations was residual (N=1 in a total of 66 zebrafish analyzed). These results had not been described before and are consistent with telomerase being essential for expansion of the germ cell compartment in late generation telomerase knockout mice (29).

Mice harboring artificially shortened telomeres display defective spermatogenesis/cell proliferation in testis, failing to produce viable offspring (29, 53, 54). Interestingly, even though we observed a marked decline in the percentage of mature spermatozoa with age, this did not correlate with the presence of short telomeres in older WT zebrafish (Figure 3.7B). Decreased spermatozoa production levels resulted in a decline in male fertility (Figure 3.7C). *tert*$^{-/-}$ mutants showed a compromised ability (~50%) to fertilize female eggs at 6 months and became completely infertile by 12 months of age (Figure 3.7C). WT male zebrafish fertility peaked from 6 to 18 months of age and progressively decreased afterwards (~50% and ~30% of fertilization capacity by 24 and 30 months, respectively; Figure 3.7C, $p< 0.0001$). Interestingly, WT zebrafish showed a tendency for inability to stimulate female spawning with age, from 24 months onwards, whereas these defects were already visible in 6-month-old *tert*$^{-/-}$ males (Supplementary Figure S3.2A). Fertility and egg spawning behavior were mildly but significantly affected with age in WT or *tert*$^{-/-}$ mutant females ($p=0.0231$ for WT females and $p=0.0357$ for *tert*$^{-/-}$ females, Supplementary Figure S3.2B-C). Thus, although *tert*$^{-/-}$ mutants anticipate the decline in spermatozoa production and fertility observed in older WT, the development of these phenotypes in a natural aging setting does not seem to depend on the presence of short telomeres in the gonads.

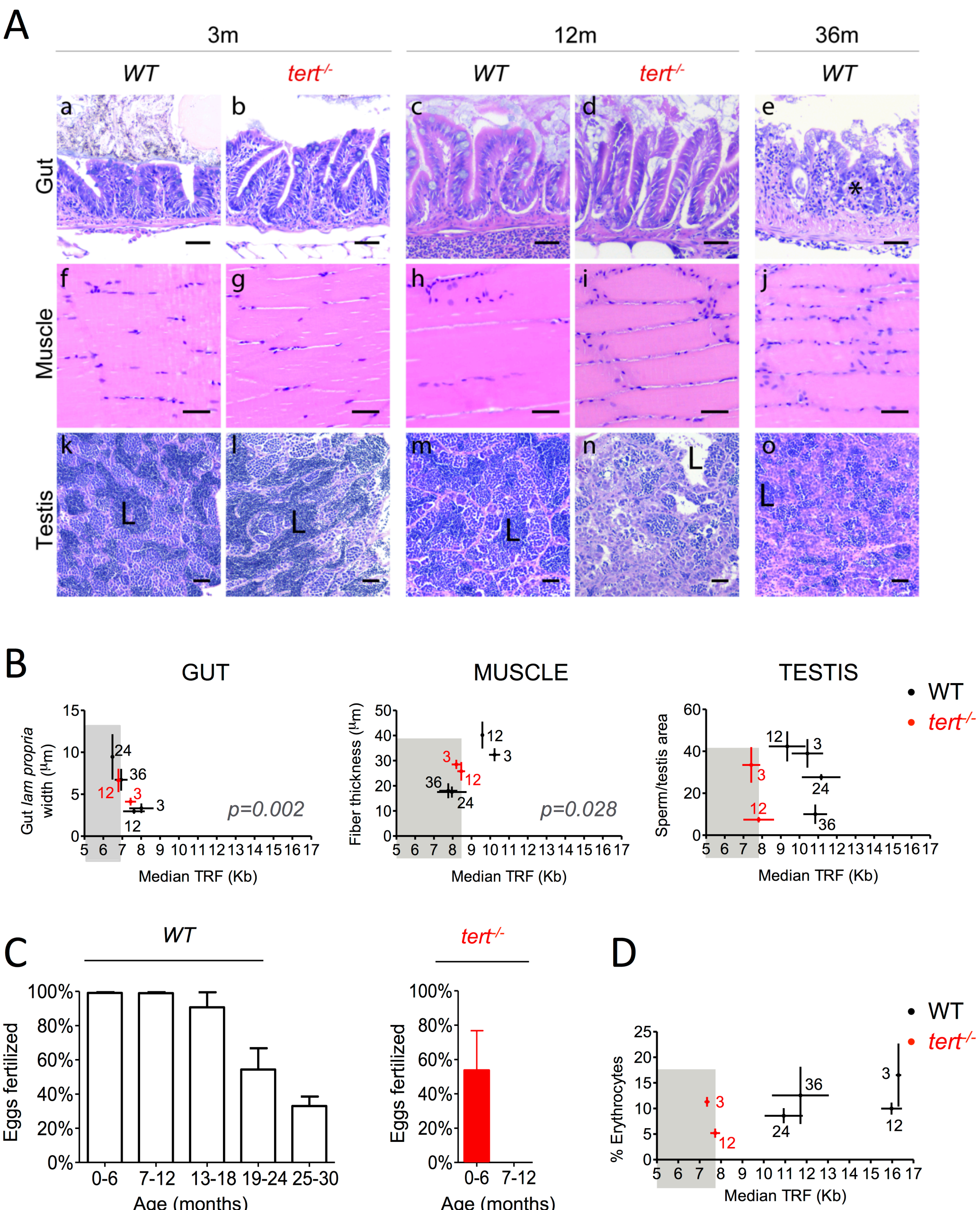

Figure 3.7 – Telomere shortening culminates in tissue defects associated with aging.

A) Representative haematoxylin and eosin-stained sections of gut, muscle and testis from WT and *tert*$^{-/-}$ siblings. By 12 months of age, *tert*$^{-/-}$ mutants (N=3) show inflammatory cell infiltration of the *lamina propria* of the gut (panel b, d), myocyte atrophy and degeneration (characteristic of

sarcopenia - panels g, i), and reduced numbers of mature spermatozoa (panels l, n). By 36 months, WT show similar lesions in the gut (N=5, panels a, c, e) and muscle (N=5, panels f, h, j); WT testis shows a gradual decrease in the number of spermatozoa with age (panels k, m, o, N=5). B) Intestinal inflammation and sarcopenia correlate with shortening of mTL (p=0.002 and p=0.028, respectively), while no correlation is found for reduction in mature spermatozoa numbers. Grey shaded area identifies the telomere length at which significant histopathological defects are observed in *tert*$^{-/-}$ mutants. WT and *tert*$^{-/-}$ age groups are indicated in each graph by black and red colored numbers. C) WT zebrafish show a decreased ability to successfully fertilize female eggs from 24 months onwards (N=4). *tert*$^{-/-}$ have impaired reproductive ability by 6 months and complete lack of function by 12 months (N=3). D) Erythrocyte levels (N=5) decrease with aging in WT and *tert*$^{-/-}$ mutants' kidney marrow, indicative of anemia, but this is not predicted by mTL decline in this tissue. Scale bar = 50 mM. N=3-6 for tissue mTL telomere length quantifications per genotype per time point (x-axis in graphs of Figures 3.7B and D). Data are represented as mean +/- SEM.

We observed that telomeres shortened with age in the kidney marrow, but this shortening never matched the length of *tert*$^{-/-}$ mutants (Figure 3.2B). We tested if, as in testis, *tert*$^{-/-}$ mutants would recapitulate kidney marrow defects associated with aging. WT zebrafish showed lower levels of red blood cells (RBC) with aging (erythrocytes declined from 17% at 3 months to 6% at 24 months, p=0.0002, Figure 3.7D and Supplementary Figure S3.3A) and also a tendency for lower lymphocyte numbers (30% at 3 months vs. 10% at 33 months, p=0.0011, Supplementary Figure S3.3A and C). Conversely, with aging, the proportion of myeloid to lymphoid cells gradually increased (2% at 3 months versus 9% at 33 months, p<0.0001, Supplementary Figure S3.3A and C). In humans the hematopoietic compartment suffers deregulation of stem cell differentiation programs with aging, resulting in higher myeloid to lymphoid progenitor ratios, an event related to "immunosenescence" (55). An increase in myeloid/lymphoid ratios is usually indicative of innate immune system activation, suggestive of chronic inflammation (56). Low levels of RBC and alteration in myeloid/lymphoid ratios surged independently of the presence of short telomeres during WT aging (Figure 3.7D, Supplementary Figure S3.3A and C). Of these phenotypes, *tert*$^{-/-}$ mutants only anticipated the low levels of RBC

(p=0.0067, Figure 3.7D, Supplementary Figure S3.3A). *tert*$^{-/-}$ myeloid/lymphoid and precursors ratios accompanied the tendencies of WT up to 12 months of age (Supplementary Figure S3.3A and C). We could not detect significant differences in total cell number per blood volume with aging in WT or *tert*$^{-/-}$ mutants (Supplementary Figure S3.3B). Thus, aging-associated deregulation of cell differentiation programs in the hematopoietic compartment is likely to be independent of kidney marrow telomere-length. Nevertheless, telomere shortening accompanies the decline in RBC levels with age.

3.2.7 – *tert*$^{-/-}$ mutants recapitulate diseases of old age

We established that telomere shortening is associated with tissue specific aging phenotypes in zebrafish. Non-tissue specific disease phenotypes also emerged with aging, namely cachexia, infection, cancer and motor behavior impairment. The onset of age-associated pathology correlated with the age at which most tissues started to display short telomeres (fin, gut and muscle, Figure 3.2A and Figure 3.3A-B). In agreement, we also observed that *tert*$^{-/-}$ mutants recapitulated these diseases prematurely.

I) Cachexia

Human aging is commonly characterized by weight loss, reduction of subcutaneous fat and sarcopenia, a condition often referred to as cachexia or wasting syndrome. This syndrome has been associated with increased frailty and mortality (57). In our cohort, we prospectively analyzed 416 WT zebrafish and found the maximum longevity to be of 43.5 months (Figure 3.8C). From 24 months onwards, we registered an increasing incidence of mortality associated with cachexia and deformation of the spine (from 13% to 62% between 24 and 42 months, Figure 3.8A-B). Cachexia was scored based on specific histopathological changes in the gut, muscle and testis (see description below,

Supplementary Figure S3.4B). Whereas 38% of older WT zebrafish died without displaying cachexia, the older the animals died, the more likely they were to be cachexic (Figure 3.8B). All first generation *tert*$^{-/-}$ homozygous mutants had significantly shorter lifespan (ca. 3x lower than WT, Figure 3.8C). This was accompanied by an accelerated incidence of cachexia, from 8 months onwards (Figures 3.8A-B; Supplementary Figure S3.4A), a feature that revealed to be common to all *tert*$^{-/-}$ zebrafish at time of death. Higher incidence of cachexia and deformation of the spine has been observed by others in older cohorts of zebrafish, by 18-24 months of age (58). However, the cause of cachexia remains unknown.

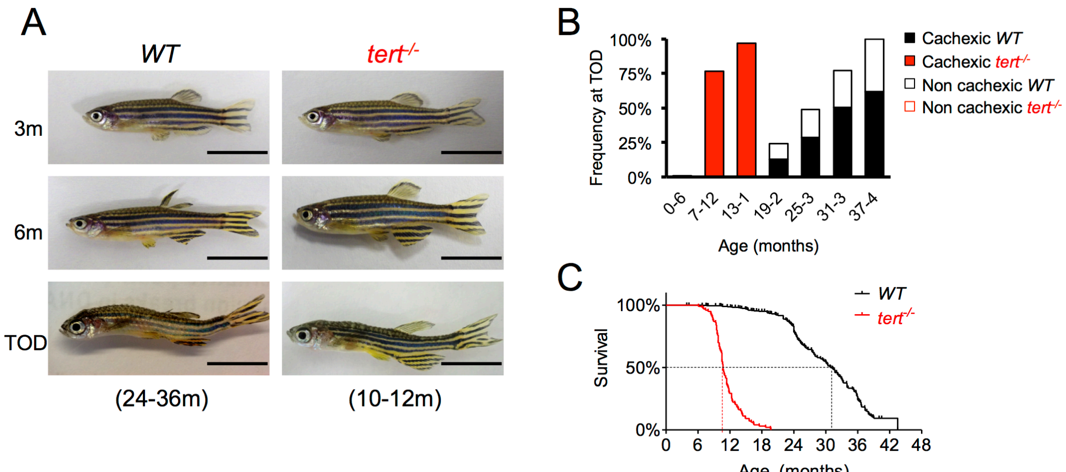

Figure 3.8 – *tert*$^{-/-}$ mutants accelerate the onset of age-associated cachexia.
A) Representative images of WT and *tert*$^{-/-}$ mutants show that, at time of death (TOD), zebrafish exhibit signs of cachexia and deformation of the spine, with very low body mass indexes. B) In the WT population the incidence of these alterations increases with age, while in *tert*$^{-/-}$ mutants, at TOD, 100% of the population is affected. C) Sustained cachexia in older zebrafish is accompanied by an increase in mortality as shown by Kaplan Meier curves of WT and *tert*$^{-/-}$ zebrafish. WT zebrafish have a half-life of 30.8 months, 2.9 times greater than the half-life of 10.6m of *tert*$^{-/-}$ ($p<0.0001$). N=426 WT; N=98 *tert*$^{-/-}$. TOD corresponds to the interval comprising the second and third quartiles of survival (25 to 75%). Scale bar = 1 cm.

Cachexia in both WT and *tert*$^{-/-}$ mutants associated with particular tissue alterations in fat, gut, muscle and testis. Aging in humans also associates with defects in lipids storage in subcutaneous adipose tissues and with an increase deposition of visceral fat in skeletal muscle, liver, heart and bone marrow (59). We observed a loss of subcutaneous and visceral fat mass with WT aging (adipocytes were ~3 times smaller at 36 months vs. 3 months, Supplementary Figure S3.5A-C), which was significantly more pronounced in cachexic stages (indicated by TOD in graphs in Supplementary Figure S3.5C). In *tert*$^{-/-}$ mutants, smaller adipocytes were visible in subcutaneous and visceral tissues by 12 months (Supplementary Figure S3.5A-C). In cachexic zebrafish, we saw a higher exhaustion of fat reserves, when comparing with non-cachexic *tert*$^{-/-}$ mutants (indicated by TOD in Supplementary Figure S3.5A-C). In the gut, we found marked decrease of villi length (Supplementary Figure S3.4B). Accordingly, intestinal crypt depletion and villus atrophy was previously observed in late generation *tert* KO mice (28). The muscle showed a higher degree of atrophy associated with myocyte degeneration and endomysial edema than that observed in non-cachexic zebrafish during aging (Supplementary Figure S3.4B). Testis also showed a more pronounced atrophy with reduced germ cell compartment, when compared with age-matched non-cachexic zebrafish (Supplementary Figure S3.4B).

To test if gut villi and muscle degeneration were direct consequences of poor nutritional states in old WT and *tert*$^{-/-}$ mutants, 12-month old WT zebrafish were put on a caloric restriction diet for 8 weeks, consisting of transition from ad libitum feeding regimen to a food intake reduced by 85%. We observed these zebrafish on caloric restriction displayed width/length ratios similar to cachectic zebrafish, maintained villi length within the normal range but showed morphological changes in the muscle similar to those described for aging WT fish (Supplementary Figure S3.6). Thus, while aging-related sarcopenia could potentially be triggered by a poor nutritional state the same does not apply to

gut villi length. Altogether, our results show that terminal *tert*$^{-/-}$ degeneration recapitulates within 12 months the macroscopic phenotypes of old WT zebrafish.

II) Swim bladder infection

We observed an increasing incidence of swim bladder infection with age (aerocystitis). By 36 months of age, up to 30% of WT population showed aerocystitis (indicated by black arrow, N=71 out of a total of 238 zebrafish analyzed, Supplementary Figure S3.7), the majority of which concomitant with cachexia. 3% of *tert*$^{-/-}$ zebrafish showed swim bladder infection (N=2 out of a total of 66 zebrafish analyzed, Supplementary Figure S3.7) and always in association with cachexia. Gram staining occasionally demonstrated the presence of bacteria and bacterial culture of the swim bladder content of 24-month old fish by fine-needle aspiration resulted in the isolation of *Vibrio alginolyticus* and *Shewanella putrefaciens* (data not shown). Our analyses suggest that bacterial aerocystitis is a prime candidate for cause of death in aged WT and *tert*$^{-/-}$ mutants. Bacterial and fungal infections are known to typically affect the swim bladder via the pneumatic duct in different fish species (60). Thus, concomitant with the appearance of cachexia, *tert*$^{-/-}$ mutants showed a tendency to anticipate the rise of infections observed during zebrafish aging.

III) Cancer

Cancer is an aging-related disease relying on mechanisms – such as accumulation of DNA damage – that emerge with time (61). Like other diseases of aging, human cancer incidence rises exponentially after mid-life (62). Telomere maintenance is a key factor for tumor progression and most commonly achieved via telomerase reactivation (11). Telomerase expression and activity are upregulated in 90% of human cancers (63). In order to explore

the role of telomerase in tumorigenesis in zebrafish, we analyzed 66 *tert*$^{-/-}$ mutants versus 238 WT zebrafish for the incidence of spontaneous tumors. Zebrafish tumors were reported to develop spontaneously at a rate of 11% (58, 64) and to resemble human tumors, in their histological and gene expression profiles (65). Accordingly, we observed a spontaneous tumor cumulative incidence of 10% in WT zebrafish up to 42 months of age (N=23 in a total of 238 zebrafish analyzed; Figure 3.9A). Surprisingly, even though *tert*$^{-/-}$ displayed approximately the same incidence, 8% (N=5 in a total of 66 zebrafish analyzed; Figure 3.9A), they had a much earlier onset, at the age of 4 months (when compared with 24 months in WT; p=0.003). 30% of all WT tumors (N=7/23), including leukemia (N=1/23) intestinal adenocarcinoma (N=3/23) hepatocellular carcinoma (N=1/23) and peripheral nerve sheath tumor (N=2/23), and 40% of all *tert*$^{-/-}$ tumors, specifically leukemia (N=2/23), were invasive. Consistent with previous studies (58), male tumors arising with aging in zebrafish comprised mainly neoplasias of the reproductive tissue (Figure 3.9B) but none of these tumors showed invasive capacities.

Tumor development in the gonads generally followed gross enlargement of the organ (with hyperplasia of spermatogonia, Supplementary Figure S3.1) and the appearance of inflammatory Periodic Acid Shift (PAS+) stained cells in the tissue stroma (Figure 3.9C-D). Cells with PAS+ staining are associated with the activation of innate and adaptive immune responses in zebrafish (66, 67). We found the appearance of PAS+ cells to be specific to pre-cancerous and cancerous lesions, since healthy testis in different time points exhibited only 0-2% of these cells (Figure 3.9D). Thus, inflammatory responses precede cancer development in zebrafish gonads. Whether inflammation is promoting cancer or is a protective (albeit insufficient) response mechanism remains to be tested.

Liver and intestinal tumors were also observed in older WT and in *tert*$^{-/-}$ male zebrafish (Figure 3.9B). WT females (N=120) showed a higher heterogeneity in their tumor spectra, with an even distribution of neoplasias between the

reproductive and hematopoietic system, liver, intestine and pancreas (Figure 3.9B). In order to confirm that tumorigenesis in *tert*$^{-/-}$ was not due to a possible reversion of the telomerase point mutation, we genotyped 3 tumors (out of the total of 5 tumors) and confirmed that the mutation background was maintained (data not shown). Thus, telomerase does not appear to be a limiting factor for initiation of tumorigenesis in zebrafish, consistent with short telomeres triggering more microadenomas in CAST/EiJ mice (22). Telomerase absence does not significantly change incidence/malignancy rates but accelerates the onset of zebrafish cancer. The appearance of DDR could imply that early genome instability events precede activation of alternative mechanisms for elongation of telomeres.

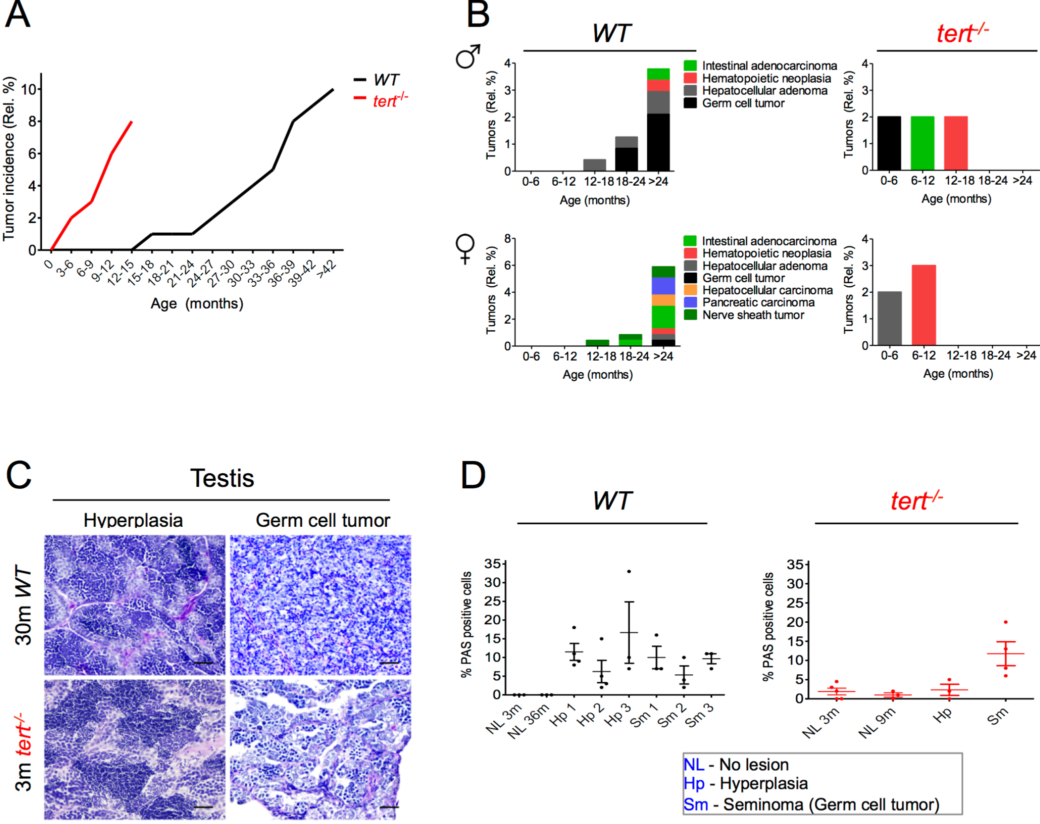

Figure 3.9 – *tert*$^{-/-}$ mutants accelerate the onset of age-associated cancer.

A) Tumorigenesis rises exponentially with age in both WT and *tert*$^{-/-}$ zebrafish, reaching cumulative incidences of ca. 10% and ca. 8%, respectively. N=23/238 WT; N=5/66 *tert*$^{-/-}$. *tert*$^{-/-}$ mutants have an earlier onset of neoplasia starting at 4 months of age (p=0.003). B) Tumors in WT male zebrafish affect mainly the reproductive system, following the appearance of Periodic Acid Shift (PAS+) stained cells in the tissue stroma (C), (quantified in D). WT females show an even distribution of tumors between reproductive and hematopoietic systems, followed by liver, intestine and pancreas. N=118 WT males; N=120 WT females; N=58 *tert*$^{-/-}$ males; N=8 *tert*$^{-/-}$ females. Quantifications of %PAS+ cells was performed in 3 fields of view for each individual in the graph. Scale bar = 50 mM. Data are represented as mean +/- SEM.

IV) Motor behavior impairment

Aging affects basic behaviors, such as response to novel stimuli (68) novelty exploration (69) and cognition (39). Zebrafish has emerged as an important model organism to study exploratory and social behaviors (70). When exposed to a novel open field arena, zebrafish typically adopt centrophobic anxious behaviors, choosing to remain on peripheral areas rather than exploring the arena's center (71). This behavior is usually more pronounced during the first periods of exploration (72). To test if zebrafish anxiety behaviors changed significantly with age and whether these changes were anticipated by *tert*$^{-/-}$ mutants, we exposed 3, 9, 18, 30 and 36-month old zebrafish as well as 3 and 9-month old *tert*$^{-/-}$ to a novel open field arena and recorded their exploratory behavior. We found aged zebrafish spent more time in the "OFT Zone", an inner central zone in the arena, and had therefore lower levels of anxiety than younger counterparts (Figure 3.10A). Similarly, *tert*$^{-/-}$ mutants moved more frequently in the OFT Zone (at 3 or 9 months), when compared with age-matched WT controls (Figure 3.10A). No differences could be detected in latency to enter the OFT Zone among the age groups tested (data not shown). Measurements of basic mobility parameters, such as velocity and travelled distance, revealed problems in locomotion after 15 months for WT and 9 months for *tert*$^{-/-}$ mutants (Figure 3.10 A). Concomitantly, significantly higher frequency

of immobility periods could be detected suggesting fear and/or stress responses increase with age/in the absence of telomerase (Figure 3.10A).

Temperament traits or coping styles such as boldness, shyness and exploration have been described as important determinants of an animal's fitness (73). As zebrafish aged, we observed a progressive lack of engagement in exploratory/bold behaviors, measured by the decreased frequency of exploration of a marble, within the context of a novelty-based paradigm (Figure 3.10B). This was obvious from 24 months onwards for WT zebrafish and, surprisingly, as early as 3 months in *tert*$^{-/-}$ (Figure 3.10B). Lack of exploration of the novel object did not seem to relate with vision problems, as *tert*$^{-/-}$ mutants did not spend less time in the vicinity of the marble than WT controls (NOT Zone, data not shown). Introduction of a novel object in the environment aggravated the defective patterns of locomotion (lower velocities/distances) and exacerbated stress/fear responses (higher immobility frequencies) in older WT and *tert*$^{-/-}$ zebrafish (Figure 3.10B).

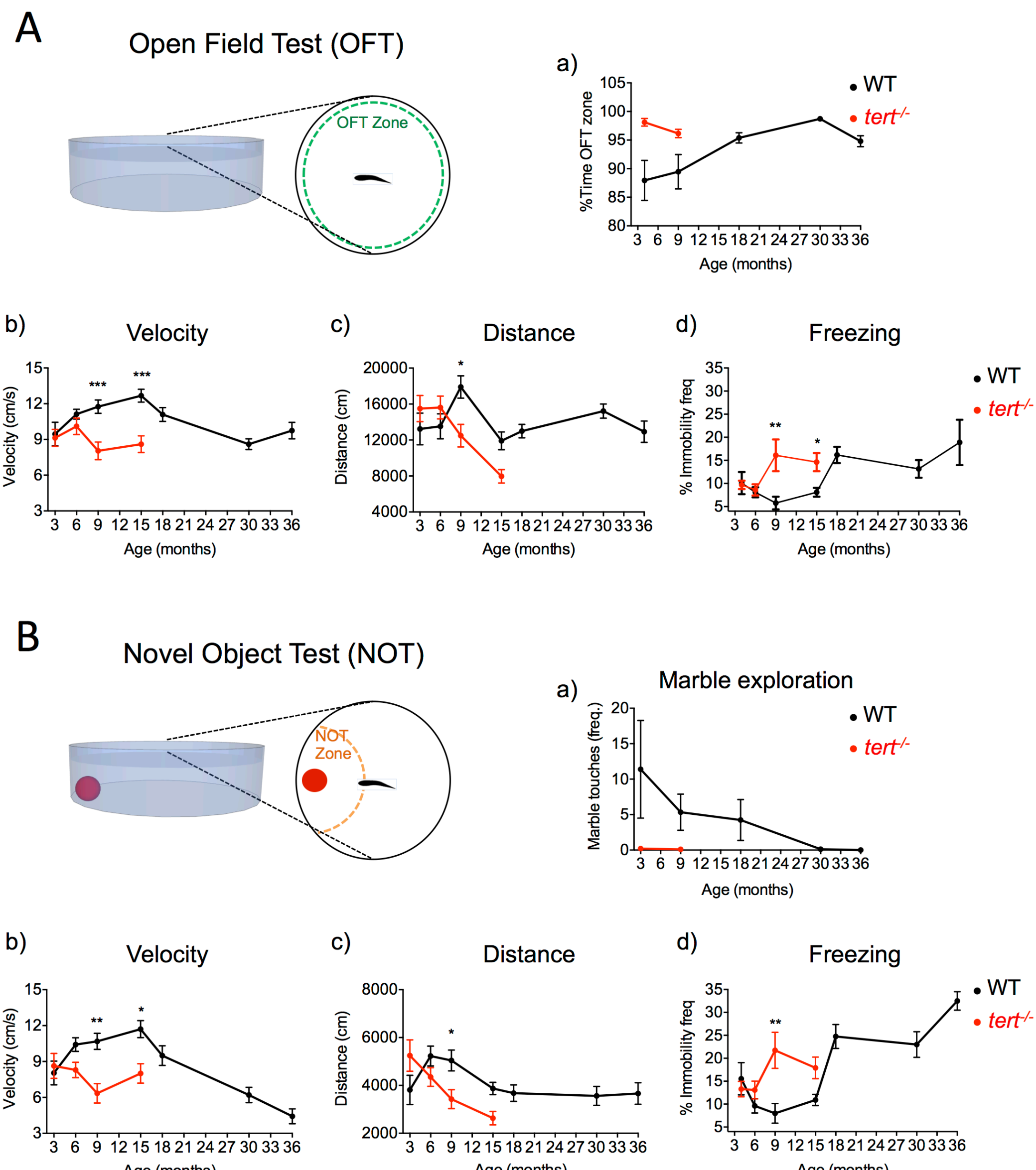

Figure 3.10 – *tert*$^{-/-}$ mutants anticipate behavior defects observed in natural aging.
A) Open Field Test (OFT) – 3D representation of side view and 2D representation of top view. The time spent in the OFT Zone, area delimited by the green dashed line, is a) higher in aged WT and in 3-month ol *tert*$^{-/-}$ zebrafish. With aging, WT and *tert*$^{-/-}$ zebrafish explore the arena at b) lower velocities, c) swim smaller distances and display d) higher periods of immobility. B) Novel Object Test (NOT) – 3D representation of side view and 2D representation of top view. Orange dashed delimited area represents the marble exploration area – NOT Zone. Older WT (30 months

onwards) and *tert*$^{-/-}$ zebrafish avoid direct exploration of the marble and their motor impairment becomes even further exacerbated (a-c). N=15-25 for 3, 6, 12, 15 (both for WT and *tert*$^{-/-}$), N=17-20 for 18 and 30 months WT and N=6 for 36 months WT. Data are represented as mean +/- SEM.

Telomerase deficient mice were also described to exhibit defective behavioral responses, potentially due to impaired neural stem cell proliferation (74). Whether the same phenomenon underlies the behavior (motor and exploratory) defects observed in zebrafish aging and promotes their acceleration in *tert*$^{-/-}$ mutants (as early as 3 months) remains to be tested.

3.3 – Discussion

In humans, progressive telomere erosion has been implicated in aging-associated diseases (23, 75). However, it is not clear whether telomere shortening is an actual cause of aging and how it contributes towards the development of degenerative phenotypes over time. The identification of tissues where intrinsic telomere shortening becomes truly limiting for optimal function and homeostasis is crucial for understanding the impact of telomere shortening on aging. Moreover, understanding whether tissue specific telomere shortening triggers systemic aging signals will help develop an integrative model of how telomere length influences the aging process as a whole.

In our current work, we show that accumulation of short telomeres and DDR anticipates dysfunction in specific tissues with natural aging. tp53-associated DDR has been shown to directly mediate some of the degenerative phenotypes associated with telomere loss in telomerase mutant zebrafish and late generation telomerase knockout mice (27, 76). Consistently with telomere shortening affecting mainly proliferative tissues (26, 27, 29), the gut appears as a leading candidate-organ where telomeres shorten over time reaching telomerase mutant length, our reference for critically short telomeres. However, similarly to the gut, the muscle also accumulates short telomeres and DDRs

with age, which anticipate severe sarcopenia phenotypes. Because the muscle is a low proliferative tissue, telomere shortening with age probably relies on factors that are independent of local cell replication. Accordingly, the rate of telomere shortening in human somatic tissues is not exclusively dictated by the respective rates of proliferation (40). As suggested for less proliferative tissues of telomerase knockout mice, telomere erosion in the zebrafish muscle could stem from the increased production of reactive oxygen species (ROS) due to mitochondrial defects (31). Interestingly, both gut and muscle follow a similar behavior of linear telomere decline until 24 months of age with stabilization of length at later ages. Telomere stabilization in older ages has been reported by others (9, 10) and goes in hand with the existence of selection processes that may favor the elimination of cells with extremely short telomeres. Alternatively, maintenance of shorter telomere lengths at older ages may rely on recombination mechanisms, described to act even in the presence of telomerase in $mTR^{+/-}$ mice (77).

Telomeres in the testis and kidney marrow do not seem to reach telomerase mutant short telomere lengths and therefore short telomeres do not anticipate dysfunction with aging. We cannot exclude, however, that a minority of cells with short telomeres do accumulate with age in these tissues, which go by undetected by methods of whole population analysis. The lack of significant detectable telomere shortening in the testis is consistent with previous work showing that zebrafish gonads retain high telomerase activity levels (36). Interestingly, testis is the tissue where the highest incidence of cancer is detected with aging, and telomerase is known to be re-activated in 90% of all human cancers (63). The significant expansion of immature germ cells (spermatogonia, spermatocytes) in detriment of mature spermatozoa in WT testis with age could also explain why we do not detect telomere shortening in this tissue. In contrast, the kidney marrow is the only tissue where significant telomere elongation occurs during development and sexual maturation of

zebrafish, from larvae to 3 months. From all WT tissues tested in adult stages, the kidney marrow shows the longest telomeres and the biggest difference between WT and *tert*$^{-/-}$ telomere length. However, kidney marrow *tert*$^{-/-}$ telomeres have lengths within the range of other *tert*$^{-/-}$ tissues. Thus, at least during development, the zebrafish kidney marrow probably has a higher necessity for telomerase activity than other tissues and perhaps retains it in some cells, as in the human hematopoietic compartment (78). Telomeres still shorten in the kidney marrow with age after 18 months, even though they do not reach telomerase mutant lengths. This data suggests that, similarly to humans, telomere shortening in certain tissues and cell types probably relates to differential restriction of telomerase expression (8).

Tissues where telomeres do not reach telomerase mutant lengths still develop aging phenotypes, namely decline in fertility and anemia. It does not seem likely, however, that these phenotypes arise in a completely telomere-independent manner as telomerase mutants recapitulate them prematurely. Thus, aging phenotypes in testis and kidney marrow are due either to: 1) non cell-autonomous effects caused by telomere shortening in the gut, muscle and/or unidentified tissues or 2) absence of non-canonical functions of telomerase required for proper tissue homeostasis, or a combination of both effects. Regarding the first point, as telomeres shorten the number of senescent cells increases in tissues. These cells may be secreting SASP molecules, such as IL-6 and IL-8, which have been described as capable of influencing growth in neighboring cells (33). However, non-catalytic telomerase functions have also been described as important determinants of tissue physiology in mammals and zebrafish (79-81). Absence of telomerase alone could explain why cell division declines with aging in the kidney marrow. TERT stimulates proliferation by acting as a β-catenin cofactor and activating Wnt signaling (82) and by binding and modulating mitochondrial RNA processing endoribonucleases levels (79). Increase in apoptosis with age could relate with the loss of telomerase

protective functions against Bcl-2 dependent pro-apoptotic stimuli (79). Recently (81), the zebrafish telomerase RNA component was shown to modulate transcriptional factors required for myeloid and erythroid differentiation, a phenomenon that may underlie the development of anemia in zebrafish aging. In CAST/EiJ mice, however, mTERT loss produces phenotypes that are directly attributable to telomere shortening and not to telomere-length independent functions of mTERT (83, 84). Finally, the observed defects in testis and kidney marrow could still rely on loss of telomere integrity with age that is not detectable using the assays we employed. In the future it will be important to dissect the mechanisms by which telomere shortening induced stress in one tissue is perceived by other tissues in the rest of the organism.

Absence of telomerase accelerates diseases of old age, including cancer. We find that zebrafish telomerase mutants show an earlier cancer onset and maintain a close to 40% tumor invasion rate. This is surprising since shutting off telomerase is thought to have evolved as a tumor suppressor mechanism by limiting cell proliferation. How tumors in *tert*$^{-/-}$ zebrafish succeed in stabilizing chromosome-ends in the absence of telomerase is currently unknown. The simplest explanation would entail the engagement of Alternative Lengthening of Telomere (ALT) mechanism, usually activated in the absence of telomerase (85, 86). It is possible that the effectiveness of ALT may vary depending on different genetic backgrounds. Absence of telomerase in mouse models may either decrease or increase tumorigenesis depending on the genetic context (30, 87-89). Our data shows that, instead of grossly altering the frequency, short telomeres accelerate the appearance of spontaneous tumors to juvenile stages in zebrafish, in agreement with previous studies (90).

Our work shows that telomere shortening and accumulation of DDR in particular tissues anticipates aging associated dysfunction. As previously proposed (91), accumulation of short telomeres and high levels of persistent DDR in one specific tissue (gut) may be enough to establish the rate of aging in

other tissues by non cell autonomous mechanisms (Figure 3.11). Strikingly, absence of telomerase triggers premature recapitulation of aging phenotypes in most tissues, even in those where telomeres do not reach *tert*$^{-/-}$ shortened telomere lengths during aging. Importantly, by using the "percentage of life" instead of absolute age to measure the progression of aging phenotypes, we find a surprising similar kinetics between the dynamics of WT and *tert*$^{-/-}$ phenotypes over time, local and systemic (Figure 3.11). Thus, rescuing telomere length of aged zebrafish in key tissues (such as the gut) may prove sufficient to restore local and systemic homeostasis in old animals. Whether systemic aging signals will be dominant over locally rejuvenated tissues remains to be tested. However, such therapies will likely only be productive before telomeres reach critical length. Previous studies have shown that reactivation of telomerase in tissues that have undergone extensive telomere erosion is a strong potentiator of tumorigenesis and malignancy (92). Our study suggests a potential therapeutic window of opportunity, in a period that correlates with mid-life in zebrafish, before age-associated defects become apparent. Time- and tissue-dependent telomerase reactivation will enable us to define and test such a useful period for telomerase therapies.

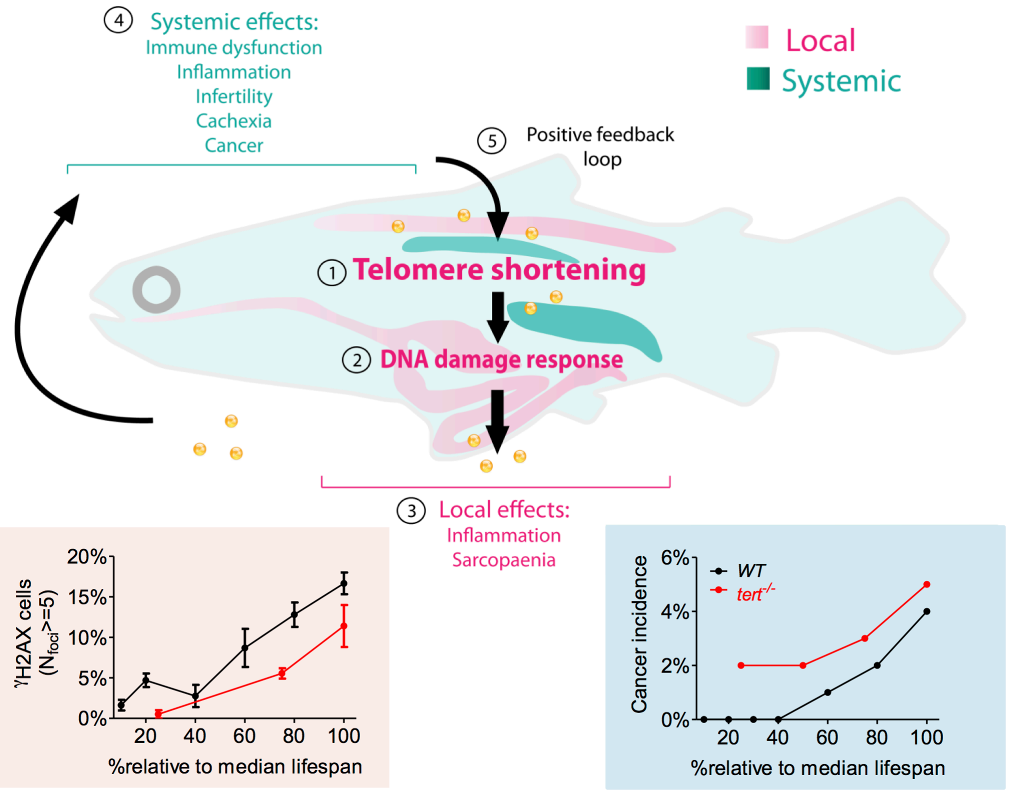

Figure 3.11 – Working model - short mTL and DDR in key tissues establish the rate of local and systemic aging.

(1) Expression of telomerase is restricted in most somatic tissues resulting in telomere shortening with aging. (2) In the gut and muscle, shortening of telomeres to critical levels results in increased DDRs with aging which (3) disrupt local homeostasis, culminating in organ and tissue-specific lesions such as inflammation and sarcopenia. (4) Gut and muscle DDRs act cell non autonomously by inducing defects in organs where telomeres do not shorten appreciably, such as testis and kidney marrow. DDR signals trigger systemic damage resulting in cachexia and fueling tumorigenesis. (5) Overall, cellular damage creates an amplifying positive feedback loop accelerating tissue dysfunction with aging.

3.4 – Materials and methods

3.4.1 – Ethics statement

All Zebrafish work was conducted according to National Guidelines and approved by the Ethical Committee of the Instituto Gulbenkian de Ciência and the DGAV (Direcção Geral de Alimentação e Veterinária, Portuguese Veterinary Authority).

3.4.2 – Zebrafish lines and maintenance

Zebrafish were maintained in accordance with Institutional and National animal care protocols. The telomerase mutant line *tert*$^{AB/\mathrm{hu3430}}$, generated by N-Ethyl-N-nitrosourea (ENU) mutagenesis (Utrecht University, Netherlands; Wienholds, 2004), has a T→A point-mutation in the *tert* gene and is available at the ZFIN repository, ZFIN ID: ZDB-GENO-100412-50, from the Zebrafish International Re-source Center – ZIRC. The *tert*$^{AB/\mathrm{hu3430}}$ used in this chapter was subsequently outcrossed six times with *tert*$^{+/+}$ AB to clear potential background mutations derived from ENU mutagenesis. The *tert*$^{hu3430/\mathrm{hu3430}}$ homozygous mutant (referred to as *tert*$^{-/-}$) was obtained by incrossing our *tert*$^{AB/hu3430}$ strain. WT siblings were used as controls. Genotyping was performed by PCR of the tert gene followed by sequencing (27). Overall characterization of *tert*$^{-/-}$ and WT zebrafish was performed in G1 animals produced by *tert*$^{+/-}$ incross. Due to a male sex bias in our crosses that affected mostly *tert*$^{-/-}$ progeny, we were unable to obtain significant numbers of females for analysis and so the majority of our data is restricted to males unless otherwise stated. All animals showing signs of morbidity that persisted for up to 5 days, such as inability to eat or swim, or macroscopic lesions/tumors were sacrificed in 200 mg/L of MS-222 (Sigma, MO, USA).

3.4.3 – Telomere restriction fragment (TRF) analysis by Southern blot

TRF analysis was performed as previously described (43). Briefly, genomic DNA was extracted from freshly isolated tissue using lysis buffer (Fermentas K0512) supplemented with 1 mg/ml Proteinase K (Sigma, MO, USA) and RNase A (1:100 dilution, Sigma, MO, USA). Samples were incubated at 50ºC for 18 h in a thermomixer and genomic DNA was extracted by equilibrated phenol-chloroform (Sigma, MO, USA) and chloroform-isoamyl alcohol extraction (Sigma, MO, USA). Genomic DNA was quantified and normalized so the same amount of DNA was digested with RSAI and HINFI enzymes (NEB, MA, USA) for 12 h at 37ºC. Samples were electrophoresed on a 20 cm 0.6% agarose gel, in 0.5% TBE buffer, at 4ºC for 17 h at 110 constant voltage. A 1.6 kb telomere probe, (TTAGGG)n, labelled with [α-32P]-dCTP using the Prime-it II random primer labelling kit (Stratagene) was used for Southern blotting. 3-6 different WT and *tert*$^{-/-}$ individuals belonging to different age groups were used for quantification of each Southern Blot experiment.

3.4.4 – Histology and phenotypic analysis

Zebrafish were sacrificed by anesthetic overdose, in 200 mg/L of MS-222 (Sigma, MO, USA), fixed for 72 hours in 10% neutral buffered formalin and decalcified in 0.5 M EDTA for 48 h. Whole fish were then paraffin-embedded and 3 micrometer midline sagittal sections were stained with hematoxylin and eosin for histopathological analysis. Sections were examined by a pathologist (TC), blinded to experimental groups, and microphotographs were acquired in a Leica DM2500 microscope coupled to a Leica MC170 HD microscope camera. A total of 238 WT and 66 *tert*$^{-/-}$ zebrafish were submitted for histopathological analysis. At least 4 animals from each age group/genotype were analyzed.

3.4.5 – Immunofluorescence (IF) and confocal analysis

Sections were deparaffinized, rehydrated and heat-induced antigen retrieval was performed in citrate buffer (10 mM Sodium Citrate, pH 6) for 20 minutes at 103ºC in an oven. Slides were left to cool down for 30 minutes at room temperature (RT), washed three times in dH20 for 5 minutes each and blocked for 1 hour at RT in 0.25% BSA in PBST (Triton 0.3%). The following primary antibodies were used: rabbit polyclonal antibodies against Proliferation Cell Nuclear Antigen (PCNA, Santa Cruz, CA, USA, 1:50 dilution) and Histone H2A.XS139ph (GTX127342, USA, 1:500). Incubation with primary antibodies was performed overnight at 4°C, followed by three 10 minute PBS washes. Incubation with the secondary antibody Alexa Fluor 568 goat anti-rabbit (Invitrogen, UK, 1:500 dilution) overnight at 4°C was followed by three 10 minute PBS washes, DAPI staining (Sigma, MO, USA) and mounting with DAKO Fluorescence Mounting Medium (Sigma, MO, USA). Apoptosis was detected using the *In Situ* Cell Death Detection Kit (Roche, SW) according to manufacturer's instructions. Briefly, deparaffinized slides were incubated with 40 µg/ml Proteinase K in 10 mM Tris-HCl pH 7.4, 45 minutes at 37°C. Slides were washed in 2×5 minutes in PBS and then incubated with TUNEL labelling mix (protocol indicated by the supplier). Washing and mounting were performed as previously described. Images were acquired on Leica SP5 Live Upright confocal microscope (Leica Microsystems, GER) equipped with Leica Las AF Lite software and with appropriate configurations for multiple color acquisition. For quantitative and comparative imaging, equivalent image acquisition parameters were used. The percentage of positive nuclei was determined by counting a total of 500-1000 cells per slide, 63x amplification (N=3-4 zebrafish per time point/genotype).

3.4.6 – Senescence-associated β-galactosidase assay

β-galactosidase assay was performed as previously described (93). Briefly, sacrificed zebrafish adults were fixed for 72h in 4% paraformaldehyde in PBS at 4ºC and then washed three times for 1 h in PBS-pH 7.4 and for a further 1 h in PBS-pH 6.0 at 4°C. β-galactosidase staining was performed for 24 h at 37°C in 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl2 and 1 mg/ml X-gal, in PBS adjusted to pH 6.0. After staining, fish were washed three times for 5 minutes in PBS pH 7 and processed for de-calcification and paraffin embedding as before. Sections were stained with nuclear fast red for nuclear detection and images were acquired in a bright field microscope (Olympus BH2, LB). To quantify SA-β-GAL staining we used an arbitrary scale of signal intensity (where weaker signal is represented by 1 and stronger signal by 4; N≥3 per time point per genotype).

3.4.7 – Fertility assays

Fish were kept in their living tanks until they were randomly selected for breeding. Briefly, breeding pairs were left overnight in external breeding tanks and collection of eggs was performed the following morning. 3-10 independent breeding trials were conducted for each age group and genotype (using either 6-month old female or male controls, tested previously for successful reproductive ability), to evaluate how aging affects male or female reproduction. Only successful breeding trials, defined as events where a female lays a clutch of eggs when paired with a male, were scored. In every trial group, a breeding box including a 6-month old couple was included to ensure the protocol in place guarantees maximum reproductive output.

Embryos were collected approximately 2 hours post fertilization (hpf) and allowed to develop at 28°C. Assessment of egg fertilization and embryo viability was conducted between 2 and 4 hpf.

3.4.8 – Flow Cytometry and cytology of head kidney marrow

Single-cell suspensions from head kidney marrow (HKM) were prepared from adult zebrafish. Freshly isolated head kidney tissue was placed in a 1.5ml eppendorff containing 200µl of complete high-glucose DMEM (10% FBS; 1% pen/strep) on ice. Tissue was maintained in the tube, on ice, while gently disrupted manually with the aid of a plastic pestle. Single-cell suspensions were obtained by gentle pipetting and filtering through a 40µM nylon membrane cell strainer (Falcon). Cells in complete DMEM were incubated for 15' on ice with 1mg/ml propidium iodide for dead cell exclusion analysis.

Single cell suspensions from HKM were examined using a CyanADP flow cytometer (Beckman Coulter), using PI for dead cell exclusion and SSC VS FSC to identify the different cell populations, as previously described (94). Data were analyzed using FlowJo software Version 9.4.11 (TreeStar). N=3-5 zebrafish per time point per genotype.

3.4.9 – Exploratory and motor behavior assays – Open Field Test (OFT) and Novel Object Test (NOT)

Prior to both trials (OFT and NOT), zebrafish were allowed to acclimate to the experimental room for 60 minutes. The tests were performed consecutively in a glass round 14 cm arena, first the OFT and second the NOT. Each zebrafish was tested only once, individually. In the OFT, zebrafish were placed in the center of the arena filled with 700 ml of water and video tracked for 30 minutes. The NOT was performed immediately after by placing a red colored marble into the water in a standardized manner, through a PVC tube. Zebrafish behavior was then video tracked for 10 min. For the 3, 6, 12 and 15-month old groups, a total of 15-25 independent zebrafish were tested for each genotype. For the 18 and 30-month old WT groups, 17-20 zebrafish were tested. For the 36 months WT group, 6 zebrafish were tested. After each round of trials, the arena was

rinsed with 70% ethanol, followed by distilled water and allowed to dry completely. All behavioral experiments were conducted between 2 and 7 PM to minimize the impact of possible daily hormonal variations in zebrafish exploratory behavior.

Video tracking and data analysis were performed using Ethovision 8.0 software (Noldus, The Netherlands). Levels of anxiety were assessed by measuring the amount of time spent in the "OFT Zone", an inner circular area distanced 1x zebrafish average width from the walls of the arena. Exploratory behavior towards a novel object was evaluated by the number of times a zebrafish directly touched the object and by the time spent in its vicinity, in the "NOT Zone". The "NOT Zone" was defined as 1x zebrafish average length from the marble to the center of the arena.

3.4.10 – Statistical and image analysis

Image edition was performed in Fiji (95). Statistical analysis was performed in *GraphPad Prism5*, using two-way ANOVA test with Bonferroni post-correction for all experiments comparing WT and *tert*$^{-/-}$ over time. Correlations between phenotypes and telomere size were tested using one-tailed Pearson's test. Comparison of WT and *tert*$^{-/-}$ survival curves was performed using a log-rank (Mantel-Cox) test. Comparison of WT and *tert*$^{-/-}$ cancer incidence curves was performed using a Kolmogorov–Smirnov test. Significance of fertility decline in *tert*$^{-/-}$ was assessed by a Mann–Whitney's test. A critical value for significance of $p<0.05$ was used throughout the study.

3.5 – Supplementary Figures

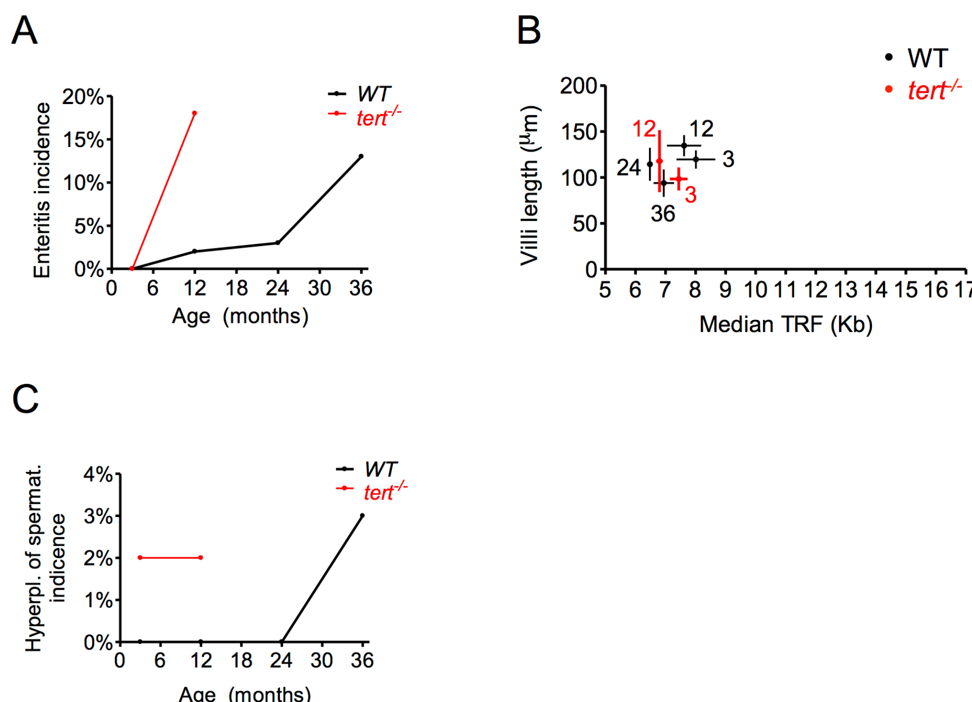

Figure S3.1 – Aging-associated enteritis and hyperplasia of spermatogonia develop prematurely in *tert*$^{-/-}$ tissues.

A) Quantification of the incidence of intestinal inflammatory cell infiltration (enteritis), gut villi length and hyperplasia of the testis in both WT and *tert*$^{-/-}$ mutants with aging. A) As WT and *tert*$^{-/-}$ mutants age, there is progressive inflammatory cell infiltration of the *lamina propria* in the gut (particularly after the age of 24 months in WT and from 6 months onwards in *tert*$^{-/-}$ mutants). WT show 13% of enteritis incidence by 36 months (N=30/238) and *tert*$^{-/-}$ have 18% incidence by 12 months (N=12/ 66). B) No differences in gut villi length are found during WT or *tert*$^{-/-}$ aging (quantification of 5 different fields of view for 4-5 different individuals per time point per genotype). WT and *tert*$^{-/-}$ age groups are indicated in each graph by black and red colored numbers, respectively. C) The percentage of zebrafish with hyperplasia of seminiferous epithelium raises to 3% in 36 months WT cohorts (N=7/238), accompanied by a progressive decrease in fertilization capacity with age (Figure 3.7C), but maintains at residual levels in *tert*$^{-/-}$ mutants (N=1/66).

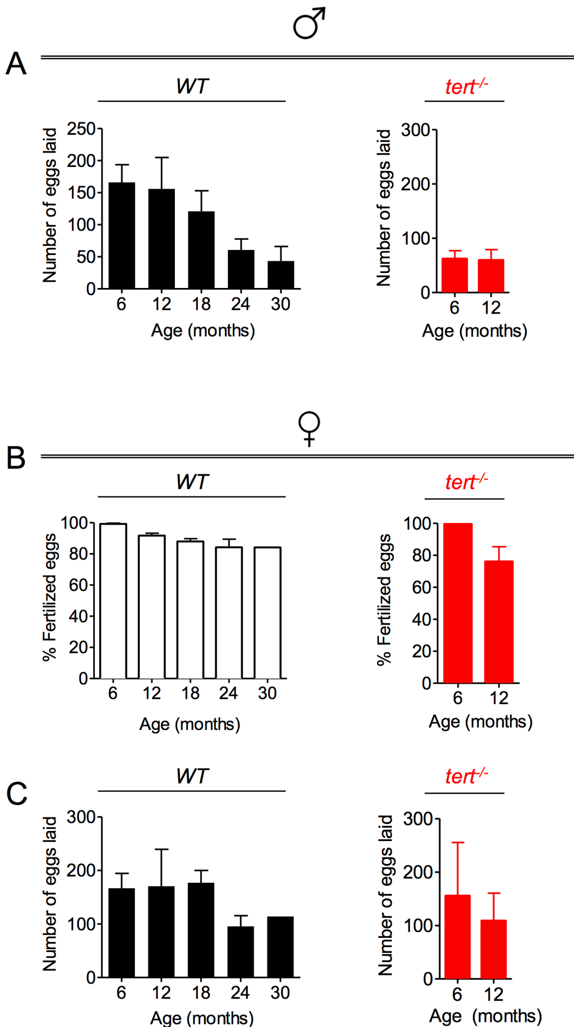

Figure S3.2 – *tert$^{-/-}$* and aged WT inhibit female spawning.

A) Fertility in WT male zebrafish declines by ca. 50% at 24 months (Figure 3.7C), and is accompanied by a reduced ability to stimulate female spawning (average 61 vs. 166 eggs layed in crosses with 24 month-old vs. 3 month-old males, 3-10 fertilization trials/time point). *tert$^{-/-}$* males show defects in egg spawning stimulation already by 6 months (average of 62 vs. 166 eggs layed in crosses with 3 month-old *tert$^{-/-}$* vs. 3 month-old WT males, 3-10 fertilization trials/time point). B) Mild reproductive function defects are seen in 24-month vs. 6-month WT females (ca. 84% vs. 99% reproductive function, respectively, 3-10 fertilization trials per time point). *tert$^{-/-}$* females show a slight defect (ca. 76%) in the percentage of fertilized eggs by 12 months of age when compared with 6 months (3 fertilization trials per time point). C) Female spawning is not significantly affected by age in WT (3-10 fertilization/per time point) or *tert$^{-/-}$* populations (3 fertilization trials/time point).

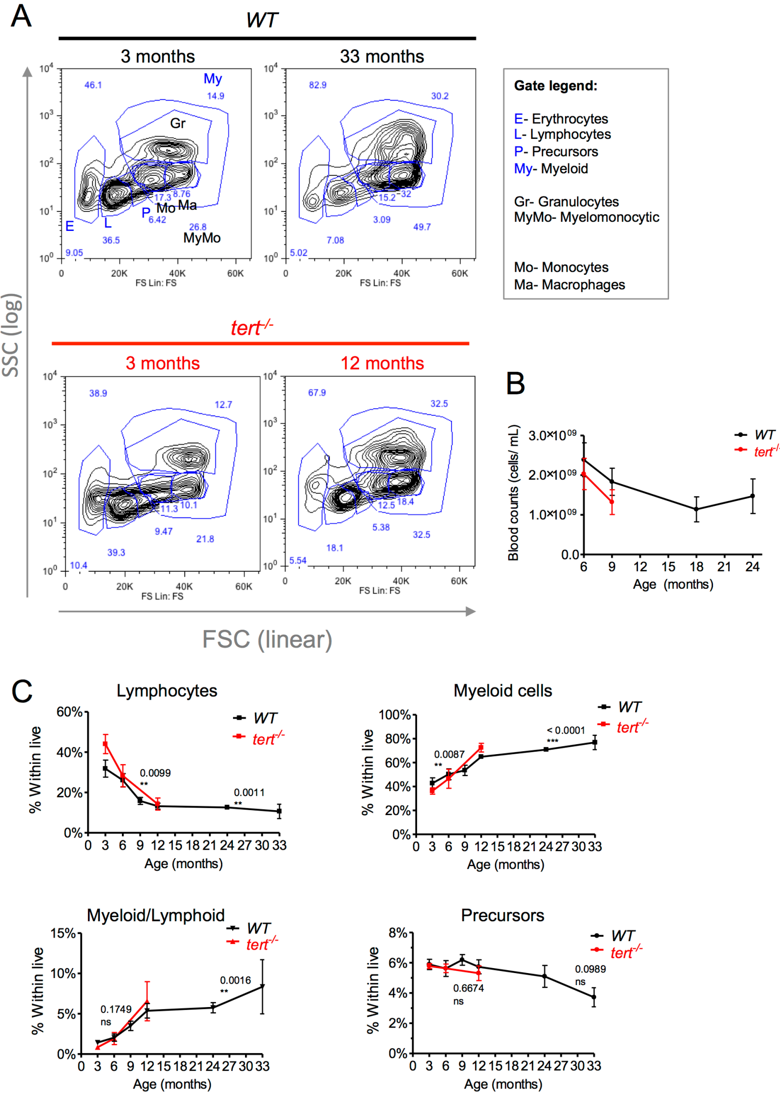

Figure S3.3 – *tert*$^{-/-}$ mutants' myeloid to lymphoid ratios accompany the progression in time found for WT aging.

A) Identification of zebrafish kidney marrow cells by flow cytometry and B) quantification of the percentage of lymphocytes, myeloid cells, myeloid/lymphoid ratios and precursors for WT and *tert*$^{-/-}$ over time. B) %Lymphocytes ("L" in A) declines from 30% to 10% from 3 to 33 months for WT (p=0.0011). *tert*$^{-/-}$ mutants accompany the decline of WT between 3 and 12 months. %Myeloid cells ("My" in A) increases gradually from 2% to 9% from 3 to 33 months (p<0.0001). *tert*$^{-/-}$ mutants' percentage of myeloid cells follows the tendency of WT from 3 to 12 months. Myeloid/lymphoid ratios increase with age in WT kidney marrow. N=3-5/time point/genotype.

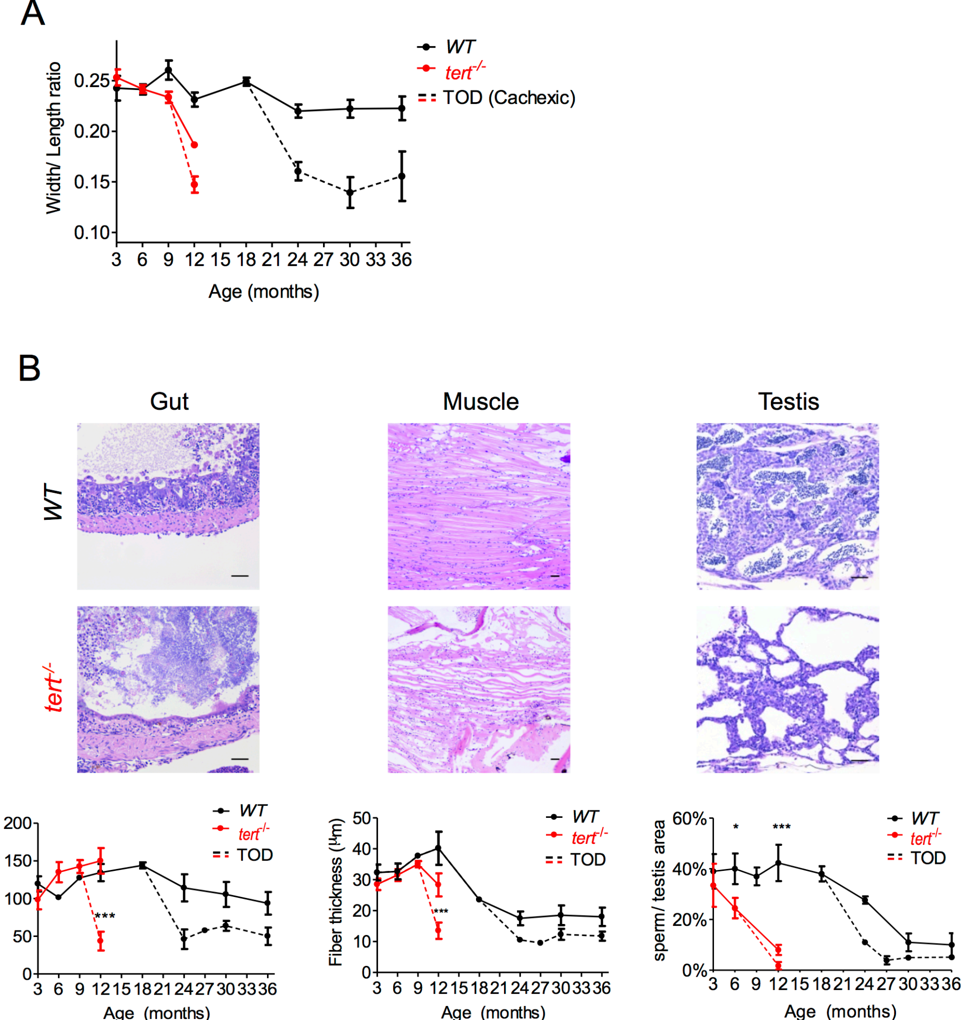

Figure S3.4 – Cachexia at time of death is associated with lower body mass, gut villi shortening and marked muscle and testis atrophy.

A) Quantification of width/length ratios shows that both WT and *tert*$^{-/-}$ zebrafish have significantly lower body mass indexes at time of death (N=4-17/time point for WT zebrafish and N=3-7 for *tert*$^{-/-}$ mutants). B) Representative hematoxilin and eosin-stained sections of gut, muscle and testis from WT and *tert*$^{-/-}$ siblings at TOD. B) Cachexia is associated with shorter/atrophic villi (N=3-6/genotype/time point) and severe myocyte atrophy and degeneration (N=3-6/genotype/time point), to a higher degree than that found for non-cachexic siblings. Testis also suffers pronounced atrophy (N=3-4/genotype/time point). TOD corresponds to the interval comprising the second and third quartiles of survival (25 to 75%). Quantifications were performed in at least 3 different fields of view for each individual. Scale bar = 50 mM. Data are represented as mean +/- SEM.

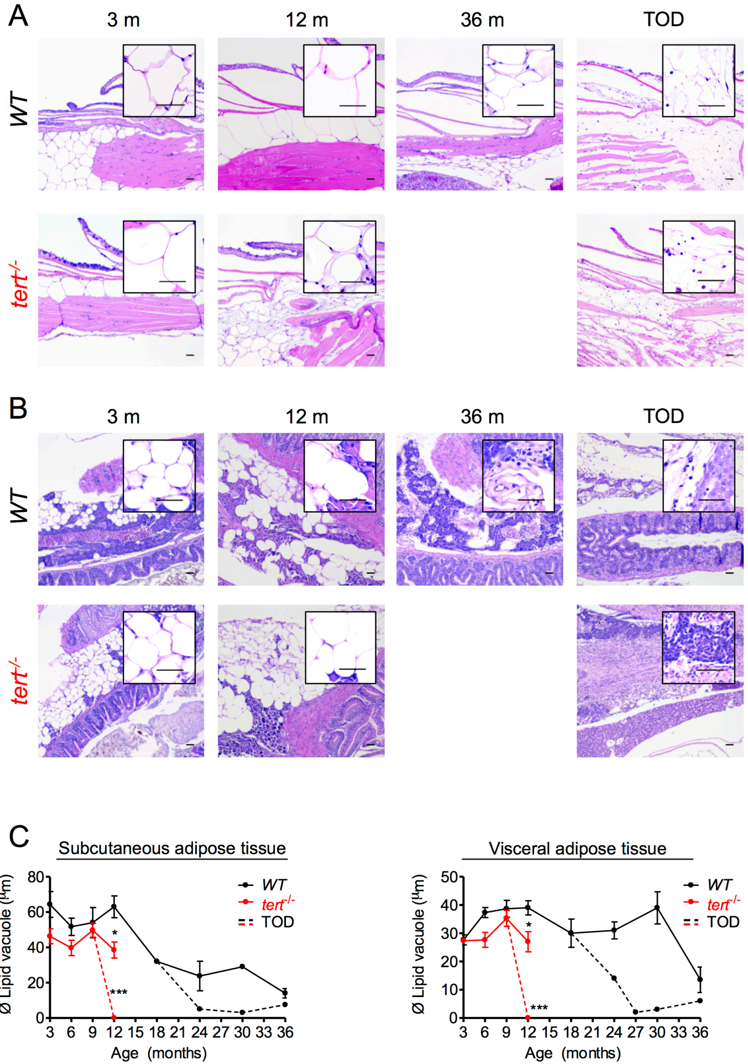

Figure S3.5 – *tert*$^{-/-}$ anticipate the age-associated loss of adipose tissue.
Representative hematoxilin and eosin-stained sections of subcutaneous (A) and visceral (B) adipose tissue depots of WT and *tert*$^{-/-}$ at the indicated ages. A) WT zebrafish show a progressive loss of the subcutaneous depot with age, C) accompanied by a reduction in the adipocytes' vacuole diameter (adipocytes are ~3.3 times smaller at 36 months vs. 3 months). *tert*$^{-/-}$ anticipate this phenotype by 12 months of age. B) Visceral adipose tissue (peri-pancreatic) is reduced or absent at 36 months in WT zebrafish and by 12 months in *tert*$^{-/-}$; similarly to subcutaneous depot, exhaustion of visceral adipose reserves associates with cachexia. N= 3-7 zebrafish/genotype/time point. Time of death (TOD) corresponds to the interval comprising the second and third quartiles of survival (25 to 75%) Scale bar = 50 mM. Data are represented as mean +/- SEM.

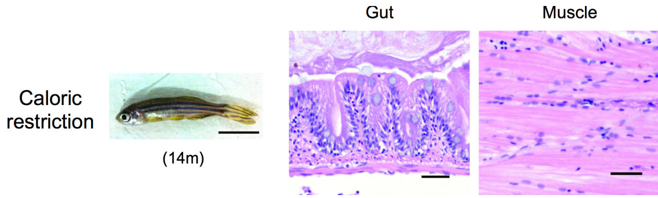

Figure S3.6 – Muscle atrophy, but not gut villi degeneration, is the consequence of a poor nutritional body state in cachexic zebrafish.

A) Representative picture and hematoxilin and eosin-stained sections of 12-month old WT after 8 weeks of caloric restriction (food intake reduced by 85%). Upon caloric restriction, gut villi length remains unaffected but myocytes undergo atrophy to a degree similar to cachexic zebrafish (Supplementary Figure S3.4). N=4. Scale bar = 50 mM.

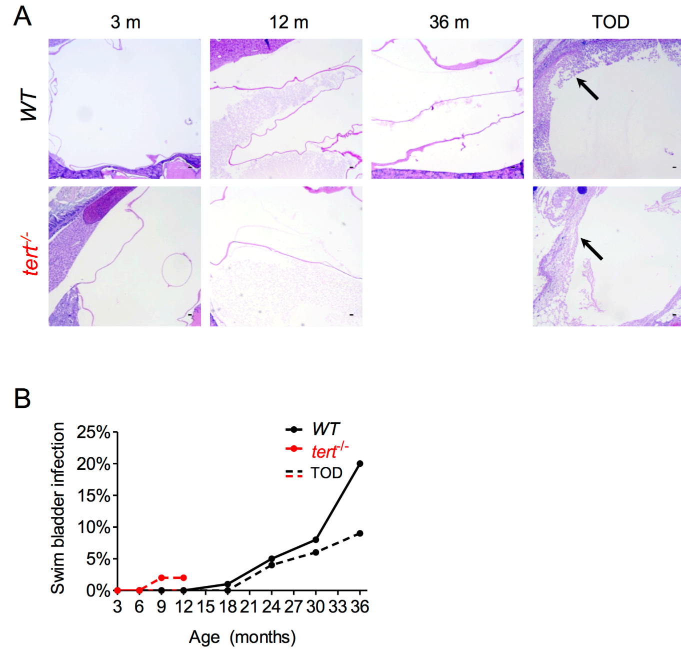

Figure S3.7 – *tert*$^{-/-}$ mutants anticipate the rise in swim bladder infection observed in aging.

A) Representative hematoxilin and eosin-stained sections of swim bladder from WT and *tert*$^{-/-}$. Inflammation of the swim bladder at time of death (indicated by black arrow) associates with cachexia and is accompanied by necrosis (that often extends throughout the visceral cavity), both in WT and *tert*$^{-/-}$ mutants. B) Incidence rates reach 30% in 24 month WT zebrafish (N=71/238) and 3% in 9-month old *tert*$^{-/-}$ (N=2/66). TOD corresponds to the interval comprising the second and third quartiles of survival (25 to 75%). Scale bar=50 μM. Data are represented as mean +/- SEM.

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Chapter 4 | Short telomeres compromise homeostasis and lifespan by repressing key oxidative defense pathways

Chapter authors

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Authors' contributions

Conceived and designed the experiments: MGF JPL MCC IPC

Performed the experiments: MCC performed most of the experiments; IPC performed measurements of ROS, cell death and collaborated with MCC in drug treatments of G2 *tert*$^{-/-}$ larvae.

Analyzed the data: MGF JPL MCC IPC

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Abstract

Short telomeres activate p53/p21-dependent mechanisms that compromise tissue function and lifespan. Examples of such mechanisms include apoptosis/senescence, which underlie the defects of high turnover tissues, and impairments in mitochondrial biogenesis, which are proposed to explain the degeneration of more quiescent organs. Abnormal accumulation of reactive oxygen species (ROS) associates with these mechanisms, appearing as a candidate that may fuel telomeric erosion, and thus sustain further tissue damage. However, the extent to which ROS may contribute to tissue failure and reduce longevity in organisms with dysfunctional telomeres is not known.

Here, we perform global transcriptomic studies of naturally aged and telomerase mutant zebrafish tissues to identify the dominant mechanisms by which short telomeres may drive natural aging phenotypes. We find prevalent tissue-specific signatures with strong repression of oxidative defense mechanisms, namely glutathione metabolism, associated with increased levels of ROS. Antioxidant treatments with the general scavenger NAC or with the superoxide dismutase mimetic MitoTEMPO, within a specific time window, are sufficient to significantly rescue homeostasis and lifespan defects caused by extreme telomere dysfunction. Thus, our study provides functional evidence that telomere-induced disruption of homeostasis relies, at least in part, in defective protection against oxidative stress.

4.1 – Introduction

Telomere erosion is an important hallmark of aging (1). Telomeres are specialized nucleoprotein structures that cap the ends of chromosomes, preventing them from being recognized as DNA double strand breaks and engaging in illicit repair (2). As the expression of telomerase, the enzyme

capable of elongating telomeres, is restricted in human somatic cells (3), telomeres shorten with each cell division (4) and with human aging (5). When telomeres reach a critical length, cells arrest proliferation and activate *tp53*-DNA damage responses that culminate in either apoptosis or senescence (6, 7). In late generation telomerase knockout mice (both catalytic enzyme, *mTert*$^{-/-}$ and RNA subunit, *mTerc*$^{-/-}$), such cellular responses put a constraint in stem cell renewal (8, 9), compromising tissue maintenance and longevity (10, 11). Notably, telomere-shortening defects of G4 *Tert*$^{-/-}$ mice can be reversed by overexpression of telomerase for a 4-week period (12) and recent evidence shows that physiological aging in mice can be delayed by telomerase gene therapy (13). While telomerase-based therapies aiming to prevent aging-associated pathologies are currently emerging, the identification of the key mechanisms acting downstream of short telomeres to promote damage is essential for the development of new and more targeted treatments.

Some of these mechanisms have been extensively characterized in telomerase knockout mice, namely those relying on p53 activation, such as downstream *Puma*-dependent apoptosis or *Cdkn1a*-dependent cell cycle arrest (8, 9). Deletion of *p53*, *Puma* or *Cdkn1a* significantly ameliorates the degenerative phenotypes caused by telomere dysfunction, particularly those of highly proliferative tissues (8, 9, 14). Recent evidence indicates that the telomere-p53 axis also disrupts homeostasis and reduces lifespan by interfering with alternative metabolic pathways, namely by repressing mitochondrial biogenesis (15), possibly via inhibition of IGF1/mTOR signaling (16), and by hyper activating glucose metabolism (16). Dietary glucose supplementation in this context is sufficient to significantly extend lifespan and rescue mitochondrial biogenesis defects (16). Concomitantly, mice tissues with short telomeres show lower oxidative protection and accumulate high levels of reactive oxygen species, ROS (15). Increased oxidative stress may in turn favor damage at telomeres, given their high content in guanine triplets (17, 18). Accumulation of

persistent damage at telomeres with age (19, 20) may accelerate further their erosion (18, 21).

Independent compelling evidence also supports a prominent role for oxidative species acting as instigators of persistent cellular damage and thus of DDR and senescence in aging of mice, worms and flies (22-25). Interestingly, specific environmental or genetic manipulations that increase longevity also confer augmented protection to oxidative stress, particularly ROS-induced. Elevated stress resistance is found in dauer adaptive states of *C. elegans* (26), in rodents under caloric restriction (27-29) and in various worm, flie and mammalian mutants that exhibit extended longevity (26, 30-32). Furthermore, naturally longer-lived mammals accumulate ROS to lower levels than shorter-lived ones (33). To neutralize unstable and reactive oxidative species, cells rely on the activity of endogenous detoxifying agents such as glutathione, GSH (34) and superoxide dismutases, SOD (35). With progressing age, reduction in the expression of such molecules causes redox unbalance and increases susceptibility of proteins, nucleic acids and lipids to damage peroxidation (36, 37). While reduced glutathione metabolism or mutations in superoxide dismutases sensitize cells to oxidative stress and reduce lifespan (38-41), stimulation of these mechanisms can retard the development of age-related phenotypes in mammals and extend longevity in *C. elegans* and *Drosophila* (42-45).

Altogether these findings have raised the hypothesis that cellular and tissue phenotypes of organisms with dysfunctional telomeres may partly rely on a continuous loop of ROS-induced damage (15, 46). However, functional experiments distinguishing cause from effect and showing the extent to which short telomere phenotypes depend on oxidative stress are currently lacking. More importantly, whether these mechanisms act in parallel with apoptotic or senescence pathways to cause dysfunction and ultimately death in natural aging remains unknown.

We previously showed that telomerase mutant (*tert*$^{-/-}$) zebrafish anticipate the tissue-specific defects and pathologies of natural zebrafish aging (Chapter 3). Thus, *tert*$^{-/-}$ zebrafish is a valid and promising model for exploring the mechanisms by which short telomeres drive aging phenotypes. Here, we identify some of those mechanisms by performing a global expression-profiling analysis of *tert*$^{-/-}$ and WT natural zebrafish aging over time. Our data reveals that short telomeres trigger expression signatures of aging in a tissue specific manner. One of the strongest responses involves repression of key oxidative defense pathways, namely glutathione metabolism. Therefore, we decided to test if improving oxidative defense responses is sufficient to rescue the degenerative phenotypes caused by extreme telomere dysfunction.

4.2 – Results

4.2.1 – Tissue-specific signatures are stronger than signals triggered by absence of telomerase/aging

To identify the molecular mechanisms by which short telomeres cause tissue defects in aging, we performed a time course transcriptional profiling analysis of WT and *tert*$^{-/-}$ tissues. We probed several time points between the age when zebrafish reach sexual maturity (3 months) and the time when WT and *tert*$^{-/-}$ populations have reached at least 50% survival (9 months for *tert*$^{-/-}$ and 36 months for WT, survival curves in Chapters 2&3). WT tissues were profiled at 3, 6, 9, 24 and 36 months and *tert*$^{-/-}$ tissues at 3, 6 and 9 months. As short telomeres may lead to tissue dysfunction by mechanisms acting non-cell autonomously, we profiled tissues where telomeres shorten, such as gut and muscle, and tissues where telomeres do not shorten with age, such as testis (Chapter 3). We started by hierarchically clustering the gene expression data for all WT and *tert*$^{-/-}$ samples (using Euclidean distances) to identify the dominant

signal in our dataset. Expression signals grouped first by tissue type (testis – green, muscle – blue, gut – brown; squares in Figure 4.1) and inside each tissue branch, older WT groups (over 24 months, blue categories in Figure 4.1) clustered separately from younger WTs (3 to 9 months). Telomerase mutants (red categories), however, clustered together with young WT controls in the gut and muscle branches. A single exception was found for telomerase mutant testis. Part of the samples belonging to this group formed a separate sub-branch inside the testis family, and the rest clustered together within the gut branch. This was the only case where clustering outside the respective tissue branch was observed, suggesting that absence of telomerase is sufficient to induce expression changes that overcome the tissue signal specifically in the testis but not in the gut or muscle.

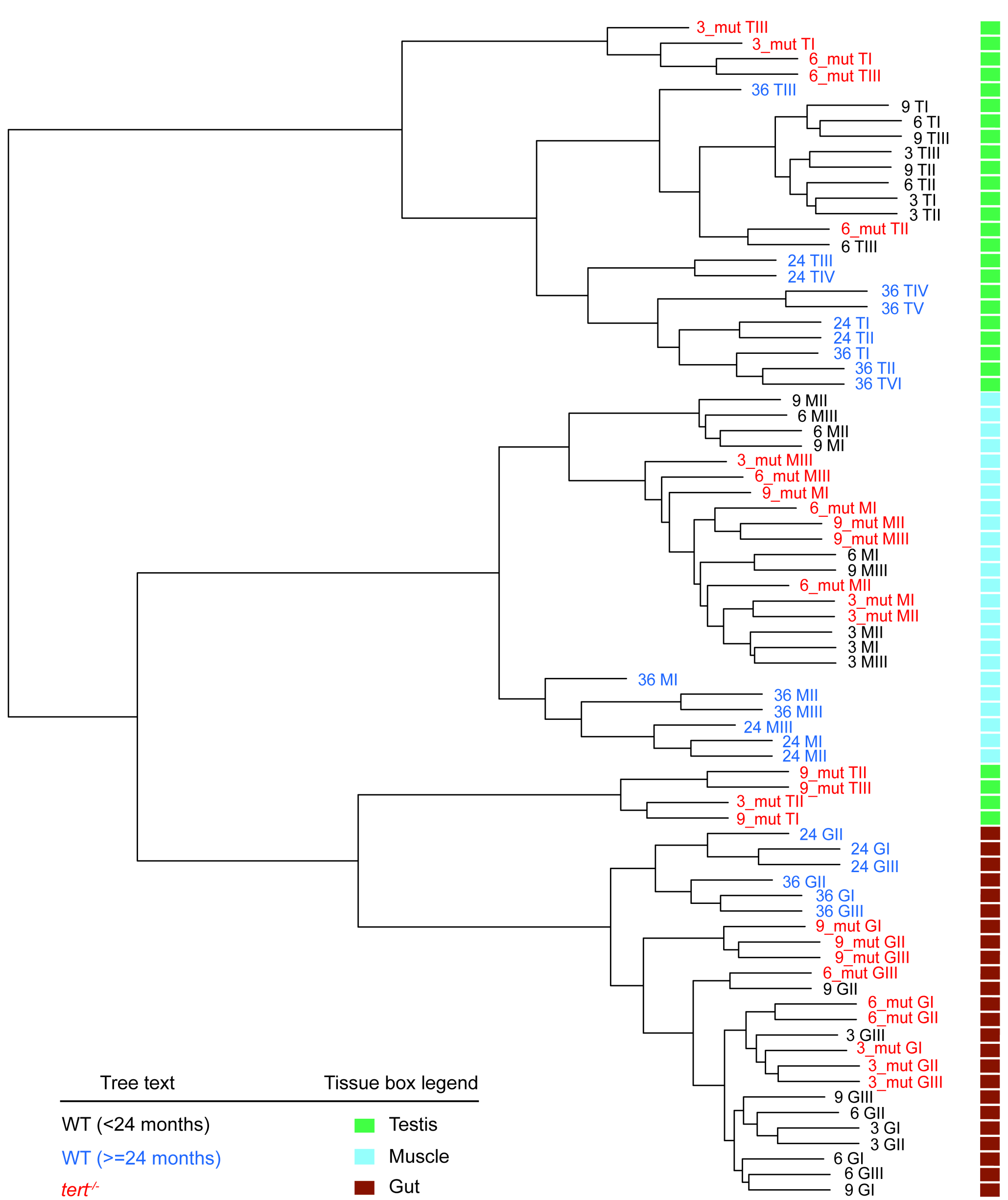


Figure 4.1 – Tissue-specific signals of gene expression prevail over signatures of aging or absence of telomerase.

Hierarchical clustering of gene expression, based on Euclidean distances, including all WT and *tert*$^{-/-}$ tissues (testis, muscle, gut) and time points (3, 6, 9, 24 and 36 months for WT and 3, 6 and 9 months for *tert*$^{-/-}$). Expression signals group first by tissue type, second by WT age (separate

clusters for samples over 24 months). Tissue type indicated by colored square placed in front of each sample branch: testis – green, muscle – blue, gut – brown. Categories labeled in black refer to WT young samples (3, 6 and 9 months), categories labeled in blue refer to WT old samples (24 and 36 months) and categories labeled in red refer to *tert*$^{-/-}$ samples (3, 6 and 9 months).

Notably, absence of telomerase did not induce a swift variation in range of gene expression values in any of the tissues tested (Figure 4.2).

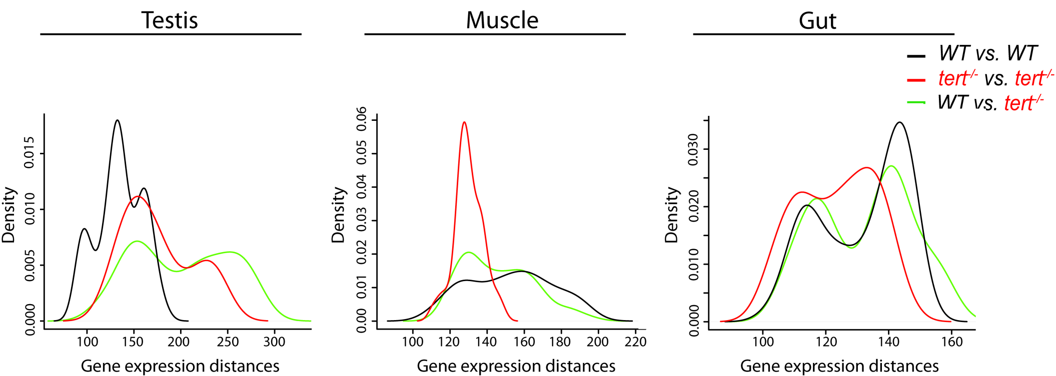


Figure 4.2 – Gene expression values fall within the same range of distributions in the presence or absence of telomerase.

Density distributions of gene expression Euclidean distances for three groups: WT vs. WT, *tert*$^{-/-}$ vs. *tert*$^{-/-}$ and WT vs. *tert*$^{-/-}$, including all time points (3, 6, 9, 24 and 36 months for WT and 3, 6 and 9 months for *tert*$^{-/-}$) for the three tissues analyzed, gut muscle and testis. The range of gene expression distances (x axis) overlaps between the three groups, even though *tert*$^{-/-}$ vs. *tert*$^{-/-}$ and WT vs. WT distributions are statistically different (p testis = 3.65×10^{-7}; p muscle = 6.21×10^{-10}, p value gut = 7.7×10^{-4}), as well as the respective medians (p testis = 3.1×10^{-9}; p muscle = 3.67×10^{-7}; p gut = 1×10^{-3}).

Together these data show that the developmental origin of each tissue is generically a stronger determinant of gene expression signatures than aging or absence of telomerase. In agreement, tissue expression signals were also shown to be dominant over aging associated transcriptomic changes in mice models (47). Therefore, rather than looking for a common generic signature of aging associated with short telomeres, we focused on finding similarities between WT and *tert*$^{-/-}$ aging signatures, within each individual tissue.

4.2.2 – Short telomeres drive aging potentially by affecting oxidative defense, lipid metabolism and inflammatory responses

We performed a time course analysis (TC) of WT and *tert*$^{-/-}$ tissues, as previously described (48), to identify genes that are differentially expressed in the absence of telomerase or in natural WT aging. The list of genes found differentially expressed at any given time point of our WT time course analysis was designated for each tissue "$TC_{WT\ TESTIS}$", "$TC_{WT\ GUT}$" or "$TC_{WT\ MUSCLE}$". The list of differentially expressed genes (DEG) found in *tert*$^{-/-}$ vs. WT controls was designated "$TC_{tert-/-\ TESTIS}$", "$TC_{tert-/-\ GUT}$" or "$TC_{tert-/-\ MUSCLE}$". TC_{WT} profiles revealed that the majority of DEG were downregulated with aging (Figure 4.3), frequently exhibiting a bimodal behavior of expression over time (downregulation after a specific time point, designated by "1 inflexion" or "1 I", Figure 4.3). $TC_{tert-/-}$ profiles also showed a high percentage of downregulated genes in the muscle and gut. These genes showed mostly a continuous behavior ("Co") of differential expression over time or again a bimodal pattern (Figure 4.3).

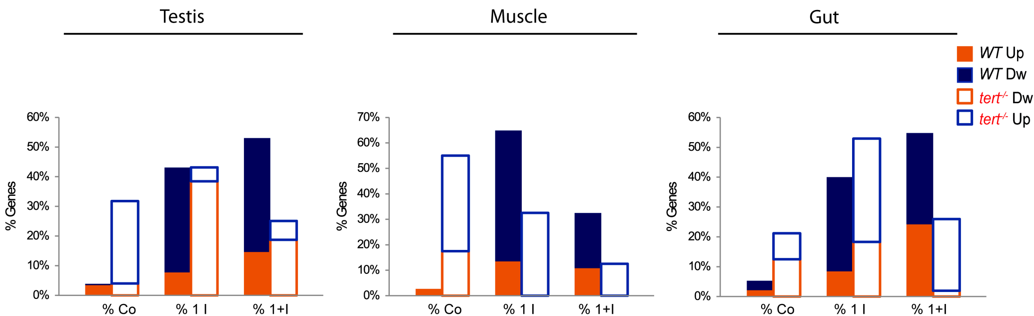


Figure 4.3 – DEG show mostly bimodial or bimodial/continuous behaviors of differential regulation over time in WT and *tert*$^{-/-}$ aging.

Proportion of genes up or downregulated in each tissue (TC analysis, $p<0.05$) showing continuous ("Co"), bimodal ("1 I", with one inflexion point) or variable ("1+I", with more than one inflexion point) behaviors of differential expression over time. Orange – upregulated genes and blue – downregulated genes. Genes are mostly downregulated in WT aging and show either bimodial behaviors, or variable behaviors (several inflexions) of differential expression over time. DEG in

tert$^{-/-}$ tissues show mainly continuous (up or downregulation from 3 to 9 months) or bimodial behaviors of differential expression over time.

Our final aim was to determine which candidate genes/mechanisms act in response to telomerase absence to promote degenerative phenotypes of aging. For that purpose we asked which DEG could be found at the intersection of TC_{WT} and $TC_{tert-/-}$ ($TC_{WT} \cap TC_{tert-/-}$), for tissues where telomeres shorten with age. In the muscle, we found 121 DEG common to TC_{WT} and $TC_{tert-/-}$ (from totals of $N_{DEG\ TC\ WT}$ = 900 $N_{DEG\ TC\ tert-/-}$ = 215). Most of these genes were downregulated in both settings (Figure 4.4A). We found enrichment for pathways related with gap junction communication ($p = 5.65\times10^{-5}$), development of megakaryocytes and production of platelets ($p = 1.39\times10^{-5}$) and folding of tubulin ($p = 2.5\times10^{-4}$) particularly via the chaperonin TCP-1 ring complex (Table 4.1).

In the gut, 99 DEG were found to be common to TC_{WT} and $TC_{tert-/-}$ (Figure 4.4B; from totals of $N_{DEG\ TC\ WT}$ = 637; $N_{DEG\ TC\ tert-/-}$ = 327). These genes enriched in pathways such as P450 metabolism of xenobiotics ($p = 1.41\times10^{-12}$), glutathione metabolism ($p = 6.16\times10^{-10}$), type I interferon (IFN-I) immune responses ($p = 7.3\times10^{-6}$), and recycling of bile acids and salts ($p = 1.97\text{x}10^{-4}$, Table 4.1). Genes involved in glutathione metabolism and recycling of bile acids and salts were generically downregulated, while genes related with IFN-I response were significantly upregulated (Table 4.1). These results stand in agreement with previous studies showing that, with aging, the expression of immune system regulators (including interferons) increases in mouse and human tissues, while the activity of glutathione reductases declines (47, 49, 50).

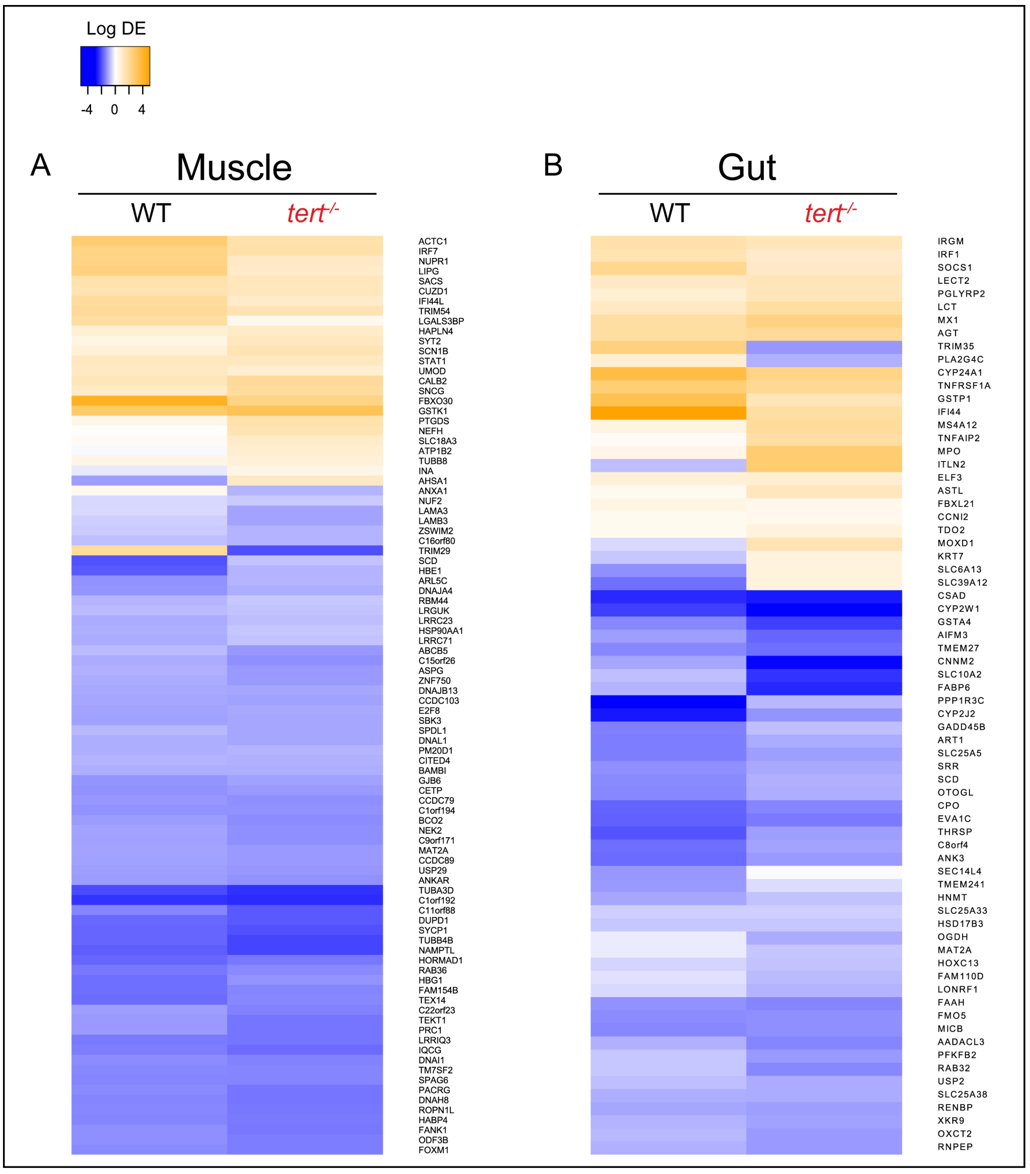


Figure 4.4 – *tert*$^{-/-}$ recapitulate the differential expression patterns of natural aging in the muscle and gut.

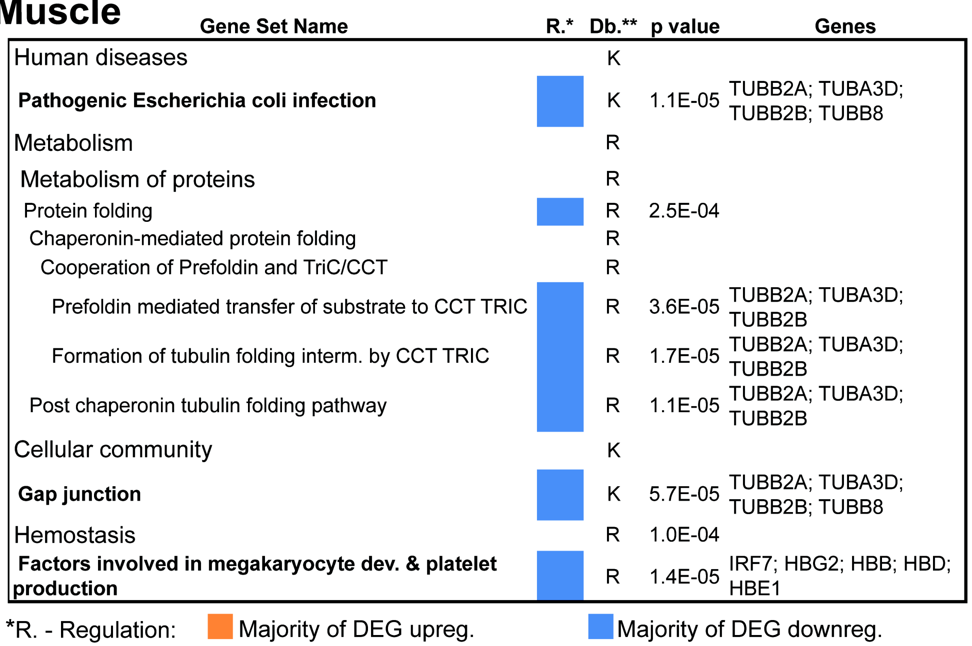
Heatmaps of differentially expressed genes (TC analysis, $p<0.05$), showing Log_2 expression for WT at 24 months and for *tert*$^{-/-}$ at 9 months, normalized to 9-months WT samples in the A) muscle and B) gut. Fold difference (Log_2 values) of expression is proportional to the intensity of coloring (orange for upregulation and blue for downregulation). Each row corresponds to a single gene, identified by the orthologous human symbol to the right. LogDE – Log_2 of differential expression.

Table 4.1 – Gene set enrichment analysis (GSEA) of $TC_{WT}∩TC_{tert-/-}$ for gut and muscle. GSEA of DEG at the intersection of WT and *tert*$^{-/-}$ time course analyses ($TC_{WT}∩TC_{tert-/-}$), for the gut and muscle. Two databases were used: REACTOME and KEGG (51, 52). Orange – upregulation; blue – downregulation. DEG in each pathway identified by the human orthologous gene symbol.

Gut

Gene Set Name	R.*	Db.**	p value	Genes
Metabolism		R		
Biological Oxidations		R	3.4E-15	
Phase 1 Functionalization of compounds		R	1.0E-05	
Drug metabolism cytochrome P450		K	1.4E-12	GSTP1; GSTA5; GSTA1; GSTA2; GSTA3; GSTA4; FMO2; FMO5
Phase II Conjugation		R	8.0E-11	
Glutathione conjugation		R	4.1E-12	GSTP1; GSTA5; GSTA1; GSTA2; GSTA3; GSTA4
Metabolism of lipids and lipoproteins		R	3.9E-05	
Recycling of bile acids and salts		R	2.0E-04	FABP6; SLC10A2
Metabolism of other amino acids		K		
Glutathione metabolism		K	6.2E-10	GSTP1; GSTA5; GSTA1; GSTA2; GSTA3; GSTA4
Immune System		R		
Cytokine signaling in immune system		R		
Interferon signaling		R	2.6E-04	
Interferon alpha beta signaling		R	7.3E-06	SOCS1; IRF1; MX1; MX2

Muscle



Gene Set Name	R.*	Db.**	p value	Genes
Human diseases		K		
Pathogenic Escherichia coli infection		K	1.1E-05	TUBB2A; TUBA3D; TUBB2B; TUBB8
Metabolism		R		
Metabolism of proteins		R		
Protein folding		R	2.5E-04	
Chaperonin-mediated protein folding		R		
Cooperation of Prefoldin and TriC/CCT		R		
Prefoldin mediated transfer of substrate to CCT TRIC		R	3.6E-05	TUBB2A; TUBA3D; TUBB2B
Formation of tubulin folding interm. by CCT TRIC		R	1.7E-05	TUBB2A; TUBA3D; TUBB2B
Post chaperonin tubulin folding pathway		R	1.1E-05	TUBB2A; TUBA3D; TUBB2B
Cellular community		K		
Gap junction		K	5.7E-05	TUBB2A; TUBA3D; TUBB2B; TUBB8
Hemostasis		R	1.0E-04	
Factors involved in megakaryocyte dev. & platelet production		R	1.4E-05	IRF7; HBG2; HBB; HBD; HBE1

*R. - Regulation: Majority of DEG upreg. Majority of DEG downreg.

**R-REACTOME; K-KEGG

While our previous clustering analysis (Figure 4.1) indicated most responses with aging/in the absence of telomerase ought to be tissue specific, we asked if we could still find conserved gene expression signatures across-tissues. We intersected the gut and muscle final lists of DEG found in ($TC_{WT} \cap TC_{tert-/-}$), since we previously knew these were tissues where telomere shortening occurs with age (Chapter 3). To avoid looking at non-cell autonomous effects of telomerase absence we excluded candidates that were also present in the profile of testis aging, a tissue where telomeres do not shorten. We did not find a single pathway that respected these conditions but identified, however, two genes whose expression changed significantly in the gut and muscle of WT and *tert*$^{-/-}$ over time and not in testis: *mat2a* (s-adenosylmethionine synthase isoform type-2) and i*fi44* (interferon-induced protein 44). *mat2a* catalyzes the synthesis of s-adenosylmethionine (AdoMet), a central methyl donor, from methionine and ATP (53). This gene was found significantly repressed with zebrafish aging in the gut (WT:36 months; *tert*$^{-/-}$: from 3 to 9 months) and muscle (WT: from 24 months; *tert*$^{-/-}$: 9 months). Inhibiting transcription of *sams-1* in *C. elegans* (homolog of *mat2a*) increases lifespan (54). This suggests that downregulation of *mat2a* with zebrafish aging may be acting as a pro-longevity compensatory mechanism, rather than being a detrimental consequence of aging itself. *ifi44* (or its paralog *ifi44-like*), an interferon-alpha (IFN-alpha)-inducible microtubular aggregate protein whose function remains unknown, was significantly upregulated with aging in zebrafish gut (WT: from 24 months; *tert*$^{-/-}$: from 6 months) and muscle (continuously, in WT and *tert*$^{-/-}$). Coincidently, we have previously reported that incidence of enteritis starts rising at the age of 24 months in WT zebrafish and earlier, by 6 months, in *tert*$^{-/-}$ (Chapter 3) and inflammatory responses, including interferon regulators, are known to be positively regulated by mammalian aging (47). Finally, transcription of *ifi44* is directly regulated by telomere length in cancer cells (55).

To get a hint on how short telomeres may be signaling non-cell autonomously between tissues in aging, we retrieved the previously excluded intersection between gut and muscle ($TC_{WT} \cap TC_{tert-/-}$) with WT testis aging. In this condition, we found co-differential expression of a single gene, *scd* (stearoyl-CoA desaturase). *scd* is a central lipogenic enzyme involved in the biosynthesis of monounsaturated fatty acids from saturated fatty acids (56). In our gene expression profiling study we found *scd* to be significantly downregulated with age in all WT/*tert*$^{-/-}$ tissues. This occurs possibly via p53 activation, a major mediator of zebrafish short-telomeres defects (57), as p53 was shown to directly bind and repress *scd* transcription (58). Lack of proper function of this enzyme most likely results in higher ratios of saturated to unsaturated fatty acids, a clear phenotype of human aging that associates with cardiovascular disease and cancer (59). Curiously, mouse models of accelerated senescence also show a significant decrease in *scd* transcription (60).

In summary, we identified several mechanisms possibly acting downstream of short telomeres in aging. We next established a rapid *in vivo* assay to functionally validate whether these pathways are important mediators of short telomere-induced defects.

4.2.3 – G2 *tert*$^{-/-}$ zebrafish: a fast model to test the influence of chemical compounds in short-telomere degenerative phenotypes

Previous studies showed second-generation (G2) *tert*$^{-/-}$ mutants are embryonic lethal (57) and exhibit various morphological defects (61). We decided to test whether G2 zebrafish could be used as a reliable model to validate the relevance of specific pathways in mediating short telomere-induced dysfunction. We started by measuring the extent to which some of the phenotypes previously observed in G1 *tert*$^{-/-}$ zebrafish (Chapters 2 and 3) – telomere shortening, reduced lifespan, increased apoptosis – were also manifested in the short lifespan of G2 *tert*$^{-/-}$.

As reported (61), G2 *tert*$^{-/-}$ larvae had extremely short telomeres (Figure 4.5A) and exhibited gross abnormalities, such as bent fin and body truncations (Fig 4.5B). However, we did not find an association between the severity of developmental defects and telomere length (Figure 4.5A-B; (61)). Curiously, live embryo staining with acridine orange revealed that both WT and G2 *tert*$^{-/-}$ had significant levels of cell death during the first 24 hours of development, but these were higher in G2 *tert*$^{-/-}$ embryos (Figure 4.5C, as previously shown, (61)). By day 3, apoptosis lowered in WT embryos but remained high in the G2 *tert*$^{-/-}$ background, particularly in the areas of the head and neural tube (Figure 4.5C).

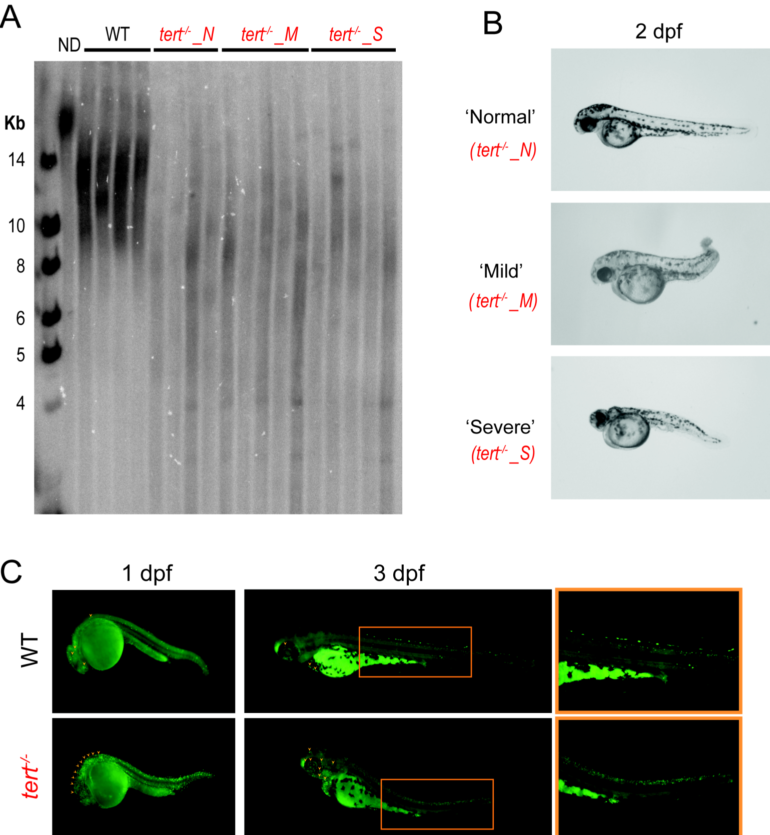


Figure 4.5 – G2 *tert*$^{-/-}$ exhibit shorter telomeres, developmental defects and increased apoptosis.

A) Representative image of restriction fragment analysis of genomic DNA by Southern Blot (random primer-labelled telomeric probe (TTAGGG)n 32P-dCTP) of 10 days post fertilization (dpf) WT and G2 *tert*$^{-/-}$ larvae. Telomeres are significantly shorter in G2 *tert*$^{-/-}$ larvae but contrary to previous reports (61) no obvious relation between telomere size and B) severity of developmental defects is observed by Southern Blot analysis (phenotypes were scored as previously described - (61)); *tert*$^{-/-}$_N – ("Normal", WT-like); *tert*$^{-/-}$_M ("mildly affected"); *tert*$^{-/-}$_S ("severely affected"). C) Representative staining of G2 larvae with acridine orange shows *tert*$^{-/-}$ larvae have higher levels of apoptosis, evident by day 3 – arrowheads point to apoptotic bodies (experiment in C) was performed by IPC).

In addition, G2 *tert*$^{-/-}$ had almost undetectable levels of proliferation and a significantly higher percentage of senescent cells (Margarida Figueira and Miguel G. Ferreira, personal communication).

Although our cohorts of G2 *tert*$^{-/-}$ showed a high degree of lethality, they only reached ca. 30% survival around day 11 (Figure 4.6A), in contrast with previous studies reporting ca. 25% survival by day 7 (61). In order to assess the degree of variability in survival, we crossed different sets of mating parents several times and analyzed their progeny individually (Parents Group A, PGA; Parents Group B, PGB; Parents Group C, PGC, Figure 4.6C-E). Within each group (PGA, PGB and PGC), where parents were siblings, G2 *tert*$^{-/-}$ progeny of different crosses had indistinguishable survival curves (Figure 4.6C-E). However, between different groups, when comparing the progeny of cousins, we detected significantly lower survival in the progeny of a particular group – PGC – in relation to PGA or PGB (p=0.0001, Figure 4.6B). Thus, G2 *tert*$^{-/-}$ survival may vary significantly when comparing the progeny of parents that have a lower degree of proximity than siblings. Still, death by 12 days was significantly different from WT controls in any of the groups tested (Figure 4.6B).

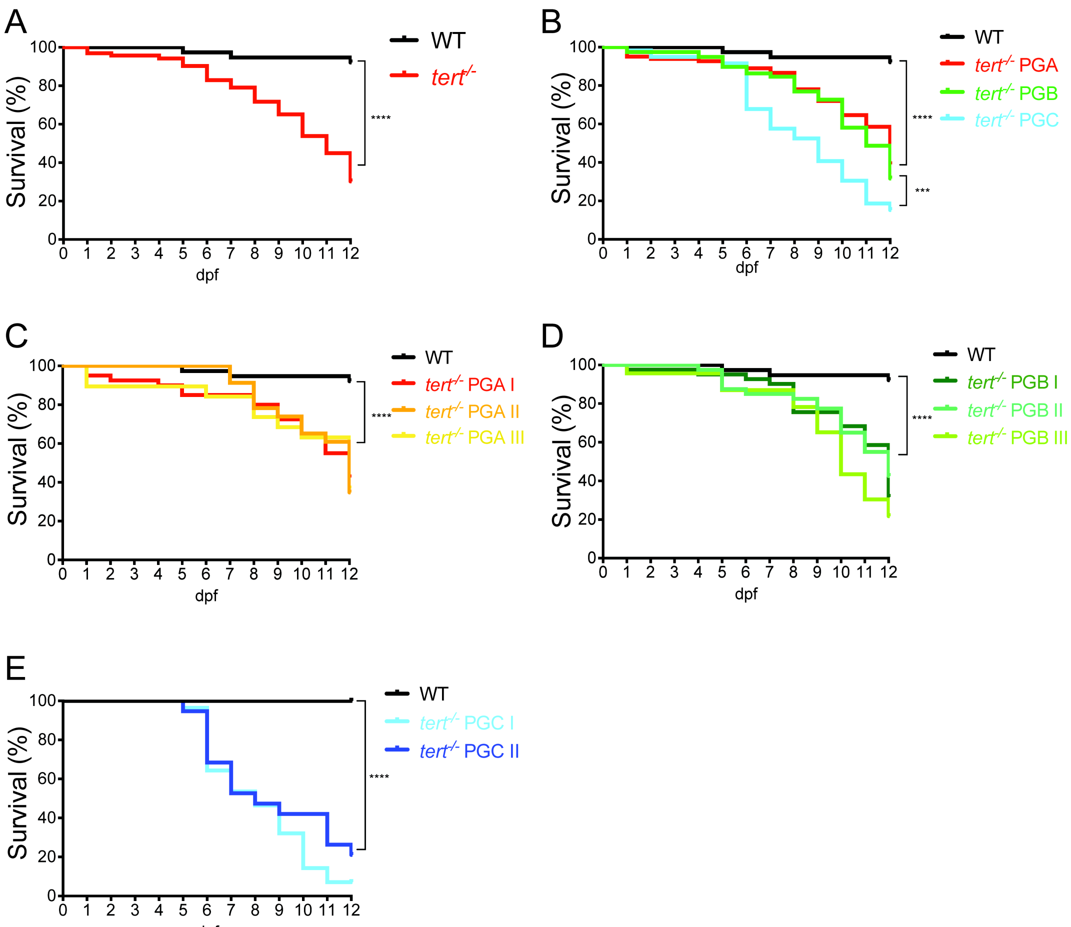


Figure 4.6 – G2 *tert*$^{-/-}$ survival is 2-3 weeks, but varies between non-sibling mating couples. A) G2 *tert*$^{-/-}$ reach ca. 30% survival by day 11 ($p<0.0001$; N=62 WT; N=264 *tert*$^{-/-}$) but B) there is significant heterogeneity in progeny survival between crosses of non-sibling parents. Parents Group A, PGA; Parents Group B, PGB; Parents Group C, PGC. Parents are siblings within each group (PGA, PGB and PGC). N=37 WT; N= 81 PGA; N= 60 PGB; N= 120 PGC. C-E) Within each group of parents the variability in progeny survival is low. N=20-40 larvae per cross.

To further establish G2 larvae as a reliable assay for validating if specific pathways mediate short telomere-induced defects, we manipulated a target already known to regulate tissue dysfunction in telomerase deficient animals. Genetic inhibition of p53 (and associated DDR) was shown to partially rescue the defects caused by short telomeres in *tert*$^{-/-}$ zebrafish and in late generation

telomerase knockout mice (14, 57, 61). More importantly, genetic inhibition or knockdown of p53, using targeting morpholinos, is able to rescue G2 *tert*$^{-/-}$ zebrafish larvae survival defects (61). We tested if a pharmacological inhibitor of zebrafish p53 function, pifithrin-α - PFTα, (62), would also rescue G2 *tert*$^{-/-}$ survival, as our goal was to target other candidate pathways using drug approaches. Because G2 *tert*$^{-/-}$ have much higher levels of apoptosis by day 3 post fertilization, which probably rely on the activation of p53 (10, 57), we treated G2 *tert*$^{-/-}$ larvae with PFTα from days 3 to 5. PFTα partially rescued the survival of G2 *tert*$^{-/-}$ and did not affect grossly WT survival (Figure 4.7).

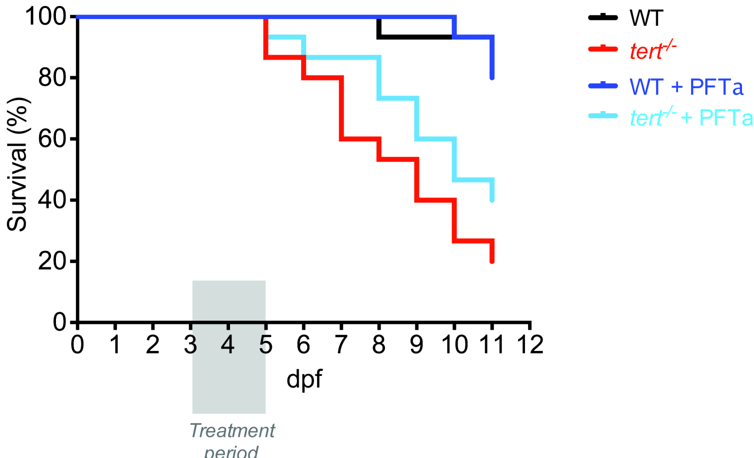


Figure 4.7 – p53 inhibitor, pifithrin-α (PFTα) partially rescues G2 *tert*$^{-/-}$ reduced survival. Treatments with PFTα were performed between days 3 and 5 (grey shaded area). N=15 per control/treated group.

In summary, G2 *tert*$^{-/-}$ larvae is a valid model to rapidly validate if specific candidate pathways act downstream of short telomeres to disrupt homeostasis. These mechanisms may prove to be conserved in a short-telomere context of natural aging.

4.2.4 – G2 *tert*$^{-/-}$ early death is rescued by acute treatments with antioxidants

Our gene expression profiling study revealed that one of the mechanisms by which short telomeres may cause tissue dysfunction in aging is by repressing glutathione metabolism, a key oxidative defense system (Table 4.1). Accordingly, G1 *tert*$^{-/-}$ mutants display higher levels of ROS already by 6 months of age in the testis and gut and by 12 months in the muscle, in agreement with previous reports in telomerase knockout mice (15). Moreover, these tissues show reduced expression of Sod-2, a mitochondrial superoxide dismutase essential for ROS detoxification (Inês P. Castro and Miguel G. Ferreira, personal communication). Thus, G1 *tert*$^{-/-}$ zebrafish tissues are subject to higher levels of oxidative stress, potentially due to the reduced expression of Sod-2 and glutathione. Coincidently, telomeres are known to be particularly sensitive to ROS-induced damage (19, 20). These observations raised the hypothesis that stimulating oxidative defense mechanisms could rescue to some extent the defects caused by lack of telomerase.

We decided to test this hypothesis using the G2 *tert*$^{-/-}$ setting. We started by assessing if G2 *tert*$^{-/-}$ larvae anticipated the defects in oxidative protection observed in G1 *tert*$^{-/-}$ tissues. Live embryo staining using the cell-permeant 5-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA), indicated G2 *tert*$^{-/-}$ larvae had significantly higher ROS levels compared with WT controls, from day 3 onwards (Figure 4.8A-B). Levels of ROS correlated directly with the severity of developmental abnormalities observed in G2 *tert*$^{-/-}$ (Figure 4.8D). Thus, G2 *tert*$^{-/-}$ larvae anticipate the abnormal production of ROS observed in G1 *tert*$^{-/-}$ zebrafish. In addition, Sod-2 levels were also reduced in G2 *tert*$^{-/-}$, and again this reduction directly correlated with the severity of developmental defects (Figure 4.8C).

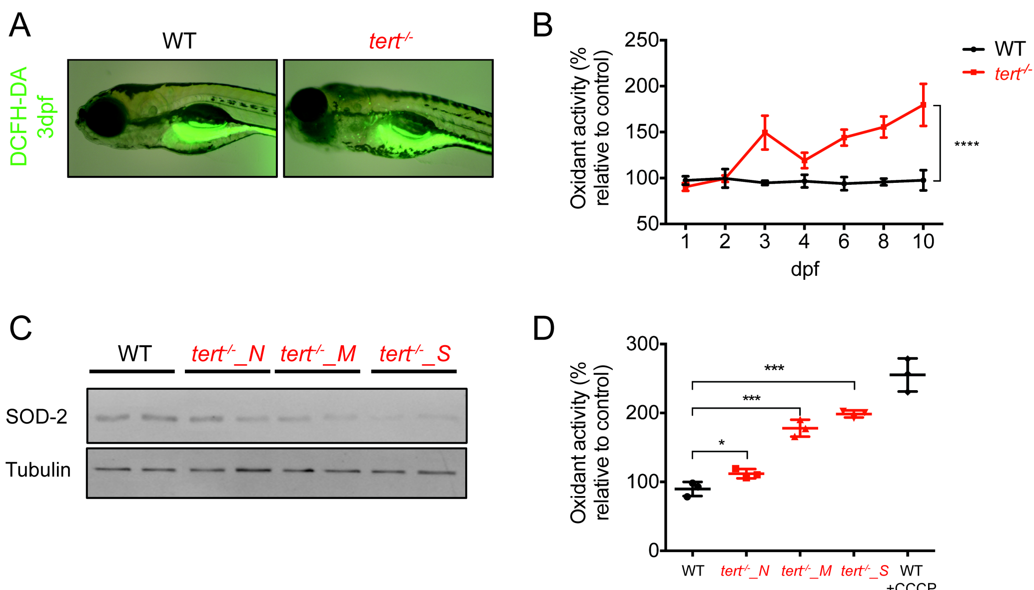


Figure 4.8 – G2 recapitulate the changes in oxidative protection of G1 *tert*$^{-/-}$ zebrafish.
A) Representative live imaging of 5-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (DCFDA) staining in G2 larvae and B) quantification of ROS levels from days 1 to 10 post fertilization. *tert*$^{-/-}$ have higher levels of ROS from day 3 onwards. N=3 pools (of 3 larvae each) per genotype per time point. C) Concomitantly Sod-2 expression levels are reduced in direct correlation with the severity of developmental abnormalities. *tert*$^{-/-}$_N – (“Normal”, WT-like); *tert*$^{-/-}$_M (“mildly affected”); *tert*$^{-/-}$_S (“severely affected”, (61)). N=2 pools (of 3 larvae each) per group. D) ROS levels (at day 7-post fertilization) also correlate with the severity of developmental defects in G2 *tert*$^{-/-}$. WT larvae treated with carbonyl cyanide m-chlorophenylhydrazone (CCCP) were used as positive control. N=3 per genotype per group (this entire figure was produced by IPC).

Therefore, we tested whether antioxidant treatments would rescue G2 *tert*$^{-/-}$ defects. We started by assessing if treatments with N-acetyl-L-cysteine (NAC), an effective precursor of L-cysteine that is necessary for *de novo* glutathione (GSH) synthesis (63), would influence the lifespan of G2 *tert*$^{-/-}$ larvae. Dietary uptake of NAC in other species such as *Drosophila*, was shown to increase WT medium lifespan by 26% (64). To ensure the variability in G2 *tert*$^{-/-}$ survival (Figure 4.6B) would not be a confounding factor when measuring drug treatment outcomes, we compared only control and test groups composed of progeny of

sibling parents. We did not detect differences between survival of G2 *tert*$^{-/-}$ and G2 *tert*$^{-/-}$-NAC treated larvae with chronic treatments (Figure 4.9A). However, when NAC was added during a specific time window, between days 5 and 10 post-fertilization, it significantly rescued the lifespan defect of G2 *tert*$^{-/-}$ larvae by ca. 39% (Figure 4.9B).

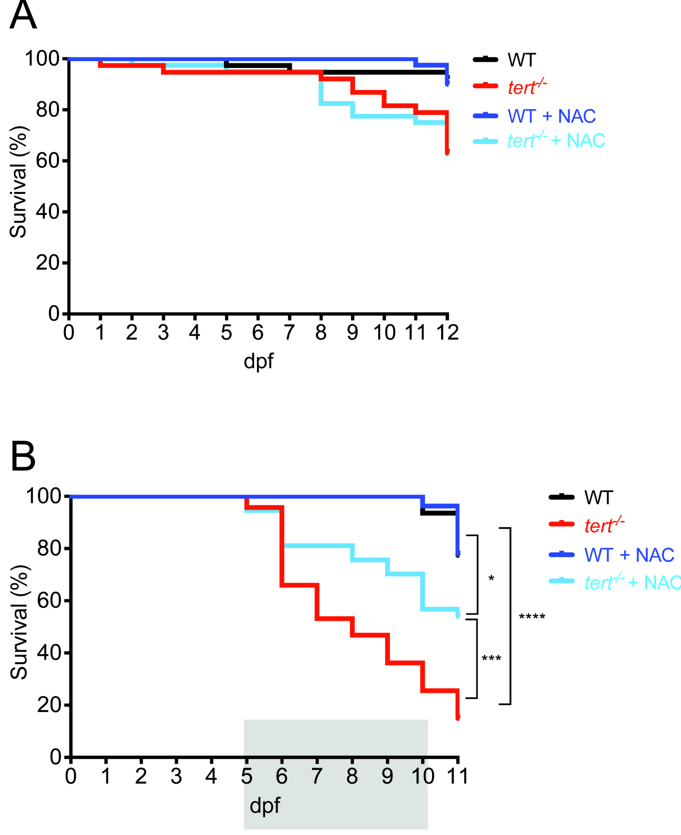


Figure 4.9 – ROS scavenger N-acetylcysteine (NAC) rescues the short lifespan of G2 *tert*$^{-/-}$. A) Chronic treatments with NAC do not rescue the reduced survival of G2 *tert*$^{-/-}$. N=40 each control/treated group. However, B) acute treatments performed between days 5 and 10 rescue G2 *tert*$^{-/-}$ survival. N=25-50 each control/treated group (two independent trials were performed).

Given these results, and because increased ROS levels in G2 *tert*$^{-/-}$ also associated with decreased expression levels of Sod-2 (Figure 4.8C), we tested if a superoxide dismutase mimetic would be sufficient to extend G2 *tert*$^{-/-}$

survival. For this effect, we treated G2 larvae with MitoTEMPO, a superoxide dismutase mimetic that contains a triphenylphosphonium moiety for mitochondria-targeting. Once more, we could not detect significant changes in survival of MitoTEMPO chronically treated larvae (Figure 4.10A), but found that acute treatments significantly extended G2 *tert*$^{-/-}$ survival (Figure 4.10B). Specifically, MitoTEMPO added to larvae media between days 3 and 5 significantly improved G2 *tert*$^{-/-}$ survival (by 32%). When added between days 5 and 10 (same period where we previously saw a rescue with NAC treatment) it had an even more robust effect of 45% increase in *tert*$^{-/-}$ survival (Figure 4.10B).

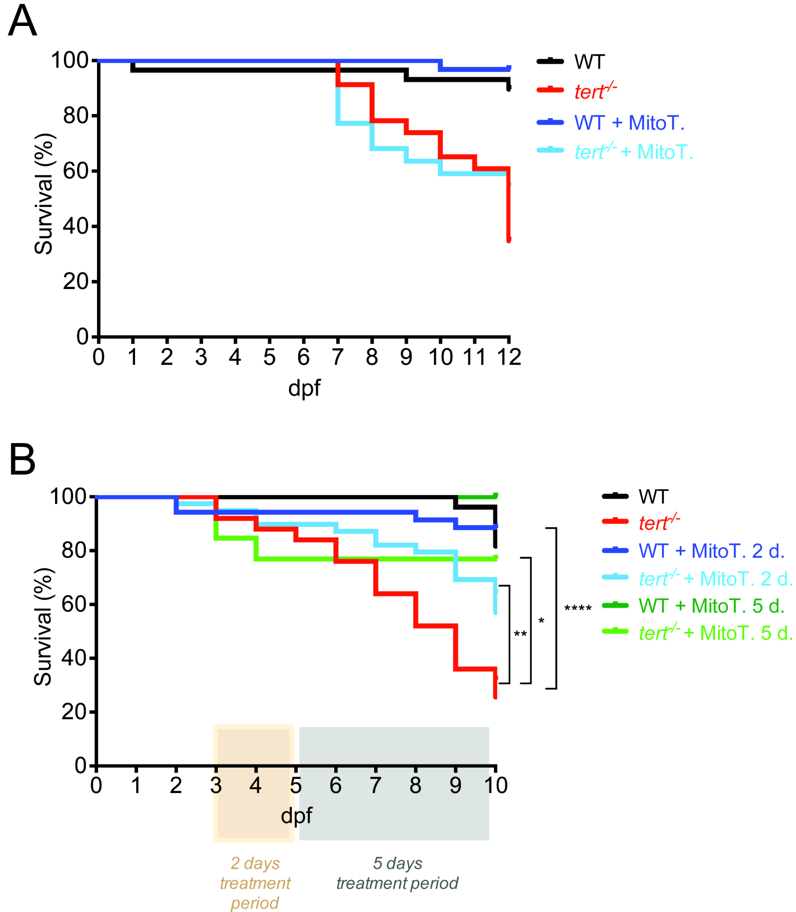


Figure 4.10 – Superoxide dismutase mimetic MitoTEMPO rescues the short lifespan of G2 *tert*$^{-/-}$.

A) Chronic treatments with MitoTEMPO do not rescue the reduced survival of G2 *tert*$^{-/-}$ (in agreement with previous observations for NAC, figure 4.9). N=30 each control/treated group. However, B) acute treatments with superoxide dismutase mimetic MitoTEMPO, from days 3 to 5 (two independent trials, N=25-40 each control/treated group) or from days 5 to 10 (two independent trials, N=12-15 each control/treated group) both rescue G2 *tert*$^{-/-}$ survival.

Our data shows that the severe defects in homeostasis and lifespan caused by telomerase absence are intimately linked with changes in the oxidative status of the organism, which in turn may partially rely in defective glutathione metabolism or low expression of Sod-2. Antioxidant treatments, when performed within a specific time window, rescue the survival defects triggered by extremely short telomeres. These findings further extend our understanding of the telomere-oxidative defense axis and provide important insights on how antioxidant treatments (with compounds currently used in the clinic) may prove useful in the context of delaying or ameliorating age-related pathologies.

4.2.5 – Aging and telomerase depletion trigger independent transcriptomic signatures

As previously mentioned, transcriptional changes found at the intersection of *tert*$^{-/-}$ and WT time courses ($TC_{WT} \cap TC_{tert-/-}$) represented a minority of the overall DEGs found in each case separately. This indicates that many responses of aging are potentially telomere-independent and that *tert*$^{-/-}$ tissues may bear alterations in biological processes that do not recapitulate those of aging. This observation prompted us to identify the main changes in transcription that were characteristic of either scenario alone: lack of telomerase or aging.

Changes in gene expression that were specific to *tert*$^{-/-}$ or WT profiles, but excluded from their intersection ($TC_{WT} \cap TC_{tert-/-}$), are hereafter designated as "*tert*$^{-/-}$ -specific" or "WT-specific". Number of DEGs in *tert*$^{-/-}$-specific profiles was highest in testis (N=3105), as previously hinted by the respective abnormal clustering pattern (Figure 4.1), and lowest in muscle (N=78). Notably, when

compared with WT-specific profiles in matching tissues, *tert*$^{-/-}$-specific responses showed a 10-fold increase of DEG in the testis (*tert*$^{-/-}$-specific=3105; WT-specific=340) and a 10-fold decrease in the muscle (*tert*$^{-/-}$ -specific=78; WT-specific= 713). *tert*$^{-/-}$- and WT-specific changes in the gut were of the same order of magnitude (*tert*$^{-/-}$-specific=209; WT-specific=475).

tert$^{-/-}$ -specific gut responses showed transcriptional activation of several immune response pathways, including the complement and toll-like receptor cascades, and JAK/STAT signaling. In addition, we found significant upregulation of genes involved in metabolism of lipids and lipoproteins (cholesterol and bile acids/salts biosynthesis, arachidonic acid metabolism and PPAR signaling), metabolism of aminoacids (taurine and hypotaurine metabolism, valine leucine and isoleucine degradation) and hemostasis (platelet activation, Appendix Table 1). Accordingly, telomere length changes were shown to directly affect the human transcription of C1S, a member of the complement cascade (65). The minority of downregulated genes overlapped in the pathway of solute carrier (SLC)-mediated transmembrane transport of small molecules (particularly ions and aminoacids, Appendix Table 1).

Similarly to the gut, *tert*$^{-/-}$ -specific testis profiles showed activation of the complement cascade and hemostasis responses (platelet activation), but also revealed elevated profiles of cytokine signaling (specifically by interleukins, Appendix Table 2). Upregulation of interleukin signaling (eg. IL-1, IL-2 and IL-6 receptors) is a typical feature of “senescence associated secretory phenotypes” (66), a phenotype likely to be found in the environment of *tert*$^{-/-}$ tissues where senescent cells accumulate (57). Surprisingly, only testis and not gut *tert*$^{-/-}$ -specific profiles showed an obvious downregulation of telomere maintenance genes (with repression of shelterin components POT1 and TRF1), cell cycle regulation (mitosis, meiosis, checkpoint signaling) and surprisingly, DNA repair (homologous recombination, mismatch repair, base and nucleotide excision repair pathways, Appendix Table 2). Checkpoint signaling processes

(particularly ATR activation in response to replication stress) and DNA repair pathways were also downregulated, contrary to what would be expected of short telomere signaling (67, 68). In addition, we observed upregulation of a series of genes involved in the IGF and mTOR pathways (Appendix Table 2), contrary to what was recently reported in telomerase deficient mice (16).

Finally, the 78 DEG present in *tert*$^{-/-}$-specific muscle profiles did not significantly overlap in any pathway. Thus, contrary to gut and testis, *tert*$^{-/-}$ -specific changes in the muscle may mostly associate with anticipation of aging phenotypes.

WT –specific profiles showed a more homogeneous pattern of differential expression across different tissues. Genes involved in telomere maintenance (packaging), meiosis, CENPA deposition at the centromere, bile acid/salt biosynthesis, arachidonic acid metabolism, pyrimidine metabolism, glutathione metabolism and transcription of pol I and III were mostly downregulated in all three tissues (Appendix Tables 3-5). When looking at a higher (and less specific) level of biological processes, we also found a common activation of cytokine signaling, aminoacid metabolism, carbohydrate metabolism and extracellular matrix organization (Appendix Tables 3-5). Notably, WT-specific muscle profiles showed overrepresentation of cell cycle regulators and apoptotic genes, contrary to what would be expected of a low turnover tissue.

Intriguingly, strong signatures previously found enriched at the intersection of *tert*$^{-/-}$ and WT profiles ($TC_{WT} \cap TC_{tert-/-}$), such as IFN-I pathway, glutathione metabolism and platelet activation (Table 4.1), prevailed overrepresented in at least 2 of the 3 WT-specific tissues (Appendix Tables 3-5). As WT-specific profiles rely solely on DEGs excluded from the intersection of *tert*$^{-/-}$ and WT aging profiles ($TC_{WT} \cap TC_{tert-/-}$), this strongly suggests that other important factors may cooperate with signals of short telomeres to regulate these pathways in aging.

4.3 – Discussion

Genetic models of telomerase deficiency have been essential tools for uncovering the molecular processes that determine how short telomeres induce tissue defects in vertebrates. Established examples of such processes include p53-dependent apoptosis and senescence (14). However, emerging evidence now reveals that alternative regulation of metabolic pathways may also dictate how telomere dysfunction impacts homeostasis and lifespan (15, 16). These studies show that a broader understanding of the molecular pathways that connect short telomeres to disruption of local physiology and reduced organism survival is warranted. More importantly, knowing how much of this mechanistic basis is conserved from extreme telomere dysfunction to natural aging scenarios remains a crucial question.

We previously showed that telomere shortening and accumulation of DDR in key tissues initiates local and systemic dysfunction events, which are characteristic of zebrafish aging (Chapter 3). We now identify the molecular responses that may be at the basis of this relation, by assessing which are the common transcriptional alterations of *tert*$^{-/-}$ and WT aging tissues and whether they have functional meaning in dictating short telomere defects. The intersection of *tert*$^{-/-}$ and WT aging transcriptomes reveals prominent repression of genes involved in glutathione metabolism. Together with higher levels of ROS and decreased Sod-2 expression, this shows that telomere shortening compromises oxidative defense mechanisms in zebrafish aging. As superoxide dismutases were proposed to synergize with GSH antioxidant function (69), repression of both pathways in short telomere contexts could be interconnected. In agreement with our data, studies in mice with dysfunctional telomeres report the same accumulation of ROS in different tissues and further suggest this relies on a cycle of p53 hyperactivation and defective mitochondrial biogenesis (15). We now further substantiate this telomere-oxidative stress correlation. Our

functional studies with two independent ROS scavengers, NAC and MitoTEMPO, provide evidence that defective ROS detoxification mechanisms are responsible (at least in part) for the loss of homeostasis and reduced lifespan caused by short telomeres. Intriguingly, the specific elimination of superoxides, by MitoTEMPO, is sufficient to rescue the lifespan of G2 *tert*$^{-/-}$. By day 10 of MitoTEMPO treatment, survival improves by 45%, having comparable effects to those of NAC (39%, Figure 4.10). Given the approximate rescue in survival by both compounds, and considering NAC is a general scavenger of oxidative species (70), our data strongly suggests the majority of ROS generated by telomere erosion are superoxides. As MitoTEMPO predominantly (but not exclusively) accumulates within the mitochondria, its effects on extension of lifespan could be largely due to prevention of specific mitochondrial oxidative damage. In accordance, targeting of other antioxidants to the mitochondria of human fibroblasts under oxidative stress counteracts telomere shortening and prolongs their replicative lifespan (71). It remains to be tested whether antioxidant treatments in G2 *tert*$^{-/-}$ larvae also prevent telomere erosion. Confirming the exact nature of damaging oxidative species that emerge upon telomere dysfunction and where they accumulate in the cell could provide important clues for selecting more effective therapies. Elevated ROS levels, either due to decreased mitochondrial function (15) or to persistent activation of DDR and CDKN1A (22), may preferentially damage telomeres (17, 18) but also fuel long-term maintenance of a cycle of generalized genome damage that culminates in cell cycle arrest (22). Thus, future experiments should directly test whether the rescue of telomere-induced damage by antioxidant treatments is attributable to a reduction in telomere attrition or rather to attenuation of generalized genome damage.

Another interesting observation is that the timing and duration of antioxidant treatments determine the dimension of their beneficial impact in rescuing short telomere defects. Specifically, longer treatments with MitoTEMPO performed

later in life (days 5 to 10), after ROS peaks have been reached, have an additional benefit of 13% increase in survival by day 10, in comparison to earlier and shorter treatments (days 3 to 5). In addition, chronic treatments with either NAC or MitoTEMPO do not improve G2 *tert*$^{-/-}$ survival (Figures 4.9 and 4.10). Accordingly, treating dysfunctional telomere mice with antioxidants from birth fails to rescue their hematopoietic stem cell defects (15). Thus, it may be that depleting ROS too early in development becomes disadvantageous for survival. Indeed, increasing evidence points towards ROS having essential signaling roles in early development (72), in stimulation of proliferation (73) and in zebrafish tissue regeneration (74). Mild increases in superoxide generation (by weak treatments with pro-oxidants) even prolong lifespan in *C. elegans* (75). Thus, the translation of antioxidant therapies to short telomere contexts of aging has to take into account the fine balance between pro-survival signals of mild ROS and detrimental damage that is generated when ROS levels go beyond a certain homeostatic threshold (76).

Our study focuses on finding the mechanisms that link short telomeres to aging phenotypes, by identifying the common differential expression patterns in *tert*$^{-/-}$ and WT aging profiles. We acknowledge however, that these patterns may represent a minority in either *tert*$^{-/-}$ or WT aging transcriptomes. Large amount of gene expression changes that do not necessarily reflect short telomere signaling would be expected of aging profiles, as loss of physiological function with time is influenced by a multitude of factors that may act independently (1). Zebrafish aging transcriptomes recapitulate features of mammalian aging such as deregulation of the somatotrophic axis – growth hormone and insulin signaling, activation of cytokine proinflammatory signaling and activation of lipid biosynthesis – cholesterol and fatty acid metabolism ((47, 77, 78), Appendix Tables 3-5). Thus, zebrafish can be established as a vertebrate model to further study what is the contribution of these processes for human aging. We also find there are several transcriptional responses exclusive of *tert*$^{-/-}$ tissues and which

do not mimic zebrafish aging. This could be due to several reasons that distinguish *tert*$^{-/-}$ from WT: 1) *tert*$^{-/-}$ cells lack catalytic and also non-catalytic functions of telomerase (57, 61); 2) *tert*$^{-/-}$ mutants exhibit a much higher synchrony in onset of tissue defects and death (Chapter 3) and therefore potentially less heterogeneity in transcriptomic alterations in time; 3) *tert*$^{-/-}$ mutants die prematurely and show rapid loss of telomere sequences, perhaps limiting the window of action of adaptive compensatory mechanisms ((57); Chapter 3).

Finally, the transcription of telomere maintenance genes seems to be affected by the absence of telomerase or natural WT aging in particular ways for different tissues. Zebrafish aging is accompanied by downregulation of core histones of the H2B and H4 families, suggesting alterations in telomeric chromatin. This occurs in the gut and muscle, tissues where telomeres shorten to *tert*$^{-/-}$ levels, but also in testis, a tissue where most telomeres do not significantly shorten with age (Chapter 3). Previous studies also reported the structure of telomeric chromatin to be highly dynamic (79) and affected by telomere shortening (80), potentially due to changes in histone modification states (81). More surprising is the fact that in the absence of telomerase significant enrichment in telomere regulatory genes, checkpoint signaling and DNA damage responses occurs exclusively in *tert*$^{-/-}$ testis. A possible explanation for this could be that as the majority of WT zebrafish testis cells do not naturally shorten their telomeres to critical levels, artificially imposing such a burden in this tissue could trigger non-physiological responses. Accordingly, of the three tissues analyzed, the *tert*$^{-/-}$ testis shows the greatest number of transcriptional changes over time. Among differentially expressed genes, we find downregulation of shelterin components POT1 and TRF1, which regulate the recruitment of telomerase (82-84). Thus, as previously proposed for *S. Cerevisiae* (85) short-telomeres could trigger an adaptive compensatory response of inhibition of the transcription of negative regulators of telomerase in *tert*$^{-/-}$ testis. An alternative explanation could be that,

with telomere shortening, less binding of shelterin proteins potentially feedbacks to less transcription of *pot1* and *terf1*.

In summary, our work provides experimental evidence that the defects in homeostasis and survival caused by short telomeres are mediated by changes in the oxidative status of the organism. At the heart of these defects could be the diminished activity of glutathione metabolism, which is reminiscent of natural aging, together with elevated production of ROS and decreased expression of Sod-2. Antioxidant therapies, with compounds currently used in the clinic, rescue significantly the short lifespan of telomere dysfunctional organisms, opening the possibility for application of these therapies in natural aging scenarios.

4.4 – Materials and methods

4.4.1 – Ethics statement

All Zebrafish work was conducted according to National Guidelines and approved by the Ethical Committee of the Instituto Gulbenkian de Ciência and the DGAV (Direcção Geral de Alimentação e Veterinária, Portuguese Veterinary Authority).

4.4.2 – Zebrafish lines and maintenance

Zebrafish were maintained in accordance with Institutional and National animal care protocols. The telomerase mutant line $tert^{AB/hu3430}$, generated by N-Ethyl-N-nitrosourea (ENU) mutagenesis (Utrecht University, Netherlands; Wienholds, 2004), has a T→A point-mutation in the telomerase (*tert*) gene. $tert^{AB/hu3430}$ line is available at the ZFIN repository, ZFIN ID: ZDB-GENO-100412-50, from the Zebrafish International Re-source Center – ZIRC. The $tert^{hu3430/hu3430}$ homozygous mutant ($tert^{-/-}$) was obtained by incrossing our $tert^{AB/hu3430}$ ($tert^{+/-}$)

strain. WT siblings were used as controls. Genotyping was performed by PCR of the *tert* gene followed by sequencing. All experiments were performed in G1 animals produced by *tert*$^{+/-}$ incross or G2 animals produced by a *tert*$^{-/-}$ incross. Due to a male sex bias in our crosses that affected mostly *tert*$^{-/-}$ progeny, the majority of our data is restricted to males.

4.4.3 – RNA isolation and purification

WT and *tert*$^{-/-}$ male zebrafish were sacrificed in MS-222 (Sigma). Gut, testis and muscle were dissected under a stereoscope (N=10-15 for each time point: 3, 6, 9, 24 and 36 months for WT and 3, 6 and 9 months for *tert*$^{-/-}$). Whole organs were transferred to 1.5 ml tubes and homogenized for 20 seconds in 1 ml of cooled Trizol (Invitrogen) using a mortar and pestle. After incubating at room temperature for 5 minutes, 200 μl of chloroform were added. Samples were vortexed for 15 seconds and incubated 10 minutes at room temperature. After centrifugation at 12,000 g for 15 minutes at 4ºC, the aqueous phase was retrieved and added to 1x volume of isopropanol. RNA was left precipitating overnight at -20ºC. The supernatant was removed and the pellet washed with 70% Ethanol and dried at 37ºC for 15 minutes. RNA was re-suspended in 50μl of RNAse-free water and purified using an RNeasy MinElute Cleanup Kit (Qiagen). RNA quality control was performed in Agilent Bioanalyzer 2100 and only samples with RIN >= 8.5 were selected for further microarray processing. RNA was quantified in NanoDrop 1000. For each genotype and tissue, 3 biological pools of RNA samples were created per time point (N=3-5 per pool), making a total of 72 arrays. RNA was stored at -80°C until use.

4.4.4 – Microarray Hybridization

Total RNA was processed and hybridized on Zebrafish Gene 1.1 ST Array Strips (Affymetrix) using GeneChip WT PLUS Reagent Kit, according to

manufacturer's protocols. Briefly, 100 ng of each RNA pool, containing Poly-A RNA controls, was used to generate first-strand cDNA in a reverse transcription reaction. After second-strand cDNA synthesis, cRNA amplification was performed by *in vitro* transcription. cRNA was purified and 15 μg were used for a second cycle of first-strand cDNA synthesis. 5.5 μg of single stranded cDNA was subsequently fragmented and end-labelled. Distribution of sizes of final cDNA was determined using Agilent Bioanalyzer 2100 with a RNA 6000 Nano Assay. 3.5 μg of fragmented and end-labelled cDNA were added to 150 μl of a hybridization cocktail, containing hybridization controls (GeneAtlas Hybridization, Wash and Stain Kit). Hybridization was performed on array strips for 20 hours at 48ºC. Post hybridization washes with streptavidin and terminal labelling with biotin were performed using a GeneAtlas system, followed by scanning of the array strips.

4.4.5 – Microarray Data Analysis

Microarray data were analyzed using R 3.1.0 and Bioconductor (http://www.R-project.org/).

- **Differential expression**

Background adjustment, normalization and summarization of probes were performed using Robust Multi-array Averaging (RMA) within the "pdInfoBuilder" package (S Falcon). Identification of differentially expressed genes over time (WT only - from 3 to 36 months, and comparison of WT with *tert*$^{-/-}$ - from 3 to 9 months) was performed using the R packages "timecourse" (encompassing a multivariate empirical Bayes model) and "plyr". This method allowed us to identify the genes that are differentially expressed at any given time point either in relation to the 3-month old WT reference, for WT datasets, or in relation to WT age-matched controls, for *tert*$^{-/-}$ datasets, taking into consideration the variance in gene expression found over time. For single pairwise comparisons,

to determine gene expression differences between two time points, the R package “limma” with moderated *t*-statistic was used. Correction for multiple testing was done using the Benjamini & Hochberg method, by applying the package “multtest”. A p-value cut-off of 0.05 was used. Heatmaps of differential expression were built using the “gplots” package and considering 9 months wild-type samples as reference. Orthologous genes between zebrafish and human were identified using the Ensembl Compara database (www.ensemble.org). Gene Set Enrichment Analysis (GSEA) and pathway analysis were performed using human orthologous differentially expressed genes as input for the *MSIG database* (Broad Institute, http://www.broadinstitute.org/gsea), without cut-off for fold difference. Pathway analysis restricted to inputs of REACTOME and KEGG databases (51, 52). Pathways were selected based on a False Discovery Rate (FDR) < 0.05 and ordered by significance (p values considered the number of differentially expressed genes vs. total number of genes within a pathway). Due to the elevated number of *tert*$^{-/-}$ -specific and WT-specific DEG/enriched pathways, we focused our pathway analysis on the top 50 pathways of each database (REACTOME and KEGG; (51, 52)).

- **Dendograms and data clustering**

Hierarchical clustering of gene expression was performed using function *hclust* applied to a matrix of Euclidean distances between all WT and *tert*$^{-/-}$ microarrays (calculated with *dist*). The final tree (dendogram) showing the hierarchy of clusters was produced using function *hist*.

- **Gene behavior classification**

Changes in expression levels of each gene over time were manually cataloged in one of three clusters of behavior: 1) Continuous, “Co” (for genes showing continuous upregulation or downregulation over time), 2) Inflexion, “1 I” (for genes showing one inflexion point in their levels of expression over time) and 3) 1+ Inflexion, 1+I” (for genes showing more than one inflexion point in their levels of expression over time).

4.4.6 – Telomere restriction fragment (TRF) analysis by Southern blot

TRF analysis was performed as previously described (86). Briefly, genomic DNA was extracted from individual larvae sacrificed in MS-222 (Sigma) using lysis buffer (Fermentas K0512) supplemented with 1 mg/ml Proteinase K (Sigma, MO, USA) and RNase A (1:100 dilution, Sigma, MO, USA). Samples were incubated at 50ºC for 18 h in a thermomixer and genomic DNA was extracted by equilibrated phenol-chloroform (Sigma, MO, USA) and chloroform-isoamyl alcohol extraction (Sigma, MO, USA). Genomic DNA was quantified and normalized so the same amount of DNA was digested with RSAI and HINFI enzymes (NEB, MA, USA) for 12 h at 37ºC. Samples were electrophoresed on a 20 cm 0.6% agarose gel, in 0.5% TBE buffer, at 4ºC for 17 h at 110 constant voltage. A 1.6 kb telomere probe, (TTAGGG)n, labelled with [α-32P]-dCTP using the Prime-it II random primer labelling kit (Stratagene) was used for Southern blotting. N=4-5 WT and G2 *tert*$^{-/-}$ individuals.

4.4.7 – In vivo detection of cell death

Apoptosis/necrosis was measured *in vivo* as previously described (87). Briefly, larvae were anesthetized in MS-222 and incubated in a 3μg/mL acridine orange solution (Sigma) prepared in E3, for 30 minutes in the dark at 28ºC, and subsequently washed 4 times with E3 containing MS-222 anesthetic. Fluorescence was observed under a 488 nm wavelength, using a Zeiss Stereo Lumar V12.

4.4.8 – Live detection of intracellular oxidant activity

Reactive oxygen species (ROS) accumulation was assessed by measuring the levels of the oxidized form of the cell-permeant 5-chloromethyl-2’,7’-

dichlorodihydrofluorescein diacetate (DCFDA) (Sigma), as previously described (88). Briefly, larvae were euthanized with MS-222 and in pools of three homogenized in 100 ml of ROS buffer (0.32 mM sucrose, 20mM hepes, 1mM $MgCl_2$ and 0.5mM Phenylmethanesulphonyl fluoride). Homogenates were centrifuged and 20 μl of the supernatant was transferred to a 96-well plate and incubated in 1 μg/ml of DCFDA for 30 minutes. Fluorescence values were measured with a Victor 3 plate reader and normalized to total protein content. N=3 pools (of 3 larvae each) per genotype per time point.

Detection of ROS generation *in vivo* was performed as previously described (89). Briefly, 3 dpf larvae were anesthetized in MS-222 (Sigma) and incubated in a 5 μM solution of DCFDA (Sigma) prepared in E3, for 20 minutes in the dark at 28ºC, and subsequently washed 5 times with E3 containing MS-222 anesthetic (5 minutes each wash). Fluorescence was observed under a 488 nm wavelength, using a Zeiss Stereo Lumar V12.

4.4.9 – Immunoblot analysis

Larvae were euthanized in MS-222 (Sigma), frozen in dry ice and homogenized in pools of three in RIPA buffer (sodium chloride 150mM; Triton X-100 1%; sodium deoxycholate 0.5%; SDS 0.1%; Tris 50 mM; pH 8.0) including complete protease and phosphatase inhibitor cocktails (Roche Diagnostics). Extracts were incubated on ice for 30 minutes and centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was collected and added to 100 μl of sample buffer with β-mercaptoethanol. The samples were heated to 95°C for 5 minutes and loaded onto a 12% SDS-PAGE gel. After electroblotting the gel onto a PVDF membrane, incubation was performed overnight at 4°C with anti-Sod2 (Sigma SAB2701618) and anti-Tubulin (Sigma T 6074) in TBS-T with 5% milk powder (both 1:1000). An IRDye 800CW secondary antibody LI-COR was used (1:10000) and detection was performed in Odyssey. N=2 pools (composed

of 3 larvae each) per genotype per group – developmental defects were classified in groups as previously proposed (61)).

4.4.10 – Drug treatments in G2 *tert*$^{-/-}$ larvae

N-Acetyl-L-Cysteine (NAC) and MitoTEMPO were purchased from Sigma-Aldrich; Pifithrin-α (PFTα) was purchased from EMD Biosciences. Stock solutions were prepared in embryo medium (E3), for NAC and MitoTEMPO and in DMSO for PFTα. NAC was applied at 40μM (as previously described in (90)) between days 5 and 10 post fertilization. PFTα was applied at 1μM (as previously described in (62)) between days 3 and 5 post fertilization. MitoTEMPO was applied at 10μM (as previously described in (91)) in two periods: between days 3 and 5 and between days 5 and 10 post fertilization. Larvae were grown at 28ºC and over the incubation periods, replacement of medium with the abovementioned compounds was performed every day, between 3 and 7 PM. For experiments where the compounds were dissolved in DMSO, controls were treated with the correspondent dilution of the solvent. Each drug was tested in at least 2 independent trials.

4.4.11 – Statistical Analysis

Statistical analysis of microarray data was performed in R 3.1.0 (http://www.R-project.org/) as previously described. Comparison of WT vs. WT and *tert*$^{-/-}$ vs. *tert*$^{-/-}$ density distributions of gene expression distances was performed using a *Kolmogorov–Smirnov* test. Differences in the respective medians were assessed using a *Mann-Whitney U/Wilcoxon Rank Sum* test, since the distributions could not be considered normal, according to *Shapiro Wilks* test.

Statistical analysis of G2 characterization parameters and drug treatments was performed in *GraphPad Prism6*. Survival curves were compared using a Log-

rank (Mantel-Cox) test. A critical value for significance of $p<0.05$ was used throughout the study.

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Chapter 5 | General discussion and future perspectives

5.1 – General discussion

5.1.1 – A new vertebrate model to study the impacts of telomere shortening in aging

The consequences of telomerase absence in vertebrate organisms have been historically addressed in the common lab mouse. Telomerase knockout mice start off with long telomeres and require several generations of inbreeding to exhibit short telomere-dependent degenerative phenotypes (1, 2). This contrasts with the immediate premature aging and decreased lifespan observed in humans that carry mutations in genes essential for telomerase maintenance (3). We believe that there is a high demand for the use of alternative vertebrate models that, like humans, require telomerase for lifespan and tissue homeostasis.

This thesis fills this gap in the field of telomere biology.

We characterize a new vertebrate model, the telomerase mutant zebrafish (*tert*$^{-/-}$), showing that it prematurely develops several tissue degenerative phenotypes and suffers a drastic reduction in lifespan already in the first generation. This contrasting difference with the mouse model most probably relates to the fact that zebrafish has human-like length telomeres. Supporting this view, removing telomerase in other models with telomere lengths equivalent to humans (such as the wild-derived Cast/EiJ mouse and the turquoise killifish) also gives rise to first generation defects (4, 5). While the number of studies using the Cast/EiJ telomerase knockout model is only residual, the use of fish models has been growing and gaining further attention (6-10). Killifish has the great advantage of being the shortest naturally lived vertebrate model bred in lab conditions. However, telomerase deficiency does not impact its lifespan or embryo telomere length (4). It remains to be shown if the lack of telomerase

causes premature aging phenotypes due to telomere shortening during killifish adulthood.

Telomerase deficient models have been used to directly infer what is the role of short telomeres in aging-related disease. However, we still do not know the extent to which the artificial shortening of telomeres reproduces the responses elicited by natural telomere erosion in physiological aging. We have performed the first extensive longitudinal study to directly address this question. Our data shows that the majority of premature degeneration events of *tert*$^{-/-}$ tissues are a tight phenocopy of natural aging. This is obvious not only for highly proliferative tissues –intestinal inflammation/atrophy, infertility and anemia – but also for more quiescent organs – sarcopenia. Furthermore, as expected of a progeroid model, we observe that *tert*$^{-/-}$ exhibit a premature onset of several age-related diseases, such as cachexia, motor disabilities, higher propensity to infection and, unexpectedly, cancer. The absence of telomerase should work as a tumor suppressor mechanism by limiting cell proliferation (11), but our work shows this is not the case for zebrafish, as telomerase mutants develop full-blown cancers. This strongly suggests that zebrafish tumors are efficient in engaging alternative mechanisms of telomere length maintenance (ALT). It would be extremely interesting to determine which of the two telomere-lengthening mechanisms, ALT or telomerase activation, has a dominant prevalence in zebrafish tumourigenesis.

5.1.2 – Intrinsic telomere shortening in specific key tissues – a limiting factor for maintenance of homeostasis in aging

Telomeres shorten with each cell division (11). Therefore, proliferative tissues are expected to accumulate short telomere-dependent damage faster than more quiescent organs.

Unexpectedly, our work demonstrates that telomeres shorten both in high turnover (gut and kidney marrow) and low turnover organs (muscle), independently of tissue replication rates. In agreement, a recent study by Daniali and colleagues (12) shows that in humans, tissues such as muscle, fat, leukocytes and skin shorten their telomeres at an equivalent pace. This raises an important question. What determines the shortening of telomeres in tissues where proliferation is more limited? We have not explored the mechanisms underlying telomere erosion in low turnover zebrafish organs, but an obvious candidate to test would be ROS. ROS are known to cause several types of genotoxic damage, and to affect particularly G-rich DNA regions, including telomeres (13, 14).

While our data shows that telomeres shorten in most tissues, a different question is whether this shortening is sufficient to cause aging-related phenotypes. We have previously shown that *tert*$^{-/-}$ zebrafish shorten their telomeres to lengths that are sufficient to trigger tissue dysfunction (Chapter 2). Therefore we decided to ask if tissues where WT zebrafish telomeres reach *tert*$^{-/-}$ critical lengths also develop equivalent dysfunctional phenotypes.

We found that telomeres reach critical lengths specifically in the gut and muscle, possibly promoting a strong induction of DDRs. The accumulation of short telomeres and DDRs anticipates the onset of aging-associated dysfunction in these tissues, including intestinal inflammation and sarcopenia. Thus, our data suggests that short telomeres are a likely cause of gut and muscle dysfunction in natural zebrafish aging.

Curiously, although natural aging phenotypes in the testis and kidney marrow also resemble those of *tert*$^{-/-}$, WT telomeres in these tissues do not reach *tert*$^{-/-}$ critical lengths. This observation is puzzling and suggests that tissue communication triggered by systemic signals of short telomeres might contribute to age-associated dysfunction in these specific organs (see future perspectives).

5.1.3 – Short telomeres limit organismal homeostasis by repressing oxidative defense mechanisms

The work developed in this thesis goes from characterizing a new telomerase deficient vertebrate model to establishing it as a model of premature aging. In order to explore the molecular mechanisms by which short telomeres drive aging-related dysfunction, we performed the first unbiased transcriptional profiling study comparing the gene expression changes of *tert*$^{-/-}$ and WT tissues over time.

Several candidate pathways were identified. To test if modulating these pathways could prevent or delay the onset of telomere dysfunctional phenotypes, we implemented a new fast assay using second generation (G2) *tert*$^{-/-}$ larvae.

G2 *tert*$^{-/-}$ zebrafish have extremely short telomeres and display several developmental defects, dying within two to three weeks. In addition, G2 *tert*$^{-/-}$ recapitulate typical signaling elicited by short telomeres in G1 *tert*$^{-/-}$ zebrafish, including apoptosis, ROS and senescence. Importantly, genetic or chemical inhibition of p53 (when performed in an optimized time window) rescues to some extent the premature death of G2 *tert*$^{-/-}$ larvae, enabling the use of other pharmacological drugs in this setting. The use of such setting for high throughput testing of chemical compounds brings great challenges, namely establishing optimal windows of action and fine tuning the concentrations for each treatment. While we acknowledge that the consequences of telomere dysfunction during development may substantially differ from those in adulthood, testing chemical modulators in G2 larvae allows for a rapid filtering of candidates that may be worth pursuing in a laborious long-term adult assay.

This assay allowed us to quickly screen some of candidate pathways identified in our gene expression profiling study. We validated one of the most prominent pathways involving repression of key oxidative stress protection mechanisms.

We found that treatments with two independent antioxidants – general scavenger NAC or mitochondrial-targeted superoxide dismutase mimetic MitoTEMPO – within a specific time window, are sufficient to rescue the premature death of G2 *tert*$^{-/-}$. These results are extremely relevant to the telomere field, because they provide functional proof that short telomere-dependent dysfunction relies heavily in higher levels of oxidative stress, particularly mitochondria derived ROS.

Future studies may attest for the relevance of these antioxidant therapies, particularly of those already used in the clinic, in preventing or delaying short telomere-induced defects in natural aging. In addition, genetic experiments should determine whether overexpression of Sod-2 or glutathione S-transferases, and consequent lowering of ROS, is sufficient to slow down telomere erosion and rescue the dysfunctional phenotypes of adult *tert*$^{-/-}$ tissues.

5.2 Future perspectives

5.2.1 – Do *tert*$^{-/-}$ zebrafish degenerative phenotypes rely on non-canonical functions of telomerase?

Besides its telomere length maintenance role, telomerase has recently been described to regulate zebrafish myelopoiesis (10) and to have a wide range of other non-canonical functions in mammals that may impact tissue physiology (e.g. affects proliferation, apoptosis, mitochondrial function) (15). Thus, it is possible that part of the degenerative phenotypes in *tert*$^{-/-}$ zebrafish is caused by the absence of non-catalytic functions of telomerase rather than by telomeric loss. Future experiments should test this directly by comparing the phenotypes of a catalytic function-defective *tert* mutant zebrafish (described to act in a dominant-negative fashion against the endogenous *tert* (6)) with our characterized telomerase mutant strain. In addition, rescue experiments carried

by reactivation of telomerase in *tert*$^{-/-}$ tissues should also overexpress a catalytically inactive form of telomerase as control (6).

5.2.2 – Is telomere shortening in specific tissues sufficient to trigger systemic aging?

Our work shows that telomere shortening and accumulation of DDR in particular tissues (gut and muscle) anticipates the onset of aging associated dysfunction, namely inflammation and sarcopenia. Interestingly, *tert*$^{-/-}$ zebrafish recapitulate prematurely not only these phenotypes but also others that develop in tissues where telomeres do not naturally shorten to critical levels (such as testis and kidney marrow). Thus, it is tempting to speculate that systemic signaling of telomere shortening in natural aging, triggered by the gut and muscle, may promote the dysfunction of tissues where telomeres do not shorten, via non cell-autonomous mechanisms. Two complementary approaches may help clarify this is the future:

A) Assess whether telomerase deficiency targeted to the gut or muscle is sufficient to cause wide spread tissue dysfunction phenotypes characteristic of a complete telomerase null;

B) Test whether telomerase overexpression specifically in the gut or muscle (compared to ubiquitous overexpression) is sufficient to prevent local and systemic damage in aging.

Dominant effects of single tissues in aging have been previously reported in telomere-independent contexts, by studies showing that inhibition of IKK-β and NF-κB specifically in the hypothalamus or of insulin signaling specifically in neurons is sufficient to delay aging and increase lifespan in mice (16, 17). On the other hand, mouse telomere dysfunctional environments are dominant and inhibit thymopoiesis in transplanted hematopoietic stem and progenitor cells with long telomeres (18). It remains to be shown whether dominance effects of

natural telomere attrition in aging can also be found in specific tissues or microenvironments.

5.2.3 – Discovering new mediators of short telomere-induced defects in aging

p53- (and PUMA) dependent apoptosis and (CDKN1a-) senescence are established mediators of short telomere-induced degeneration of high turnover organs in late generation telomerase knockout mice (19, 20). However, the mechanistic basis underlying loss of homeostasis in the majority of other telomere dysfunctional tissues is still not entirely understood. Furthermore, deciphering whether these mechanisms are also relevant in contexts of natural aging is crucial for the development of targeted rejuvenation therapies.

The last part of this thesis introduces an unbiased transcriptional profiling study, comparing the gene expression changes occurring in *tert*$^{-/-}$ and WT tissues over time. This study provides a solid basis for the identification of new mechanisms that may dictate how short telomeres drive aging phenotypes. While still in a very preliminary stage, our study strongly suggests that various (to our knowledge) unreported alterations in molecular pathways are caused by telomere shortening, such as activation of type I interferon signaling and repression of monounsaturated fatty acid (MUFA) synthesis (among many others). As a first approach to validate if these alterations are meaningful for the loss of homeostasis in short telomere contexts, one may consider modulating these pathways using described chemical compounds in a G2 *tert*$^{-/-}$ setting (e.g. inhibiting IFN-I signaling using *fludarabine* or stimulating synthesis of MUFAs with *oleic acid*). Only the chemical or genetic manipulation of these pathways will determine whether their altered expression in aging and in short telomere contexts acts as a primary cause of loss of function or as a secondary bystander effect.

5.2.4 – Which molecular mechanisms for which telomere dysfunctional cells?

The identification of new molecular mechanisms regulating organismal homeostasis in response to short telomeres may have enormous implications for the future development of aging therapeutics. In tandem, this will create a growing demand for understanding the extent to which each individual mechanism contributes to the degeneration of single telomere dysfunctional tissues. Perhaps a key step to help clarify this involves escalating from the generalized perspective of a tissue to a detailed analysis at the cellular level. Delineating which cells or cellular characteristics (lineage, replication status, etc) will determine if pathway X or Y is activated upon telomere shortening, and defining how this affects cell maintenance and from there contributes to whole organ degeneration.

There is already compelling evidence that senescence and apoptosis checkpoints are typically elicited by telomere dysfunctional stem cells in intestinal, skin, brain and hematopoietic tissues (21), particularly when these cells are stimulated to progress from a quiescent to actively dividing state (22). However, how other individual cells cope with telomere-induced DDRs in these or other tissues remains a mystery. The G2 *tert*$^{-/-}$ zebrafish model may be an ideal experimental setting to help clarify this in an initial stage. Given that zebrafish larvae are transparent, the combination of transgenic zebrafish lines with fluorescent reporters for specific molecular players deregulated by short telomeres and for different cell types will allow monitoring the interplay between these two *in vivo*, as telomeres shorten.

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Appendix

Appendix Table 1 – GSEA of DEG in *tert*$^{-/-}$ -specific gut profiles

GSEA of DEG specific to *tert*$^{-/-}$ gut, and not present in $TC_{WT} \cap TC_{tert-/-}$ profiles (TC analysis, $p<0.05$), with input of two databases: REACTOME and KEGG. Differentially expressed genes in each pathway are indicated by the human orthologous gene symbol – orthologs between zebrafish and human were predicted using the Ensembl Compara database (www.ensemble.org).

Gene Set Name	Datab.*	p value	Genes
Metabolism	R		
Metabolism of lipids and lipoproteins	R	1.0E-16	
Lipid digestion, mobilization, and transport	R	9.3E-04	
Lipoprotein metabolism	R	2.1E-04	
HDL-mediated lipid transport	R	1.7E-03	ABCA1; LCAT
Fatty acid, triacylglycerol, and ketone body met.	R	7.0E-04	
PPARA Activates Gene Expression	R	7.6E-05	FDFT1; HMGCR; HMGCS1; CYP7A1; ABCA1
Cholesterol biosynthesis	R	4.5E-23	CYP51A1; FDFT1; HMGCR; HMGCS1; EBP; DHCR24; FDPS; IDI1; HSD17B7; SC5DL; SQLE; IDI2
Steroid biosynthesis	K	3.5E-13	HSD17B7; EBP; CYP51A1; DHCR24; FDFT1; SC5DL; SQLE
Arachidonic acid metabolism	K	1.8E-03	GGT1; GGT5; PTGES
Primary bile acid biosynthesis	K	2.0E-03	CYP7A1; CYP39A1
Metabolism of terpenoids and polyketides	K		
Terpenoid backbone biosynthesis	K	2.2E-11	ACAT2; HMGCS1; FDPS; HMGCR; IDI1; IDI2
Biological oxidations	R	2.0E-06	
Phase 1 - Functionalization of compounds	R	2.1E-04	
Cytochrome P450 - arranged by substrate type	R	6.1E-05	
Endogenous sterols	R	3.1E-05	CYP51A1; CYP7A1; CYP39A1

Gene Set Name	Datab.*	p value	Genes
Drug metabolism - other enzymes	K	6.1E-05	UPP2; CES1; CES5A; CES2
Phase II Conjugation	R		
Glutathione conjugation	R	1.2E-04	GGT1; GGT5; GGCT
Amino acid metabolism	K		
Valine, leucine and isoleucine degradation	K	8.1E-04	ACAT2; HMGCS1; IL4I1
Metabolism of other amino acids	K		
Taurine and hypotaurine metabolism	K	7.5E-04	GGT1; GGT5
Glutathione metabolism	K	1.2E-03	GGT1; GGT5; GGCT
Seleno amino acid metabolism	K	5.2E-03	GGT1; GGT5
Muscle contraction	R	1.2E-09	
Striated Muscle Contraction	R	1.3E-09	MYBPC1; MYH6; MYL1; TNNC2; TNNI2; TNNT2
Cardiac Muscle Contraction	K	4.5E-03	MYH6; MYH7; TNNT2
Immune System	R	1.4E-08	
Innate Immune System	R	3.0E-07	
Toll Receptor Cascades	R	1.5E-03	CTSL1; IRF7; SAA1; TLR5
Complement cascade	R	3.8E-09	
Initial triggering of complement	R	3.7E-05	C3; CFB; C4A
Regulation of Complement cascade	R	2.5E-05	C3; CFB; C4A
Adaptive Immune System	R	9.2E-05	
Class I MHC mediated antigen processing & presentation	R	6.5E-04	CYBB; NCF2; PSMB9; MRC1; SOCS3; ASB5
Antigen processing-Cross presentation	R	2.9E-04	CYBB; NCF2; PSMB9; MRC1
Cytokine signaling in immune system	R		
Interferon gamma signaling	R	2.3E-03	IRF7; SOCS3; IRF8
Interferon alpha/beta signaling	R	2.4E-03	IRF7; SOCS3; IRF8

Gene Set Name	Datab.*	p value	Genes
Leukocyte transendothelial migration	K	1.0E-05	MYLPF; CLDN15; CLDN11; ITGB1; NCF2; CYBB
Transmembrane transport of small molecules	R	6.2E-05	
SLC-mediated transmembrane transport	R	7.0E-05	
Amino acid and oligopeptide SLC transporters	R	5.2E-05	SLC7A9; SLC6A18; SLC1A1; SLC15A2
Transport of inorganic cations/anions and amino acids/oligopeptides	R	6.5E-04	SLC7A9; SLC6A18; SLC1A1; SLC15A2
Hemostasis	R	1.5E-04	
Platelet activation, signaling and aggregation	R	1.8E-03	
Response to elevated platelet cytosolic Ca2+	R	3.6E-05	FN1; ALDOA; SERPINA1; PLEK; SERPINF2
Environmental Information Processing	K		
Signaling molecules and interaction	K		
ECM-receptor interaction	K	5.2E-03	ITGB1; FN1; VTN
Signal transduction	R		
Signaling by GPCR	R		
Class A/1 (Rhodopsin-like receptors)	R		
Peptide ligand-binding receptors	R	1.2E-03	C3; C5; SAA1; BDKRB2; C3AR1
GPCR downstream signaling	R		
G alpha (i) signalling events	R	1.4E-03	C3; C5; SAA1; BDKRB2; C3AR1
Jak-STAT signaling pathway	K	3.6E-02	CSF3R; LIFR; SOCS3; IL13RA2
Calcium signaling pathway	K	6.6E-03	BDKRB2; EGFR; P2RX4; TNNC2
Metabolism of proteins	R		
Post-translational protein modification	R	1.2E-03	
O-linked glycosylation of mucins	R	1.1E-04	MUC5AC; MUC5B; B3GNT7; C1GALT1

Gene Set Name	Datab.*	p value	Genes
Transport and catabolism	K		
Lysosome	K	1.7E-03	CTSL1; MAN2B1; GM2A; PPT1
Cell motility	K		
Regulation of actin cytoskeleton	K	2.1E-03	BDKRB2; MYLPF; ITGB1; FN1; EGFR
Cellular community	K		
Tight junction	K	1.6E-06	MYLPF; CLDN15; CLDN11; MYH6; MYH7; MYH11; MYH7B
Focal Adhesion	K	1.6E-03	MYLPF; ITGB1; FN1; EGFR; VTN
Human diseases	K		
Cardiovascular diseases	K		
Viral myocarditis	K	2.5E-04	MYH6; MYH7; MYH11; MYH7B
Dilated cardiomyopathy	K	6.0E-04	MYH6; MYH7; ITGB1; TNNT2
Hypertrophic cardiomyopathy (HCM)	K	4.4E-04	MYH6; MYH7; ITGB1; TNNT2
Immune diseases	K		
Systemic lupus erythematosus	K	2.7E-05	C5; C6; C9; C3; C4A; C4B
Neurodegenerative diseases	K		
Prion diseases	K	4.1E-04	C5; C6; C9
Infectious diseases: parasitic	K		
Leishmania infection	K	3.4E-03	C3; ITGB1; NCF2

Appendix Table 2 – GSEA of DEG in *tert*$^{-/-}$ -specific testis profiles

GSEA of DEG specific to *tert*$^{-/-}$ testis, and not present in $TC_{WT} \cap TC_{tert-/-}$ profiles (TC analysis, p<0.05), with input of two databases: REACTOME and KEGG. Differentially expressed genes in each pathway are indicated by the human orthologous gene symbol – orthologs between zebrafish and human were predicted using the Ensembl Compara database (www.ensemble.org).

Gene Set Name	Datab.*	p value	Genes
Cell cycle	R	3.5E-82	
Cell cycle mitotic	R	6.2E-65	
Mitotic G1-G1/S phases	R	1.0E-19	
G0 and early G1	R	3.4E-09	
G1/S Transition	R	3.1E-15	PSMB7; PSMB9; PSMC1; PSMC5; PSMD4; PSMD5; CDK2; RPA1; RPA2; RPA3; POLE; PRIM1; PRIM2; MCM8; MCM5; MCM6; MCM7; CDC45; CDC6; ORC3; ORC5; DBF4; CDC7; RPA4; FBXO5; CDKN1B; CCNA1; CCNA2; CDK1; CCNB1; CKS1B; TFDP1; TYMS
S phase	R	1.7E-16	
Mitotic G2-G2/M phases	R	4.5E-18	
G2/M transition	R		
Centrosome maturation	R		
Recruitment of mitotic centrosome proteins and complexes	R	9.4E-16	PLK1; MAPRE1; PAFAH1B1; CKAP5; DYNLL1; CDK1; TUBA4A; CCNB1; HSP90AA1; CNTRL; FGFR1OP; NEDD1; NEK2; CETN2; PLK4; CEP164; AZI1; PCM1; PCNT; CENPJ; CEP76; CEP290; CEP70; OFD1; TUBGCP2; NUMA1

Gene Set Name	Datab.*	p value	Genes
Loss of Npl from mitotic centrosomes	R	5.6E-14	PLK1; MAPRE1; PAFAH1B1; CKAP5; DYNLL1; CDK1; TUBA4A; HSP90AA1; CNTRL; FGFR1OP; NEDD1; NEK2; CETN2; PLK4; CEP164; AZI1; PCM1; PCNT; CENPJ; CEP76; CEP290; CEP70; OFD1
M phase	R		
Mitotic prometaphase	R	6.5E-29	KIF2C; KIF2A; KIF18A; PPP2R5B; CDC20; STAG1; SMC3; CENPP; CENPN; CENPK; CENPH; CENPO; PLK1; MAPRE1; PAFAH1B1; CKAP5; XPO1; MAD2L1; BUB1B; AURKB; NDC80; NUDC; SGOL1; SKA1; NSL1; BIRC5; INCENP; APITD1; ERCC6L; ZWILCH; CDCA8; SPC25; BUB1; MIS12; CENPM; DSN1; CENPT; B9D2; NDEL1; NUF2; CENPL; KNTC1
M/G1 transition	R	2.1E-12	PSMB7; PSMB9; PSMC1; PSMC5; PSMD4; PSMD5; PSME4; CDK2; RPA1; RPA2; RPA3; POLE; PRIM1; PRIM2; MCM8; MCM5; MCM6; MCM7; CDC45; CDC6; ORC3; ORC5; DBF4; CDC7; RPA4
Regulation of mitotic cell cycle	R	3.1E-10	CDC20; PSMB7; PSMB9; PSMC1; PSMC5; PSMD4; PSMD5; PSME4; PLK1; MAD2L1; BUB1B; AURKB; CDK2; FBXO5; CCNA1; CCNA2; ANAPC10; ANAPC11; CDC27; CDK1; CCNB1; AURKA; CDC14A
Cell cycle checkpoints	R	5.7E-18	
G2/M checkpoints	R	1.9E-18	
Activation of ATR in response to replication stress	R	1.9E-15	CDK2; RPA1; RPA2; RPA3; MCM8; MCM5; MCM6; MCM7; CDC45; CDC6; ORC3; ORC5; DBF4; CDC7; RFC5; CHEK1; ATRIP; RAD1; RAD9A; RAD17
Chromosome maintenance	R	8.7E-25	
Telomere maintenance	R	3.5E-14	

Gene Set Name	Datab.*	p value	Genes
Extension of telomeres	R	1.0E-08	RPA1; RPA2; RPA3; POLE; PRIM1; PRIM2; RFC5; LIG1; POLD1; FEN1; RUVBL1; RUVBL2
Nucleosome assembly	R		
Deposition of new CENPA containing nucleosomes at the centromere	R	4.3E-13	CENPP; CENPN; CENPK; CENPH; CENPO; HIST3H2BB; HIST2H4B; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST1H4D; HIST1H4J; HIST1H4C; HIST1H4H; HIST1H4B; HIST1H2BJ; RUVBL1; OIP5; MIS18A; MIS18BP1; RBBP7; SMARCA5
Meiosis	R	5.2E-20	
Meiotic recombination	R	2.3E-14	CDK2; RPA1; RPA2; RPA3; HIST3H2BB; HIST2H4B; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST1H4D; HIST1H4J; HIST1H4C; HIST1H4H; HIST1H4B; HIST1H2BJ; RAD51C; BRCA2; RAD51; DMC1; SPO11; MLH1; MSH4; MSH5; RBBP8; BLM; TOP3A; MND1
Meiotic synapsis	R	1.0E-11	STAG1; SMC3; HIST3H2BB; HIST2H4B; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST1H4D; HIST1H4J; HIST1H4C; HIST1H4H; HIST1H4B; HIST1H2BJ; POT1; TERF1; SYCP2; STAG3; SUN2; SMC1B; SYCP3; SYCP1; REC8
DNA replication	R	8.6E-49	
M/G1 transition	R	2.1E-12	
DNA replication pre-initiation	R		
Activation of the pre-replicative complex	R	4.7E-15	CDK2; RPA1; RPA2; RPA3; POLE; PRIM1; PRIM2; MCM8; MCM5; MCM6; MCM7; CDC45; CDC6; ORC3; ORC5; DBF4; CDC7; RPA4
Synthesis of DNA	R	3.6E-17	

Gene Set Name	Datab.*	p value	Genes
DNA strand elongation	R	4.8E-14	
Unwinding of DNA	R	1.7E-08	MCM8; MCM5; MCM6; MCM7; CDC45; GINS2; GINS4; GINS1
Switching of origins to a post-replicative state	R		
Orc1 removal from chromatin	R	2.9E-08	PSMB7; PSMB9; PSMC1; PSMC5; PSMD4; PSMD5; PSME4; CDK2; MCM8; MCM5; MCM6; MCM7; CDC6; ORC3; ORC5; CDKN1B; CCNA1; CCNA2
DNA repair	R	2.1E-14	
Homologous recombination	K	2.3E-13	RAD51; BRCA2; POLD1; RPA4; RPA1; RPA2; RPA3; EME1; RAD54B; RAD51C; RAD52; BLM; TOP3A; XRCC3; MUS81; RAD54L
Mismatch repair	K	5.0E-11	MLH1; MSH6; MSH2; POLD1; RPA4; RPA1; RPA2; RPA3; LIG1; RFC1; RFC5; PMS2; EXO1
Base excision repair	K	2.7E-10	POLD1; LIG1; POLE3; POLE; POLE4; FEN1; UNG; PARP2; PARP1; APEX2; HMGB1; MUTYH; POLB; TDG; XRCC1
Nucleotide excision repair	K	1.2E-08	POLD1; RPA4; RPA1; RPA2; RPA3; LIG1; RFC1; RFC5; POLE3; POLE; POLE4; CETN2; ERCC1; GTF2H5; XPA
Programmed Cell Death	R		
Cell growth and death	K		
p53 signaling pathway	K	2.8E-07	CCND1; CDK2; CCND2; CDK1; CCNB1; CCNB2; CCNB3; GADD45A; CHEK1; CHEK2; SIAH1; SESN3; SESN1; CD82; PERP; SESN2; CCNG1
Apoptosis	R		
Apoptosis execution phase	R		
Apoptosis induced DNA fragmentation	R	4.0E-09	KPNA1; HMGB1; DFFA; H1F0; HIST1H1C; HIST1H1D; HIST1H1E; HIST1H1B; HIST1H1A

Gene Set Name	Datab.*	p value	Genes
Metabolism	R		
Metabolism of lipids and lipoproteins	R	3.4E-14	
Phospholipid metabolism	R	1.1E-08	
Glycerophospholipid metabolism	K	3.0E-05	PPAP2B; DGKA; DGKD; PLA2G12B; GPAT2; AGPAT3; AGPAT4; AGPAT9; CHPT1; PHOSPHO1; GPD1; LCAT; ACHE; ETNK1; PCYT1B
Biosynthesis of unsaturated fatty acids	K	1.9E-05	FADS2; YOD1; ACOT2; ACOT4; ELOVL2; ELOVL5; ELOVL6; TECR
Amino acid metabolism	K		
Tyrosine metabolism	K	5.6E-09	ADH1A; ADH1B; ADH1C; ADH4; ADH5; ADH6; ADH7; MAOA; MAOB; AOC2; AOC3; GOT2; TAT; GSTZ1; COMT
Phenylalanine metabolism	K	3.8E-05	MAOA; MAOB; AOC2; AOC3; GOT2; TAT; PAH
Glycine, serine and threonine metabolism	K	6.7E-07	MAOA; MAOB; AOC2; AOC3; CTH; SHMT2; DAO; GATM; ALAS2; SARDH; SRR
Carbohydrate metabolism	K		
Glycolysis/Gluconeogenesis	K	7.6E-09	ADH1A; ADH1B; ADH1C; ADH4; ADH5; ADH6; ADH7; PCK2; G6PC; TPI1; LDHAL6A; LDHA; LDHC; LDHAL6B; ACSS1; ALDOB; PGAM2; BPGM
Pyruvate metabolism	K	1.1E-05	PCK2; LDHAL6A; LDHA; LDHC; LDHAL6B; ACSS1; MDH2; GRHPR; PC; ACOT12; LDHD
Inositol phosphate metabolism	K	9.1E-06	PIK3CB; PIK3CD; PIKFYVE; PIP5K1A; PIP4K2A; PLCB3; PLCD3; TPI1; INPP5K; INPP5J; INPP5E; ALDH6A1; IPMK
Nucleotide metabolism	K		

Gene Set Name	Datab.*	p value	Genes
Purine metabolism	K	4.9E-06	POLD1; POLE3; POLE; POLE4; PRIM1; PRIM2; ADCY2; POLR2G; POLR3GL; NME6; NME7; NME4; NME5; ENTPD1; ADA; NPR2; ENPP1; GUCY2D; AK7; PDE11A; PDE2A; PDE4C; PDE6D; ALLC; PDE5A
Pyrimidine metabolism	K	1.2E-05	POLD1; POLE3; POLE; POLE4; PRIM1; PRIM2; POLR2G; POLR3GL; NME6; NME7; NME4; NME5; ENTPD1; DPYD; TYMS; TK1; UPP1; CDA
Immune System	R	3.0E-31	
Adaptive Immune System	R	1.0E-24	
Class I MHC mediated antigen process. & presentation	R	4.3E-11	
Antigen processing: ubiquitination & proteasome degrad.	R	2.0E-08	CDC20; PSMB7; PSMB9; PSMC1; PSMC5; PSMD4; PSMD5; PSME4; ANAPC10; ANAPC11; CDC27; SOCS3; CBLB; FBXO6; SAE1; UBE2E3; LRRC41; UBE2J2; ASB6; ASB7; UBR2; MKRN1; FBXO2; UBE2S; TRIM37; UBE2R2; UBE2W; UBE2B; UBE2H; ZBTB16; RNF34; CUL3; SPSB4; ASB16; FBXO44
MHC class II antigen presentation	R	1.4E-09	KIF2C; KIF2A; KIF18A; KIF20A; KIF23; DYNLL1; KIF4A; KIF4B; RACGAP1; KIFAP3; KIF26A; KIF11; KIF22; KIF15; KIF3B; SEC24D; HLA-DQA2; DYNLL2; CTSO; DYNC1I1; DYNC2LI1; DYNC2H1; DCTN5
Cytokine signaling in immune system	R	4.6E-08	
Signaling by ILs	R	1.1E-05	CDK1; PIK3R1; PIK3CB; VAV1; SYK; PIK3CD; SOCS3; RIPK2; STAT1; STAT5A; STAT5B; GAB2; SQSTM1; PTK2B; IL2RG; HCK; PELI1; IL6R; IL1R1

Gene Set Name	Datab.*	p value	Genes
Leukocyte transendothelial migration	K	3.6E-16	PIK3CB; PIK3CD; PIK3R5; PIK3R1; RAC1; CDC42; ITGB1; PRKCB; VAV3; VAV1; ROCK2; ACTN4; ITGA4; MYL9; MYL12B; MYL12A; ITGAM; ITGB2; EZR; VASP; RAP1B; MAPK11; GNAI2; PTK2B; CXCL12; CYBA; NCF2; NCF4; OCLN; F11R; JAM3; CLDN3; CLDN15; CDH5; CYBB
Fc gamma R-mediated phagocytosis	K	6.6E-13	PIK3CB; PIK3CD; PIK3R5; PIK3R1; RAC1; CDC42; PRKCB; VAV3; VAV1; PIKFYVE; PIP5K1A; WAS; ARPC5; SCIN; VASP; SPHK2; INPP5D; DOCK2; HCK; WASF3; MARCKSL1; DNM1L; ASAP3; SYK; PTPRC; PPAP2B; GAB2; PLA2G4F
Chemokine signaling pathway	K	5.3E-07	GSK3B; PIK3CB; PIK3CD; PIK3R5; PIK3R1; RAC1; CDC42; PRKCB; STAT1; NFKBIA; STAT5B; VAV3; VAV1; ROCK2; WAS; TIAM1; RAP1B; GNAI2; PTK2B; CXCL12; DOCK2; HCK; ADCY2; PLCB3; ARRB2; RASGRP2; FOXO3; CCR9; FGR; GSK3A
B cell receptor signaling pathway	K	2.1E-05	GSK3B; PIK3CB; PIK3CD; PIK3R5; PIK3R1; RAC1; PRKCB; NFKBIA; VAV3; VAV1; INPP5D; SYK; PPP3R1; NFATC2; BTK
Fc epsilon RI signaling pathway	K	4.0E-05	PIK3CB; PIK3CD; PIK3R5; PIK3R1; RAC1; PRKCB; MAPK10; VAV3; VAV1; MAPK11; INPP5D; SYK; GAB2; PLA2G12B; BTK
Transmembrane transport of small molecules	R	2.4E-19	
SLC-mediated transmembrane transport	R	5.1E-18	
Transport of inorganic cations/anions and amino acids/oligopeptides	R	2.7E-09	SLC7A9; SLC7A11; SLC3A2; SLC6A19; SLC6A6; SLC9A1; SLC38A2; SLC43A2; SLC1A4; SLC1A5; SLC3A1; SLC7A1; SLC38A5; SLC15A1; SLC9A6; SLC12A7; SLC24A4; SLC26A3; SLC24A3; SLC4A1; SLC20A1;

Gene Set Name	Datab.*	p value	Genes
			SLC20A2; SLC4A8
Transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds	R	8.4E-10	SLC16A1; SLC2A2; SLC6A19; SLC6A6; SLC2A1; SLC5A1; SLC22A2; SLC22A1; SLC13A5; SLC47A1; SLC13A1; SLC13A2; SLC2A6; SLC2A9; SLC2A10; SLC6A2; RHAG; SLC30A5; SLC30A8; SLC30A1; SLC40A1; SLC22A16; SLC5A2
Transport and catabolism	K		
Endocytosis	K	2.0E-05	CDC42; EGFR; FGFR2; CBLB; NTRK1; KIT; CSF1R; RET; PIKFYVE; PIP5K1A; KDR; FLT1; DNM1L; ASAP3; ADRB2; ERBB3; ARRB2; HSPA8; IL2RG; EHD1; IQSEC2; SH3KBP1; EHD4; EHD2; ACAP1; IQSEC1
Folding sorting and degradation	K		
Ubiquitin mediated proteolysis	K	1.4E-05	ANAPC10; ANAPC11; CDC27; FZR1; CDC20; CBLB; PIAS4; SIAH1; SOCS3; UBE2J2; SAE1; UBE2E3; UBE2QL1; FBXO2; UBE2S; TRIM37; UBE2R2; FANCL; UBE2W; UBE2B; UBE2H; CUL3
Hemostasis	R	6.4E-28	
Factors involved in megakaryocyte development and platelet production	R	5.4E-13	
Kinesins	R	6.8E-15	KIF2C; KIF2A; KIF18A; KIF20A; KIF23; KIF4A; KIF4B; RACGAP1; KIFAP3; KIF26A; KIF11; KIF22; KIF15; KIF3B; KIFC1; KIF9

Gene Set Name	Datab.*	p value	Genes
Platelet activation signaling and aggregation	R	1.7E-11	TUBA4A; CD36; RAC1; CDC42; PIK3R1; PIK3CB; VAV1; SYK; PRKCB; RASGRP2; RAP1B; CFD; RHOG; CAP1; COL1A1; TRPC6; TGFB1; PIK3R6; PIK3R5; VAV3; PDGFB; FN1; ACTN4; VEGFA; CLU; MMRN1; HABP4; BRPF3; SCG3; SERPINA1; PLEK; SERPINF2; SPARC; CD9; APBB1IP; P2RY1; DGKA; DGKD; GNAI2; ARRB2
Environmental Information Processing	K		
Signaling molecules and interaction	K		
ECM-receptor interaction	K	2.2E-05	ITGB1; ITGA6; FN1; ITGA4; ITGA1; ITGA5; ITGB4; THBS2; COL1A1; COL6A1; COL6A2; COL6A3, CD36; CD44; HMMR; SV2B
Neuroactive ligand-receptor interaction	K	1.2E-06	CHRM2; ADRB2; GRIN2A; AGTR1; EDNRA; GRIN2D; CHRNA7; HTR4; OXTR; P2RX1; TACR3; CCKAR; C3AR1; C5AR1; DRD2; LPAR1; TSHR; LPAR6; NMUR1; CHRNA2; DRD4; APLNR; GABRR2; GCGR; NPBWR1; UTS2R; LPAR4; GPR35; P2RY1; PTGER2; PTH1R; RXFP1; VIPR1; VIPR2; TRPV1; S1PR4; GLP2R
Cell adhesion molecules (CAMs)	K	2.8E-05	ITGB1; ITGA6; ITGA4; ITGAM; ITGB2; OCLN; F11R; JAM3; CLDN3; CLDN15; CDH5; PTPRC; PTPRF; PVRL2; HLA-DQA2; NLGN1; MPZ; NLGN2; CADM3; PVR; CD276
Membrane transport	K		
ABC transporters	K	3.0E-05	TAP2; ABCD3; ABCA10; ABCA9; ABCA8; ABCC2; ABCA13; ABCA6; ABCB1; ABCB4; ABCG2
Signal transduction	R		

Gene Set Name	Datab.*	p value	Genes
Signaling by SCF-KIT	R	2.0E-09	CDKN1B; CDK1; CHEK1; RAC1; PIK3R1; VAV1; FOXO1; FOXO3; TRIB3; BAD; GSK3A; GRAP2; STAT1; STAT5A; STAT5B; GAB2; CMA1; GRB10; KIT; SOCS6; GRAP
Signaling by Rho GTPases	R	5.2E-09	RAC1; CDC42; RACGAP1; VAV1; RHOG; VAV3; ARHGEF9; RHOC; ECT2; TIAM1; ARHGEF2; CHN2; RHOQ; HMHA1; ARHGAP30; ARHGDIG; MYO9B; GMIP; ARHGAP15; ARHGAP20; ARHGAP19; STARD8; ARHGAP11A; G3BP2; ARHGAP25
Calcium signaling pathway	K	1.6E-10	EGFR; PDGFRB; PRKCB; ERBB2; CHRM2; PTK2B; SPHK2; ADCY2; CALML6; ITPR1; PPP3R1; CAMK2G; PLCB3; PLCD3; ADRB2; GRIN2A; AGTR1; EDNRA; GRIN2D; CHRNA7; HTR4; OXTR; P2RX1; TACR3; CCKAR; CACNA1A; CACNA1H; ERBB3; ATP2A2; RYR2; CD38; ATP2A3; BST1; PPID; ATP2B1
Phosphatidylinositol signaling system	K	2.4E-07	PIK3CB; PIK3CD; PIK3R5; PIK3R1; PRKCB; PIKFYVE; PIP5K1A; PIP4K2A; INPP5D; CALML6; ITPR1; PLCB3; PLCD3; INPP5K; INPP5J; INPP5E; DGKA; DGKD
VEGF signaling pathway	K	2.4E-07	PIK3CB; PIK3CD; PIK3R5; PIK3R1; RAC1; CDC42; PRKCB; BAD; VEGFA; PTGS2; MAPK11; KDR; SPHK2; PPP3R1; NFATC2; PLA2G12B; HSPB1; SH2D2A
Wnt signaling pathway	K	5.4E-07	CCND1; GSK3B; CCND2; RAC1; PRKCB; MAPK10; DVL1; FZD4; FZD9; ROCK2; PPP3R1; CAMK2G; PPP2R5B; PLCB3; NFATC2; SIAH1; CSNK2A1; CSNK2A2; DAAM1; LRP5; SFRP1; SFRP2; TBL1X; FOSL1; RUVBL1; TBL1Y

Gene Set Name	Datab.*	p value	Genes
MAPK signaling pathway	K	5.4E-06	TGFB1; GADD45A; RAC1; CDC42; EGFR; PDGFRB; PDGFB; FGFR2; FGF1; FGF2; FGF7; FGF10; PRKCB; MAPK10; NTRK1; RAP1B; MAPK11 RPS6KA1; PPP3R1; CACNA1A; CACNA1H; NFATC2; PLA2G12B; HSPB1; ARRB2; RASGRP2; NTRK2; HSPA8; TNFRSF1A; CACNB1; IL1R1; RASGRP4; DUSP6; STMN1; PPM1B
ErbB signaling pathway	K	3.4E-05	GSK3B; CDKN1B; PIK3CB; PIK3CD; PIK3R5; PIK3R1; EGFR; PRKCB; MAPK10; ERBB2; BAD; CBLB; STAT5B; STAT5A; CAMK2G; ERBB3
Developmental biology	R	1.6E-17	
Axon guidance	R	2.1E-13	CDK1; HSP90AA1; KIF4A; KIF4B; RAC1; CDC42; ITGB1; PTPRC; VASP; RPS6KA1; RHOG; CAP1; COL1A1; TRPC6; ITGA5; RHOC; GPC1; COL6A1; COL6A2; COL6A3; ERBB2; ROCK2; MYL12B; MYL9; MYH11; MYH9; ITGA1; SEMA6D; SEMA4A; SPTBN1; EGFR; DLG3; EZR; CSNK2A1; CSNK2A2; SPTB; ANK3; SCN2A; SCN4A; KIAA1598; CACNB1; ABLIM1; TRPC4; RGMB; SIAH1; SLIT3; GFRA1; CACNA1H
Encondrine system	K		
PPAR signaling pathway	K	1.5E-06	RXRG; PCK2; FADS2; CD36; ACADM; ACSL4; CPT1A; PPARA; SCP2; CYP8B1; CYP27A1; LPL; SLC27A2; FABP2; SLC27A1; PLTP
Insulin signaling pathway	K	2.9E-07	GSK3B; PIK3CB; PIK3CD; PIK3R5; PIK3R1; MAPK10; BAD; FOXO1; CBLB; INPP5D; CALML6; PCK2; G6PC; INPP5K; PTPRF; SOCS3; PRKAG2; EIF4E; EIF4E1B; PRKAR1B; PRKAR2A; GYS2; FLOT2; RHOQ; LIPE

Gene Set Name	Datab.*	p value	Genes
Progesterone-mediated oocyte maturation	K	1.3E-12	CDK2; CCNA1; CDK1; CCNB1; CCNB2; ANAPC10; ANAPC11; CDC27; MAD2L2; MAD2L1; PLK1; BUB1; CCNB3; FZR1; CCNA2; PIK3CB; PIK3CD; PIK3R5; PIK3R1; MAPK10; HSP90AA1; MAPK11; GNAI2; ADCY2; RPS6KA1; CPEB1
Nervous system	K		
Neurotrophin signaling pathway	K	1.1E-05	GSK3B; PIK3CB; PIK3CD; PIK3R5; PIK3R1; RAC1; CDC42; MAPK10; BAD; NFKBIA; NTRK1; RAP1B; MAPK11; RPS6KA1; CALML6; CAMK2G; FOXO3; NTRK2; NGFR; RIPK2; MAGED1
Cell motility	K		
Regulation of actin cytoskeleton	K	2.0E-17	PIK3CB; PIK3CD; PIK3R5; PIK3R1; RAC1; CDC42; ITGB1; EGFR; PDGFRB; PDGFB; ITGA6; FN1; FGFR2; FGF1; FGF2; FGF7; FGF10; VAV3; VAV1; ROCK2; ACTN4; ITGA4; MYL9; MYL12B; MYL12A; ITGAM; ITGB2; EZR; ITGA1; ITGA5; ITGB4; PIKFYVE; PIP5K1A; WAS; ARPC5; SCIN; CHRM2; PIP4K2A; TIAM1; BAIAP2; MYH9; SLC9A1; IQGAP3; NCKAP1L; PFN3; ITGAD; ITGAE; ITGAX; SSH2; ARHGEF7
Cellular community	K		
Focal Adhesion	K	1.4E-14	CCND1; GSK3B; CCND2; PIK3CB; PIK3CD; PIK3R5; PIK3R1; RAC1; CDC42; ITGB1; EGFR; PDGFRB; PDGFB; ITGA6; FN1; PRKCB; MAPK10; ERBB2; BAD; VEGFA; PGF; VAV3; VAV1; ROCK2; ACTN4; ITGA4; MYL9; MYL12B; MYL12A; ITGA1; ITGA5; ITGB4; VASP; RAP1B; KDR; FLT1; THBS2; COL1A1; COL6A1; COL6A2; COL6A3; CAPN2; CAV1; CAV2
Adherens junction	K	4.8E-06	RAC1; CDC42; EGFR; ERBB2; ACTN4; WAS; BAIAP2; WASF3; PTPRF; CSNK2A1; CSNK2A2; PVRL2; SSX2IP; LMO7; PTPRB;

Gene Set Name	Datab.*	p value	Genes
			PTPRJ
Human diseases	K		
Pathways in cancer	K	7.9E-18	
Pancreatic cancer	K	1.0E-08	CCND1; TGFB1; PIK3CB; PIK3CD; PIK3R5; PIK3R1; RAC1; CDC42; EGFR; MAPK10; ERBB2; BAD; VEGFA; PGF; RAD51; BRCA2; STAT1; RALGDS; RALA
Prostate cancer	K	2.8E-06	CCND1; GSK3B; CDK2; CDKN1B; PIK3CB; PIK3CD; PIK3R5; PIK3R1; EGFR; PDGFRB; PDGFB; FGFR2; ERBB2; BAD; HSP90AA1; FOXO1; NFKBIA; CREB3L3
Colorectal cancer	K	1.8E-06	CCND1; GSK3B; TGFB1; PIK3CB; PIK3CD; PIK3R5; PIK3R1; RAC1; MAPK10; BAD; MLH1; MSH6; MSH2; RALGDS; BIRC5
Acute myeloid leukemia	K	6.1E-06	CCND1; CCNA1; PIK3CB; PIK3CD; PIK3R5; PIK3R1; BAD; STAT5B; KIT; STAT5A; JUP; CEBPA; RARA; ZBTB16
Infectious diseases: parasitic	K		
Leishmania infection	K	1.3E-05	TGFB1; ITGB1; PRKCB; STAT1; PTGS2; NFKBIA; ITGA4; ITGAM; ITGB2; MAPK11; CYBA; NCF2; NCF4; MARCKSL1; HLA-DQA2
Infectious diseases: bacterial	K		
Pathogenic Escherichia coli infection	K	2.5E-05	CDC42; ITGB1; ROCK2; EZR; WAS; ARPC5; OCLN; TUBA3D; TUBA8; TUBA4A; HCLS1; KRT18; ARHGEF2

Appendix Table 3 – GSEA of DEG in WT-specific gut profiles

GSEA of DEG specific to WT gut, and not present in $TC_{WT} \cap TC_{tert-/-}$ profiles (TC analysis, $p<0.05$), with input of two databases: REACTOME and KEGG. Differentially expressed genes in each pathway are indicated by the human orthologous gene symbol – orthologs between zebrafish and human were predicted using the Ensembl Compara database (www.ensemble.org).

Gene Set Name	Datab.*	p value	Genes
Cell cycle	R	1.3E-05	
Chromosome maintenance	R	8.4E-07	
Telomere maintenance	R	2.0E-07	
Packaging of telomere ends	R	5.5E-09	HIST3H2BB; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST2H4A; HIST1H2BJ
Deposition of new CENPA containing nucleosomes at the centromere	R	5.8E-08	HIST3H2BB; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST2H4A; HIST1H2BJ
Meiosis	R	5.5E-07	
Meiotic recombination	R	5.9E-07	HIST3H2BB; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST2H4A; HIST1H2BJ
Meiotic synapsis	R	9.6E-09	HIST3H2BB; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST2H4A; HIST1H2BJ; HSPA2
Programmed Cell Death	R		
Cell growth and death	K		
p53 signaling pathway	K	2.7E-04	CDK1; CDKN1A; GADD45G; BAX; DDB2
Metabolism	R		
Metabolism of lipids and lipoproteins	R	2.7E-12	
Lipid digestion, mobilization, and transport	R	5.6E-04	ABCA1; SCARB1; APOB; ABHD5
Fatty acid, triacylglycerol, and ketone body metabolism	R	1.2E-05	
PPARA Activates Gene Expression	R	2.5E-06	SULT2A1; CD36; NR1D1; ARNTL; CLOCK; ABCA1; FHL2; ME1
Phospholipid metabolism	R	4.3E-05	

Gene Set Name	Datab.*	p value	Genes
Glycerophospholipid biosynthesis	R		
Synthesis of PE	R	8.7E-05	LPIN1; CHKB; ETNK2
Sphingolipid metabolism	K	3.3E-04	GLA; ENPP7; ASAH2; SGPL1
Arachidonic acid metabolism	K	1.4E-03	GGT1; PLA2G3; CYP2J2; ALOX5
Primary bile acid biosynthesis	K	7.5E-03	CYP7B1; HSD3B7
Biological oxidations	R	2.8E-15	
Phase 1 - Functionalization of compounds	R	2.5E-05	
Metabolism of xenobiotics by cytochrome P450	K	2.5E-05	GSTM1; GSTM2; GSTM3; GSTM4; GSTM5; MGST1
Drug metabolism - other enzymes	K	6.3E-05	DPYD; UPB1; CDA; NAT2; NAT1
Phase II Conjugation	R	1.4E-11	
Glutathione conjugation	R	1.1E-06	GGT1; GSTM1; GSTM4; GSTM5; MGST1
Cytosolic sulfonation of small molecules	R	1.9E-04	SULT2A1; SULT1C2; PAPSS2
Amino acid metabolism	R	1.2E-07	
Amino acid synthesis and interconversion (transamination)	R	3.5E-04	GLS2; GLS; OAT
Tyrosine metabolism	K	5.0E-03	MAOA; MAOB; TAT
Phenylalanine metabolism	K	1.3E-05	MAOA; MAOB; TAT; PAH
Glycine, serine and threonine metabolism	K	2.7E-12	CTH; MAOA; MAOB; DAO; GATM; CBS; AGXT; AGXT2; ALAS2
Cysteine and methionine metabolism	K	2.7E-03	CTH; CBS; TAT
Alanine aspartate and glutamate metabolism	K	1.4E-04	AGXT; AGXT2; GLS2; GLS
Arginine and proline metabolism	K	6.0E-10	MAOA; MAOB; DAO; GATM; GLS2; GLS; ARG2; OAT; PRODH2
Metabolism of other amino acids	K		
Glutathione metabolism	K	1.8E-07	GSTM1; GSTM2; GSTM3; GSTM4; GSTM5; MGST1; GGT1
Seleno amino acid metabolism	K	5.8E-05	CTH; CBS; GGT1; PAPSS2
Carbohydrate metabolism	R	2.3E-04	
Pyruvate metabolism	K	4.4E-03	PCK1; PCK2; ME1
Fructose and mannose metabolism	K	2.7E-03	KHK; PFKFB1; PFKFB3

Gene Set Name	Datab.*	p value	Genes
Nucleotide metabolism	R	3.3E-04	
Pyrimidine metabolism	R	4.2E-05	
Pyrimidine catabolism	R	1.2E-04	AGXT2; DPYD; UPB1
Metabolism of cofactors and vitamins	K		
Retinol metabolism	K	1.9E-03	DHRS4; CYP26A1; DHRS4L2; DHRS3
Pantothenate and CoA biosynthesis	K	7.5E-03	DPYD; UPB1
Energy metabolism	K		
Nitrogen metabolism	K	5.3E-10	CTH; GLS2; GLS; CA2; CA13; CA1; CA3
Reversible hydration of carbon dioxide	R	2.1E-06	CA13; CA1; CA2; CA3
Immune System	R	3.3E-12	
Innate Immune System	R	1.2E-04	
RIG-I/MDA5 mediated induction of IFN-alpha/beta pathways	R	3.5E-04	
Negative regulators of RIG-I/MDA5 signaling	R	5.2E-06	UBA52; UBA7; HERC5; RNF125; NLRC5
Adaptive Immune System	R	3.1E-06	
Class I MHC mediated antigen processing & presentation	R	2.6E-04	
Antigen processing cross presentation	R	4.0E-05	CD36; B2M; UBA52; CALR; TAP2; CTSS
Antigen Presentation: Folding, assembly and peptide loading of class I MHC	R	2.4E-05	B2M; CALR; TAP2; HSPA5
Cytokine signaling in immune system	R	1.4E-10	
Interferon signaling	R	8.8E-10	B2M; UBA52; HLA-DQA1; HLA-DQA2; GBP2; GBP4; GBP6; GBP1; CIITA; UBA7; HERC5; KPNA2; IFIT3
Interferon gamma signaling	R	5.1E-08	B2M; HLA-DQA1; HLA-DQA2; GBP2; GBP4; GBP6; GBP1; CIITA
Intestinal immune network for IgA production	K	4.7E-05	HLA-DRA; HLA-DMB; HLA-DOA; HLA-DQA1; HLA-DQA2
Transmembrane transport of small molecules	R	7.9E-08	
SLC-mediated transmembrane	R	2.0E-09	

Gene Set Name	Datab.*	p value	Genes
transport			
Transport of inorganic cations/anions and amino acids/oligopeptides	R	1.2E-06	SLC6A19; SLC38A4; SLC43A1; SLC15A1; SLC26A3; SLC4A5; SLC26A6; SLC9A3
Transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds	R	7.7E-07	SLC6A19; SLC2A5; SLC2A12; SLC22A6; SLC22A8; SLC40A1; SLC39A5; SLC13A3
Environmental Information Processing	K		
Signaling molecules and interaction	K		
Cell adhesion molecules (CAMs)	K	5.2E-03	HLA-DRA; HLA-DMB; HLA-DOA; HLA-DQA1; HLA-DQA2
Cytokine-cytokine receptor interaction	K	6.9E-03	TNFRSF1B; GHR; IL10RB; OSMR; CCR7; TNFRSF9; TNFRSF14
Circadian clock	R	1.3E-08	
BMAL1:CLOCK, NPAS2 activates circadian gene expression	R	1.6E-08	NR1D1; ARNTL; CLOCK; CRY1; DBP; PER1; BHLHE40
Gene expression	R		
RNA polymerase I RNA Polymerase III and mitochondrial transcription	R	8.3E-06	
RNA polymerase I transcription	R	7.7E-07	
RNA polymerase I promoter clearance	R		
RNA polymerase I promoter opening	R	4.5E-08	HIST3H2BB; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST2H4A; HIST1H2BJ
Transcription	R	3.8E-04	HIST3H2BB; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST2H4A; HIST1H2BJ
Metabolism of proteins	R		
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	R	2.9E-04	IGFBP1; IGFALS; IGFBP2
Transport and catabolism	K		
Peroxisome	K	4.7E-04	DAO; AGXT; DHRS4; PMVK; PHYH
Encondrine system	K		
PPAR signaling pathway	K	2.7E-04	PCK1; PCK2; CD36; ME1; FABP7
Excretory system	K		

Gene Set Name	Datab.*	p value	Genes
Proximal tubule bicarbonate reclamation	K	2.7E-08	GLS2; GLS; CA2; PCK1; PCK2; SLC9A3
Adipocytokine signaling pathway	K	2.3E-04	PCK1; PCK2; CD36; TNFRSF1B; IRS2
Human diseases	K		
Cardiovascular diseases	K		
Viral myocarditis	K	3.2E-05	HLA-DRA; HLA-DMB; HLA-DOA; HLA-DQA1; HLA-DQA2; PRF1
Immune diseases	K		
Asthma	K	4.4E-06	HLA-DRA; HLA-DMB; HLA-DOA; HLA-DQA1; HLA-DQA2
Systemic lupus erythematosus	K	5.4E-14	HLA-DRA; HLA-DMB; HLA-DOA; HLA-DQA1; HLA-DQA2; C3; HIST3H2BB; H2BFWT; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST2H4A; HIST1H2BJ; H2AFY
Autoimmune thyroid disease	K	4.9E-06	HLA-DRA; HLA-DMB; HLA-DOA; HLA-DQA1; HLA-DQA2; PRF1
Allograft rejection	K	6.6E-07	HLA-DRA; HLA-DMB; HLA-DOA; HLA-DQA1; HLA-DQA2; PRF1
Graft versus host disease	K	1.2E-06	HLA-DRA; HLA-DMB; HLA-DOA; HLA-DQA1; HLA-DQA2; PRF1
Infectious diseases: parasitic	K		
Leishmania infection	K	2.9E-05	HLA-DRA; HLA-DMB; HLA-DOA; HLA-DQA1; HLA-DQA2; C3
Amyloids	R	9.6E-11	B2M; HIST3H2BB; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST2H4A; HIST1H2BJ; APCS; TTR
Endocrine and metabolic diseases	K		
Type I diabetes mellitus	K	1.6E-06	HLA-DRA; HLA-DMB; HLA-DOA; HLA-DQA1; HLA-DQA2; PRF1

Appendix Table 4 – GSEA of DEG in WT -specific testis profiles

GSEA of DEG specific to WT testis, and not present in $TC_{WT} \cap TC_{tert-/-}$ profiles (TC analysis, p<0.05), with input of two databases: REACTOME and KEGG. Differentially expressed genes in each pathway are indicated by the human orthologous gene symbol – orthologs between zebrafish and human were predicted using the Ensembl Compara database (www.ensemble.org).

Gene Set Name	Datab.*	p value	Genes
Cell cycle	R		
Chromosome maintenance	R	1.1E-04	
Telomere maintenance	R	7.2E-06	
Packaging of telomere ends	R	5.0E-07	HIST3H2BB; HIST2H4B; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H2BJ
Deposition of new CENPA containing nucleosomes at the centromere	R	2.8E-06	HIST3H2BB; HIST2H4B; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H2BJ
Meiosis	R	8.5E-05	
Meiotic recombination	R	1.6E-05	HIST3H2BB; HIST2H4B; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H2BJ
Meiotic synapsis	R	6.1E-06	HIST3H2BB; HIST2H4B; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H2BJ
Metabolism	R		
Metabolism of lipids and lipoproteins	R	1.9E-06	
Metabolism of steroid hormones and vitamin D	R	6.3E-05	
Steroid biosynthesis	K	2.3E-05	HSD11B2; AKR1D1; CYP17A1; HSD3B1; HSD3B2
Androgen biosynthesis	R	2.7E-05	CYP17A1; HSD3B1; HSD3B2
Arachidonic acid metabolism	K	3.0E-05	GPX6; GPX3; GPX5; ALOX5; PTGIS
Bile acid and bile salt metabolism	R	6.0E-04	
Primary bile acid biosynthesis	K	4.3E-03	

Gene Set Name	Datab.*	p value	Genes
Synthesis of bile acids and bile salts via 24-hydroxycholesterol	R	1.6E-03	CYP27A1; AKR1D1
Biological oxidations	R	2.6E-05	
Phase 1 - Functionalization of compounds	R	9.3E-04	
Cytochrome P450 - arranged by substrate type	R	2.8E-04	CYP17A1; CYP27A1; CYP26A1; PTGIS
Amino acid metabolism	R	3.6E-05	
Cysteine and methionine metabolism	K	1.2E-03	CTH; BHMT; IL4I1
Arginine and proline metabolism	K	2.1E-05	NOS1; GLUL; CKM; CKMT2; P4HA3
Metabolism of other amino acids	K		
Glutathione metabolism	K	3.6E-03	GPX6; GPX3; GPX5
Nucleotide metabolism	R	8.4E-05	
Synthesis and interconversion of nucleotide di- and triphosphates	R	2.1E-04	NME1; NME2; NME1-NME2
Pyrimidine metabolism	K	3.2E-03	PNP; NME1; NME2; NME1-NME2
Purine metabolism	K	4.7E-04	
Purine salvage	R	2.8E-03	PNP; AMPD1
Muscle contraction	R	1.5E-11	
Striated Muscle Contraction	R	1.3E-08	TPM3; VIM; MYL1; NEB; TNNC2; TNNI2
Smooth Muscle Contraction	R	1.6E-05	TPM3; ACTA2; ACTG2; CALD1
Immune System	R	2.1E-03	
Innate Immune System	R		
Complement cascade	K	2.4E-07	C8A; C1S; PLG; F5; SERPIND1; KNG1; PROC
Hemostasis	R		
Formation of Fibrin Clot (Clotting Cascade)	R	9.9E-04	KNG1; PROC; F5
Environmental Information Processing	K		

Gene Set Name	Datab.*	p value	Genes
Signaling molecules and interaction	K		
Cytokine-cytokine receptor interaction	K	1.4E-03	PRLR; CXCL12; CCR4; CCR8; IL10RB; INHBA; TNFRSF14
Signal transduction	R		
Signaling by GPCR	R		
Class A/1 (Rhodopsin-like receptors)	R	2.9E-03	
Peptide ligand-binding receptors	R	1.1E-03	KNG1; CXCL12; CCR4; CCR8; AGTR2; SST
GPCR downstream signaling	R		
G alpha (i) signalling events	R	4.1E-07	KNG1; CXCL12; CCR4; CCR8; AGTR2; SST; OPN3; CASR; ADCY8; RGS4
Signaling by NOTCH	R		
Pre-NOTCH Expression and Processing	R	2.5E-03	JUN; MOV10; ATP2A1
Circadian clock	K	2.8E-03	
BMAL1:CLOCK, NPAS2 activates circadian gene expression	R	1.4E-03	NR1D1; CCRN4L; BHLHE41
Gene expression	R		
RNA polymerase I RNA Polymerase III and mitochondrial transcription	R	1.1E-04	
RNA polymerase I transcription	R	1.9E-05	
RNA polymerase I promoter clearance	R		
RNA polymerase I promoter opening	R	2.3E-06	HIST3H2BB; HIST2H4B; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H2BJ
Transcription	R	2.0E-03	HIST3H2BB; HIST2H4B; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H2BJ
Metabolism of proteins	R		
Peptide hormone metabolism	R		
Peptide hormone biosynthesis	R		

Gene Set Name	Datab.*	p value	Genes
Glycoprotein hormones	R	2.4E-03	INHA; INHBA
Encondrine system	K		
Insulin signaling pathway	K	1.6E-03	INS; MKNK2; SOCS1; PYGM; PPP1R3C
Excretory system	K		
Aldosterone-regulated sodium reabsorption	K	2.2E-03	HSD11B2; INS; KCNJ1
Cellular community	K		
Adherens junction interactions	R	6.0E-04	CDH1; PVR; CDH3
Extracellular matrix organization	R	2.1E-04	
Degradation of the extracellular matrix	R	2.9E-05	PLG; CTRB1; CTRB2; PRSS1
Cell-Cell communication	R		
Cell junction organization	R	1.4E-03	CDH1; PVR; CDH3; LAMC2
Human diseases	K		
Immune diseases	K		
Systemic lupus erythematosus	K	2.4E-07	C8A; C1S; HLA-DQA1; HIST3H2BB; HIST2H4B; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H2BJ
Neurodegenerative diseases	K		
Alzheimers disease	K	4.0E-03	NOS1; UQCRC1; ATP5J; ATP2A1; SNCA
Amyloids	R	2.4E-09	HIST3H2BB; HIST2H4B; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H2BJ; INS; APCS; SNCA
Endocrine and metabolic diseases	K		
Type I diabetes mellitus	K	2.5E-03	HLA-DQA1; INS; PRF1

Appendix Table 5 – GSEA of DEG in WT -specific muscle profiles

GSEA of DEG specific to WT muscle, and not present in $TC_{WT} \cap TC_{tert-/-}$ profiles (TC analysis, p<0.05), with input of two databases: REACTOME and KEGG. Differentially expressed genes in each pathway are indicated by the human orthologous gene symbol – orthologs between zebrafish and human were predicted using the Ensembl Compara database (www.ensemble.org).

Gene Set Name	Datab.*	p value	Genes
Cell cycle	R	3.5E-13	
Cell cycle mitotic	R	1.7E-07	
Regulation of mitotic cell cycle	R	2.6E-08	PSMA6; PSMB9; PSME4; CDC20; CDK1; CDK2; MAD2L1; BUB1B; PLK1; AURKB; CCNA2
Chromosome maintenance	R	1.1E-06	
Telomere maintenance	R	6.9E-09	
Packaging of telomere ends	R	4.4E-11	HIST3H2BB; HIST2H4B; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST1H4D; HIST1H4B; HIST1H4L; HIST1H2BJ
Deposition of new CENPA containing nucleosomes at the centromere	R	1.2E-09	HIST3H2BB; HIST2H4B; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST1H4D; HIST1H4B; HIST1H4L; HIST1H2BJ
Meiosis	R	7.9E-08	
Meiotic recombination	R	2.5E-09	HIST3H2BB; HIST2H4B; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST1H4D; HIST1H4B; HIST1H4L; HIST1H2BJ; CDK2
Meiotic synapsis	R	5.1E-09	HIST3H2BB; HIST2H4B; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST1H4D; HIST1H4B; HIST1H4L; HIST1H2BJ
DNA replication	R	5.3E-07	
Programmed Cell Death	R		

Gene Set Name	Datab.*	p value	Genes
Cell growth and death	K		
p53 signaling pathway	K	4.7E-05	IGF1; CDK2; CDKN1A; CDKN2A; CDK1; CASP9; CCNG2
Apoptosis	R	1.6E-07	
Apoptosis execution phase	R		
Apoptosis induced DNA fragmentation	R	5.7E-07	HIST1H1C; HIST1H1D; HIST1H1E; HIST1H1B; HIST1H1A
Metabolism	R		
Metabolism of lipids and lipoproteins	R	1.6E-15	
Fatty acid, triacylglycerol, and ketone body metabolism	R	1.0E-07	
PPARA Activates Gene Expression	R	1.9E-06	TRIB3; CYP7A1; CYP1A1; SULT2A1; HMGCR; RXRA; SMARCD3; ACSL1; CPT2; ANKRD1
Cholesterol biosynthesis	R	7.3E-07	CYP51A1; HMGCR; EBP; HSD17B7; SC5DL; SQLE
Metabolism of steroid hormones and vitamin D	R		
Steroid biosynthesis	K	1.6E-09	HSD17B7; CEL; EBP; CYP51A1; SC5DL; SOAT1; SQLE
Arachidonic acid metabolism	K	8.4E-03	PTGS2; GGT1; PLA2G12B; CYP2J2
Bile acid and bile salt metabolism	R		
Primary bile acid biosynthesis	K	1.3E-03	CYP7A1; CYP27A1; HSD3B7
Biosynthesis of unsaturated fatty acids	K	1.1E-05	
Linoleic acid metabolism	K	7.3E-03	CYP1A2; PLA2G12B; CYP2J2
Biological oxidations	R	6.9E-15	
Phase 1 - Functionalization of compounds	R	2.2E-10	
Cytochrome P450 - arranged by substrate type	R	3.1E-08	CYP7A1; CYP1A1; CYP51A1; CYP27A1; CYP24A1; CYP1A2; CYP2D6; CYP2J2; CYP2W1

Gene Set Name	Datab.*	p value	Genes
Drug metabolism - other enzymes	K	4.7E-07	GUSB; DPYS; UPP1; NAT2; CES1; CES5A; CES2; NAT1
Phase II Conjugation	R	5.2E-07	SULT2A1; CYP1A2; MAT1A; SULT1C2; PAPSS2; GGT1; MGST3; NAT2; NAT1
Amino acid metabolism	R	3.3E-09	
Tyrosine metabolism	K	2.3E-05	MAOA; MAOB; DDC; IL4I1; HPD; TH
Phenylalanine metabolism	K	1.1E-07	MAOA; MAOB; DDC; IL4I1; HPD; PAH
Glycine, serine and threonine metabolism	K	8.4E-04	MAOA; MAOB; AGXT; ALAS2
Alanine aspartate and glutamate metabolism	K	9.6E-03	IL4I1; GLS2; AGXT
Arginine and proline metabolism	K	8.9E-04	MAOA; MAOB; GLS2; P4HA1; AGMAT
Tryptophan metabolism	K	1.3E-10	MAOA; MAOB; DDC; IL4I1; CYP1A1; CYP1A2; AADAT; TDO2; KMO; KYNU
Histidine metabolism	K	7.3E-03	MAOA; MAOB; DDC
Metabolism of other amino acids	K		
Glutathione metabolism	K	5.0E-03	ANPEP; MGST3; GGT1; TXNDC12
Seleno amino acid metabolism	K	5.3E-03	GGT1; PAPSS2; MAT1A
Carbohydrate metabolism	R	2.1E-06	
Glycolysis/Gluconeogenesis	R		
Digestion of dietary carbohydrate	R	3.6E-07	AMY1A; AMY1B; AMY1C; AMY2A; AMY2B
Starch and sucrose metabolism	K	7.9E-05	GUSB; AMY1A; AMY1B; AMY1C; AMY2A; AMY2B
Nucleotide metabolism	R		
Pyrimidine metabolism	K	2.4E-03	DPYS; UPP1; TYMS; NT5E; ENTPD8; ENTPD5
Retinol metabolism	K	1.9E-03	CYP1A1; CYP1A2; DGAT2; RDH16; ALDH1A2

Gene Set Name	Datab.*	p value	Genes
Energy metabolism	K		
Nitrogen metabolism	K	3.8E-03	GLS2; CA4; CA12
Immune System	R	4.9E-16	
Innate Immune System	R		
Complement cascade	K	1.8E-10	C3; C9; C1S; C4A; C4B; F2; FGA; FGB; FGG; SERPINC1; SERPINA1; SERPING1
Adaptive Immune System	R	7.5E-12	
Class I MHC mediated antigen processing & presentation	R	9.6E-08	
Antigen processing: ubiquitination & proteasome degradation	R	3.1E-07	PSMA6; PSMB9; PSME4; CDC20; SOCS1; SOCS3; FBXO6; ASB15; MKRN1; FBXO2; ASB2; ZBTB16; TRIM63; HERC2; FBXO44
Cytokine signaling in immune system	R	1.4E-06	
Interferon alpha/beta signaling	R	1.8E-08	SOCS1; SOCS3; IFITM1; IRF1; MX1; MX2; IFITM3; IFITM2; IFI27; IFIT3
Transmembrane transport of small molecules	R	2.1E-13	
SLC-mediated transmembrane transport	R	5.3E-08	
Amino acid and oligopeptide SLC transporters	R	2.1E-08	
Transport of inorganic cations/anions and amino acids/oligopeptides	R	7.6E-08	SLC7A8; SLC7A10; SLC3A2; SLC38A4; SLC1A5; SLC7A3; SLC43A1; SLC1A2; SLC17A5; SLC26A2; SLC24A3
Amino acid transport across the plasma membrane	R	1.8E-07	SLC7A8; SLC7A10; SLC3A2; SLC38A4; SLC1A5; SLC7A3; SLC43A1
Hemostasis	R	1.2E-10	
Formation of Fibrin Clot (Clotting Cascade)	R	4.5E-06	FGA; F2; FGB; FGG; SERPING1; SERPINC1
Platelet activation, signaling and aggregation	R	2.4E-07	

Gene Set Name	Datab.*	p value	Genes
Thrombin signalling through proteinase activated receptors (PARs)	R	4.5E-06	GNG3; GNG7; GNG13; F2; GNA11; ARRB1
Environmental Information Processing	K		
Signaling molecules and interaction	K		
ECM-receptor interaction	K	1.5E-10	ITGA7; THBS2; CHAD; COL1A1; COL1A2; COL2A1; COL5A2; COL6A1; COL6A2; COL11A1; COL11A2; TNC; SV2C
Signal transduction	R		
Signaling by PDGF	R	1.6E-08	TRIB3; CDKN1A; CDK1; CASP9; THEM4; RICTOR; COL1A1; COL1A2; COL2A1; COL5A2; COL6A1; COL6A2; THBS2
Gene expression	R		
RNA polymerase I RNA Polymerase III and mitochondrial transcription	R	1.4E-07	
RNA polymerase I transcription	R	3.8E-09	
RNA polymerase I promoter clearance	R		
RNA polymerase I promoter opening	R	8.4E-10	HIST3H2BB; HIST2H4B; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST1H4D; HIST1H4B; HIST1H4L; HIST1H2BJ
Transport and catabolism	K		
Peroxisome	K	4.5E-03	ACAA1; ACSL1; SLC27A2; AGXT; PHYH
Encondrine system	K		
PPAR signaling pathway	K	2.8E-09	CYP7A1; ACAA1; FADS2; RXRA; CYP27A1; ACSL1; SLC27A2; CPT2; UCP1; FABP7; PLTP
Progesterone-mediated oocyte maturation	K	1.2E-03	IGF1; CDK2; CDK1; MAD2L1; PLK1; CCNA2
Renin-angiotensin system	K	2.6E-06	ANPEP; AGTR1; CMA1; CPA3; NLN

Gene Set Name	Datab.*	p value	Genes
Excretory system	K		
Proximal tubule bicarbonate reclamation	K	1.3E-05	ATP1A3; ATP1B1; ATP1B2; GLS2; CA4
Vasopressin-regulated water reabsorption	K	3.1E-03	CREB3L3; AVPR2; DYNLL2; AQP3
Cellular community	K		
Focal Adhesion	K	9.2E-07	ITGA7; THBS2; CHAD; COL1A1; COL1A2; COL2A1; COL5A2; COL6A1; COL6A2; COL11A1; COL11A2; TNC; IGF1; MYLK2
Extracellular matrix organization	R	6.7E-14	
Collagen formation	R	3.9E-10	COL1A1; COL1A2; COL2A1; COL5A2; COL6A1; COL6A2; COL10A1; COL11A1; COL11A2; COL28A1; ADAMTS2
Neuronal system	R	2.1E-06	
Transmission across Chemical Synapses	R	2.2E-06	
Neurotransmitter Release Cycle	R	1.6E-08	CHAT; MAOA; SLC6A11; GLS2; SNAP25; STXBP1; RAB3A; SYN1
Human diseases	K		
Pathways in cancer	K	7.1E-04	
Bladder cancer	K	2.6E-03	CDKN1A; CDKN2A; MMP2; DAPK2
Prostate cancer	K	7.9E-03	IGF1; CDK2; CDKN1A; CASP9; CREB3L3
Cardiovascular diseases	K		
Viral myocarditis	K	6.8E-05	HLA-DMB; HLA-DOA; HLA-DQA2; CASP9; MYH6; MYH7; MYH7B
Dilated cardiomyopathy	K	9.0E-03	ITGA7; IGF1; MYH6; MYH7; MYBPC3
Hypertrophic cardiomyopathy (HCM)	K	1.2E-03	ITGA7; IGF1; MYH6; MYH7; MYBPC3; PRKAG3
Immune diseases	K		
Asthma	K	8.0E-03	HLA-DMB; HLA-DOA; HLA-DQA2

Gene Set Name	Datab.*	p value	Genes
Systemic lupus erythematosus	K	6.2E-16	C3; C9; C1S; C4A; C4B; HLA-DMB; HLA-DOA; HLA-DQA2; HIST3H2BB; H2BFWT; HIST2H4B; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST1H4D; HIST1H4B; HIST1H4L; HIST1H2BJ; H2AFY
Neurodegenerative diseases	K		
Amyotrophic lateral sclerosis (ALS)	K	6.1E-03	CASP9; NEFM; ALS2; SLC1A2
Infectious diseases: parasitic	K		
Leishmania infection	K	3.2E-03	C3; HLA-DMB; HLA-DOA; HLA-DQA2; PTGS2
Amyloids	R	5.3E-13	HIST3H2BB; HIST2H4B; HIST1H4I; HIST1H2BH; HIST1H2BO; HIST2H2BE; HIST1H4A; HIST1H4D; HIST1H4B; HIST1H4L; HIST1H2BJ; FGA; CALCA; LTF; APCS

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