UNIVERSIDAD DE MURCIA

DEPARTAMENTO DE BIOLOGÍA CELULAR
E HISTOLOGÍA

The Zebrafish (*Danio rerio*) New Model to Study the Role of Telomerase in Aging and Regeneration

El Pez Cebra (*Danio rerio*) Nuevo Modelo para el Estudio de la Función de la Telomerasa en Envejecimiento y Regeneración

Dña. Monique Anchelin Flageul

2016

UNIVERSIDAD DE MURCIA

DEPARTAMENTO DE BIOLOGÍA CELULAR E HISTOLOGÍA

THE ZEBRAFISH (*Danio rerio*) NEW MODEL TO STUDY THE ROLE OF TELOMERASE IN AGING AND REGENERATION

EL PEZ CEBRA (*Danio rerio*) NUEVO MODELO PARA EL ESTUDIO DE LA FUNCIÓN DE LA TELOMERASA EN ENVEJECIMIENTO Y REGENERACIÓN

Memoria que presenta

Dña. Monique Anchelin Flageul

Para optar al grado de Doctor

por la Universidad de Murcia

Enero 2016

Index

Abbreviations

a.u.f.	arbitrary unit of fluorescence
ALT	Alternative Lengthening of Telomeres
ATR	ATM and Rad3-related
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CTC1	Conserved Telomere maintenance Component 1
dCTP	Deoxycytidine Triphosphate
DC	Dyskeratosis congenita
DDR	DNA Damage Response
DKC1	Dyskerin 1
D-loop	Displacement-loop
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide Triphosphate
dpa	days post-amputation
dpf	days post-fertilization
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EDTA	Ethylenediaminetetraacetic acid
ENU	*N*-ethyl-*N*-nitrosourea
F	Direct primer
FSC	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
FISH	Fluorescence *In Situ* Hybridization
G	Generation
gDNA	genomic DNA
hpa	hours post-amputation

hpf	hours post-fertilization
hpi	hours post-injection
H&E	Hematoxylin & Eosin
MFI	Medium Fluorescence Intensity
MO	Morpholino
mRNA	messenger RNA
MTS	Multiple Telomeric signals
PAS	Periodic Acid Schiff
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PNA	Peptide Nucleic Acid
POT1	Protection of Telomeres 1
Q-FISH	Quantitative-FISH
Q-TRAP	Quantitative-TRAP
R	Reverse primer
RNA	Ribonucleic Acid
RNase	Ribonuclease
rps11	ribosomal protein S11
rRNA	ribosomal RNA
RTA	Relative Telomerase Activity
RT-PCR	Reverse Transcription-quantitative Polymerase Chain Reaction
SA β-Gal	Senescence Associated β-galactosidase
SEM	Standard Error of the Mean
Std	Standard
TBE	Tris/Borate/EDTA
TEN1	CST complex subunit TEN1
TERC	Telomerase RNA Component

TERRA	Telomere-Repeat-containing RNA
TERT	Telomerase catalytic subunit
TIN2	TRF1-Interacting Nuclear factor2
t-circle	telomeric DNA circle
t-loop	telomeric DNA-loop
TMM	Telomere Maintenance Mechanism
TR	Telomere RNA Component
TRAP	Telomeric repeat amplification protocol
TRF1	Telomeric Repeat-binding Factor 1
TRF2	Telomeric Repeat-binding Factor 2
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling
zf	zebrafish

Summary

The aim of this Doctoral Project is to contribute to the undestanding of the processes of how telomerase activity and telomere length determine and maintain telomere length througout life during aging and regeneration processes. Towards this aim, we have exploited the unquestionable advantages of the zebrafish vertebrate model.

First to set the basis for further assessment of telomerase role on telomere length maintenance, we have characterized telomerase function and telomere length in distinct strains of wild-type zebrafish and we concluded that the expression of telomerase and telomere length are closely related during the entire life cycle of the fish and that these two parameters can be used as biomarkers of aging in zebrafish. Our results also reveal a direct relationship between the expression of telomerase, telomere length and the efficiency of tissue regeneration.

In a second part of this work, we have performed an exhaustive characterization of the telomere and telomerase behaviour in the telomerase-deficient zebrafish, establishing the usefulness of this model for aging studies and for the discovery of new drugs able to reactivate telomerase in individuals with DC.

Following our research line, using the mutant zebrafish line, we have investigated the telomere length maintenance mechanism during the caudal fin regeneration throughout life of the fish, showing that an alternative and telomerase-independent telomere lengthening mechanism is implicated in the telomere maintenance during the regeneration process.

Taken together, this Doctoral Thesis provides a complete description of telomerase activity, telomerase expression and telomere length in distinct strains of wild-type zebrafish, as in telomerase-deficient zebrafish providing a basis for the usage of zebrafish as a model system for aging and regeneration studies and telomere/ telomerase research.

Introduction

1. Telomeres

Genomic stability is the prerequisite of species survival to ensure that all required information will be passed on to the next generations. In contrast to single-cell-species, higher order organisms, in order to preserve their genomic information, require more efficient DNA repair mechanisms due to later onset of reproduction. Therefore, a remarkable ability of cells to recognize and repair DNA damage and progress through the cell cycle, in a regulated and orderly manner, has been developed.

A vulnerable portion of the genome, especially in eukaryotic organisms whose genome is organized in linear chromosomes, is their edges called telomeres (after the greek words '*τέλος*' (*télos*) and '*μέρος*' (*méros*) meaning 'the ending part'). For this, telomeres form specialized structures at the ends of linear chromosomes that ensure their integrity by 'hiding' the free-ends of the chromosome from the mechanisms within the cell that monitor DNA damage. They are also needed to overcome the 'end-replication problem' [Greider, 1996].

Telomeres are long tracts of DNA at the linear chromosome's ends composed of tandem repeats of a guanine rich sequence motif that vary in length according to species. This motif is conserved in lower eukaryotes and in mammalian cells [Greider, 1998]. Usually, but not always, the telomeric DNA is heterochromatic and contains direct tandemly repeated sequences of the form $(T/A)_xG_y$ where *x* is between 1 and 4 and *y* is greater than 1 (**Table I**).

Table I: The following table shows the diversity of telomeric DNA. *Adapted from Sfeir (2012).*

Organism	Sequence	Length
Homo sapiens	TTAGGG	5-15 kb
Mus musculus	TTAGGG	20-100 kb
Danio rerio	TTAGGG	12-20 kb
Schizosaccharomyces pombe	$GGTTACA_{0-1}C_{0-1}G_{0-1}$	5 kb
Saccharomyces cerevisiae	TG_{1-3}	300 bp
Organism	**Sequence**	**Length**
Trypanosoma brucei	GGGTTA	2-26 kb
Tetrahymena thermophila	TTGGGG	120-420 bp
Coenorhabditis elegans	TTAGGC	4-9 kb
Arabidopsis thaliana	TTTAGGG	2-5 kb
Oxytricha	TTTTGGGG	20 bp

Exceptionally, the chromosome ends of a few insect species (*Drosophila* and some dipterans), instead of telomeric motifs, possess tandem arrays of retrotransposons [Abad *et al.*, 2004].

1.1. Structure and function

Telomeric DNA is double stranded with a single-stranded terminus that is on average 50-300 nucleotides (nt) long in human cells. Under normal conditions, in most somatic cells of an adult organism, telomeres shorten in each cell division (i.e. in humans by about 50-150 nt). The basic telomere DNA repeat unit in vertebrates is the hexamer TTAGGG, in which the strand running 5'→ 3'outwards the centromere is usually guanine-rich and referred to as G-tail (**Fig. 1**).

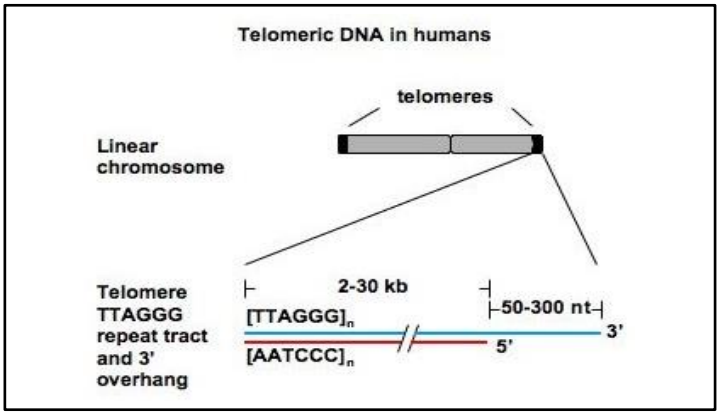


Figure 1: Telomere primary structure scheme. *Adapted from the website http://gallus.reactome.org*

In order not to leave exposed a single stranded overhang, this G-rich strand protrudes its complementary DNA-strand and, by bending on itself, it folds back to form a telomeric DNA loop (t-loop), while the G-tail 3' end invades into the double strand forming a triple-stranded structure called displacement loop (D-loop) inside the t-loop. As a result, the t-loop protects the G-tail from being recognized as a double-stranded DNA (dsDNA) break by sequestering the 3'-overhang into a higher order DNA structure [Griffith *et al.*, 1999], (**Fig. 2**).

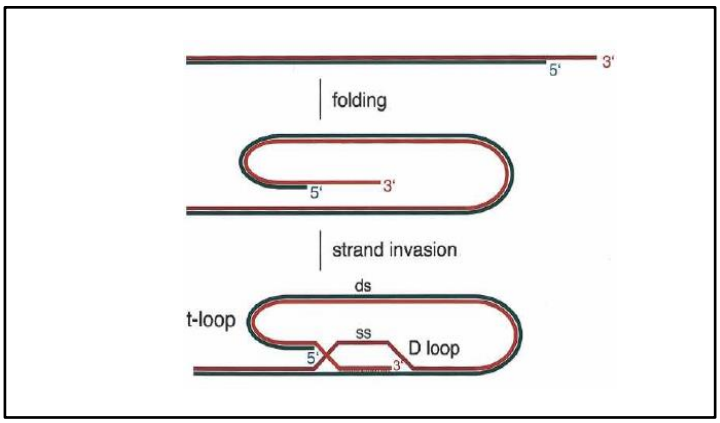


Figure 2: Telomere secondary structure scheme. The single stranded overhang folds back and forms a telomeric DNA loop, t-loop. Then, the 3' overhang is strand-invaded into the adjacent duplex telomeric repeat array, forming a D-loop. The size of the loop is variable. *Adapted from de Lange (2005).*

In vertebrates, the role of chromosome end protection in order to be distinguished from chromosome breaks is attributed to a specific complex of proteins collectively referred to as shelterin. Shelterin complex is basically composed by six proteins: TRF1 (telomeric repeat-binding factor 1), TRF2 (telomeric repeat-binding factor 2), POT1 (protection of telomeres 1), RAP1 (repressor activator protein 1), TIN2 (TRF1-interacting nuclear factor 2) and TPP1 (TIN2 and POT1-interacting protein 1). TRF1 and TRF2 bind directly to double stranded telomeric sequence, while POT1 binds single-stranded DNA (ssDNA). TRF2 interacts with and recruits RAP1, while TIN2 mediates TPP1-POT1 binding to the TRF1/TRF2 core complex. POT1 binds to and protects the 3´ ssDNA overhang of telomeres (G-tail), while TIN2 likely links the ssDNA and dsDNA binding complexes, especially in the area of the telomeric D-loop formation [reviewed in de Lange, 2005 and Stewart *et al.*, 2012], (**Fig. 3**).

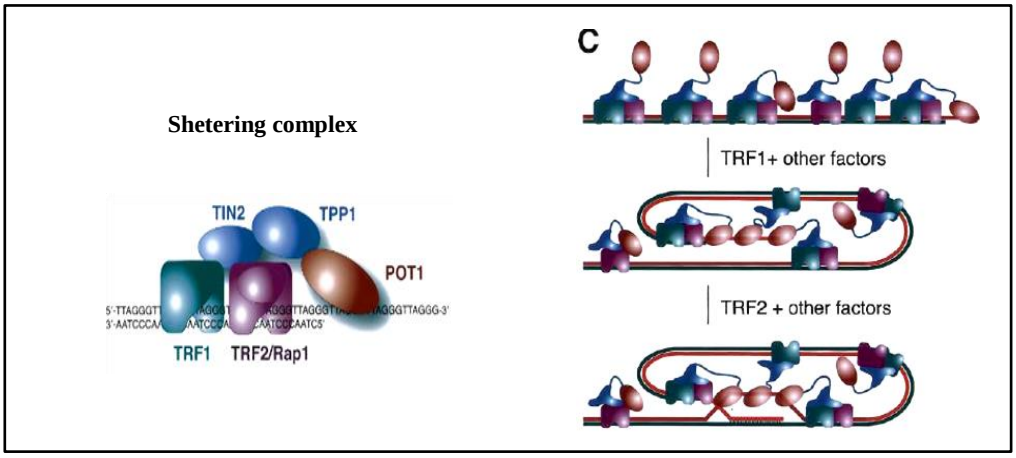


Figure 3: Speculative model for t-loop formation by shelterin. TRF1 has the ability to bend, loop, and pair telomeric DNA *in vitro* and could potentially fold the telomere. *Adapted from de Lange (2005).*

It seems that this core shelterin complex is mainly located at the telomere end (also referred to as telosome) and serves both to stabilize t-loop structure and to protect it from being recognized by the DNA damage detection machinery as dsDNA breaks and repaired by Non-Homologous-End-Joining (NHEJ) repair activities. Additionally, shelterin regulates access to restoration processes of telomeric DNA after each genome replication. In general, shelterin complex seems to function as a platform regulating recruitment of a growing list of factors involved in chromatin remodelling, DNA replication, DNA damage repair, recombination and telomerase function, thus regulating telomere access/modification by diverse cellular processes, as reviewed by Diotti and Loayza (2011).

Apart from shelterin and interacting partners, another significant complex has recently emerged to be also involved in telomere biology, the CST complex. The CST complex is composed of three subunits: CTC1 (conserved telomere maintenance component 1), STN1 (suppressor of cdc thirteen homolog) and TEN1 (CST complex subunit TEN1), and has been attributed the rescue of stalled replication forks during replication stress. The CST complex interconnects telomeres to genome replication and protection independently of the POT1 pathway [Miyake *et al.*, 2009].

RNA molecules called telomeric-repeat-containing RNA (TERRA), has been identified as the third entity of the telomere nucleoprotein complex. TERRA transcription is mediated by DNA-dependent RNA polymerase II and is initiated from

the sub-telomeric regions that are found near chromosome ends [Azzalin *et al*., 2007; Schoeftner & Blasco, 2008]. TERRA levels are regulated during the cell cycle, and its localization at telomeres is modulated by the nonsense-mediated decay machinery [Porro *et al*., 2010; López de Silanes *et al.*, 2010]. It has been recently demonstrated that this non-coding RNA acts as a bimolecular regulator to turn telomerase and the telomere on (replication-competent state) and off (protected state) during the cell cycle [Redon *et al.*, 2013].

The structure of telomeres is intrinsically dynamic, as chromosome ends should relax during genome replication and then re-establish their 'capped' state after replication. Consequently, telomeres may switch between off/protected and on/replication competent states during the cell cycle. Each state is governed by a number of interactions with specific factors and can lead the cell to either cell division or senescence/apoptosis under normal conditions, or to disorders/cancer in abnormal cases. Moreover, during development and in certain cell types in adults, telomere length should be preserved. Thus, multiple physiological processes guarantee functional and structural heterogeneity of telomeres concerning their length and nucleoprotein composition. A functional chromosome end structure is essential for genome stability, as it must prevent chromosome shortening and chromosome end fusion as well as degradation by the DNA repair machinery. Hence, structure and function of telomeres are highly conserved throughout evolution [review by Galati *et al.*, 2013].

1.2. The 'end-replication problem'

The primary difficulty with telomeres is the replication of the lagging strand. Because DNA synthesis requires a RNA template (that provides the free 3'-OH group) to prime DNA replication, and this template is eventually degraded, a short single-stranded region would be left at the end of the chromosome (referred to as the 'end-replication problem'). This region would be susceptible to enzymes that degrade ssDNA. In the absence of any compensatory mechanism, the result would be that the length of the chromosome would be shortened after every cell division, resulting in cumulative telomere attrition during aging [Watson, 1972; Olovnikov, 1973]. In addition, loss of telomere DNA also occurs due to post-replicative degradation of the 5' strand that generates long 3' G-rich overhangs [Wellinger *et al.*, 1996], (**Fig.4**).

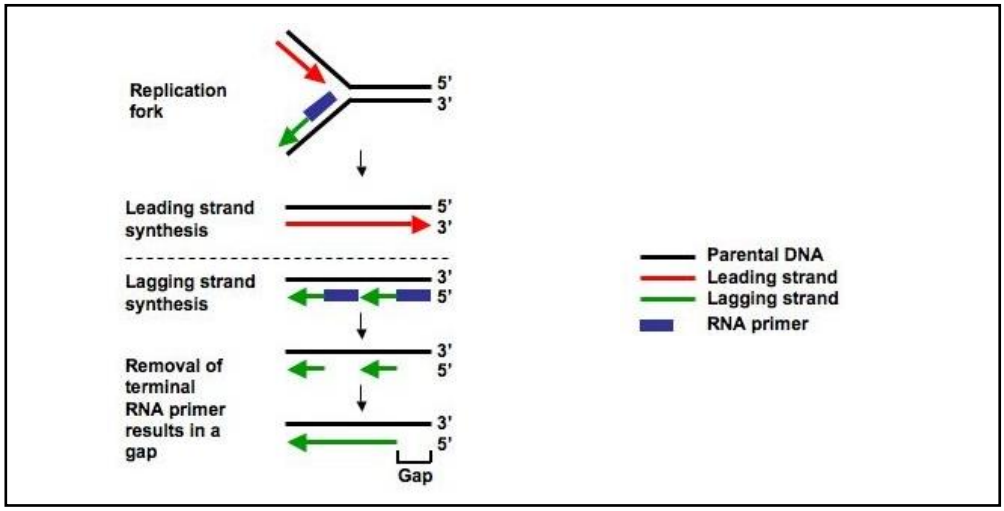


Figure 4: The 'end-replication problem'. DNA polymerase requires an RNA primer to initiate synthesis in the 5'→3' direction. At the end of a linear chromosome, DNA polymerase can synthesize the leading strand until the end of the chromosome. In the lagging strand, however, DNA polymerase's synthesis is based on a serie of fragments, called Okazaki, each requiring an RNA primer. Without DNA to serve as template for a new primer, the replication machinery is unable to synthesize the sequence complementary to the final primer event. The result is an end-replication problem in which sequence is lost at each round of DNA replication. *Adapted from the website http://gallus.reactome.org*

Upon each genome duplication, cells would otherwise keep losing genetic material, eventually resulting in premature cell death or replicative senescence, a critical problem for both the species and an individual's survival. This issue is even more prominent especially in multi-cellular organisms with late onset of reproduction.

2. Telomerase

2.1. Structure and function

During ontogenesis, eukaryotic organisms solved the 'end replication problem' by preventing telomere attrition in dividing cells, through recruitment of telomerase, a specialized and unique RNA-dependent DNA polymerase that synthesizes telomeric repeats at the end of eukaryotic chromosomes, thereby maintaining them at a 'safe' length, as a limited telomere length is a prerequisite for cell replication [Blackburn, 2005], (**Fig. 5**).

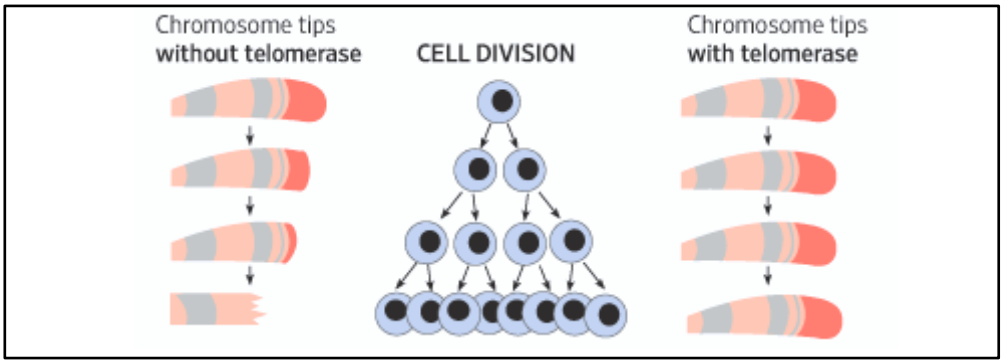


Figure 5: Telomerase synthesizes telomeres. Without telomerase, chromosomes get shorter over time and cells eventually stop dividing. *Adapted from The Nobel Committee for Physiology or Medicine 2009.*

The telomerase complex is a ribonucleoprotein (RNP) composed by a catalytic subunit (telomerase reverse transcriptase, TERT), a RNA component (*TR*) which acts as a template for the addition of the telomere sequence in the 3' end of the telomere, and species-specific accessory proteins that regulate telomerase biogenesis, subcellular localization and its function *in vivo* [Wyatt *et al.*, 2010]. In human telomerase, seven associated proteins have been identified: Dyskerin, NHP2 (H/ACA ribonucleoprotein complex subunit 2), NOP10 (nucleolar protein 10), GAR1 (H/ACA ribonucleoprotein complex subunit 1), TCAB1 (telomerase Cajal body protein 1), Pontin and Reptin, [reviewed by Gómez *et al*., 2012], (**Fig. 6**).

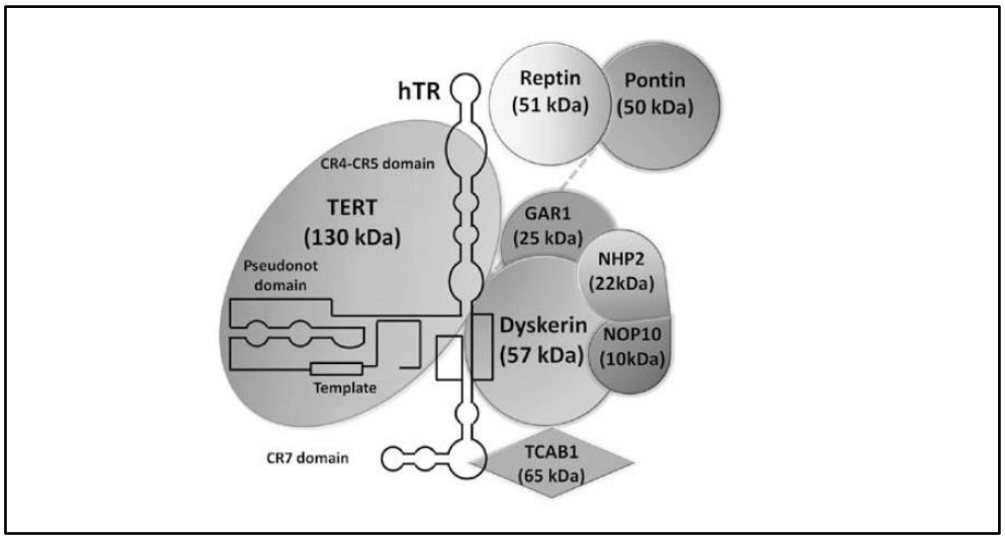


Figure 6: Schematic representation of human telomerase and its associated proteins. *Adapted from Gómez et al. (2012).*

The action of the telomerase complex ensures that the ends of the lagging strands are replicated correctly. Elongation of the telomere by telomerase is a process that happens in different stages. First, the nucleotides of the 3' extreme of the telomeric DNA are hybridized to the end of the RNA template, inside the RNA domain of the telomerase complex. The template sequence of 11 nucleotides is complementary to almost two telomeric repeats. Next, the gap in the extreme of the template is completed by synthesis, using triphosphate nucleotides in the catalytic site of the enzyme (TERT). In this way, a complete hexanucleotidic repeat is assembled in the template. Then, telomerase relocates and the cycle is repeated, extending the telomere in the 3'-direction. Finally, the DNA polymerase can synthesize the lagging strand and thus, the end of the chromosome is faithfully replicated [reviewed by Sfeir, 2012], (**Fig. 7**).

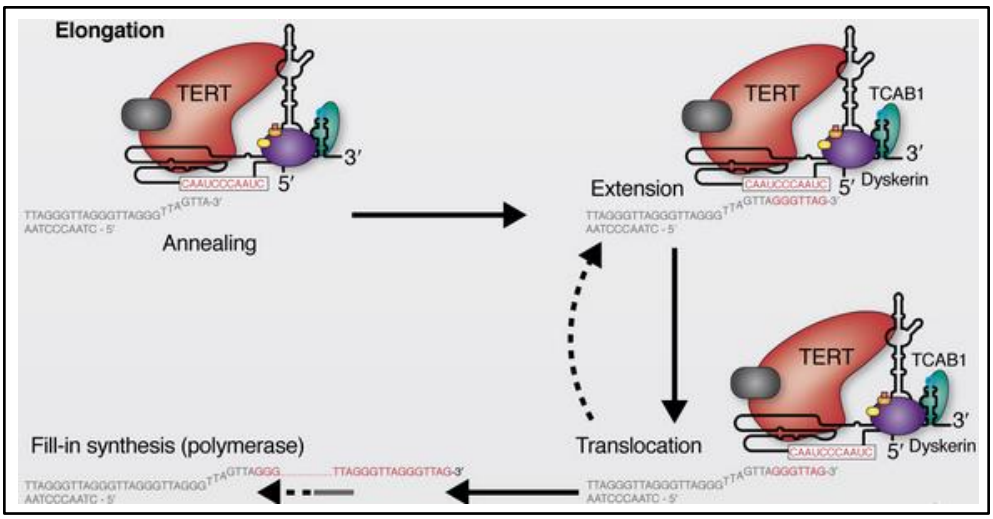


Figure 7: Telomere maintenance. Telomerase, consisting mainly of the protein (TERT) and RNA subunit (*TR*), binds to 3' flanking end of telomere that is complementary to *TR*, and bases are added using *TR* as template. Then, telomerase relocates and telomere is extended in the 3'-direction. Finally, the DNA polymerase can synthesize the lagging strand. This process can maintain telomere length or lead to telomere lengthening. *Adapted from Sfeir (2012).*

Telomerase is routinely active only during embryogenesis and development, while in adults is expressed only to rapidly dividing cells (i.e. epithelial cells, activated lymphocytes, specific bone marrow stem cells and dividing male germ cell lineages) [Ulaner & Giudice, 1997]. In most adult cells, telomerase is not expressed. Consequently, after a number of cell divisions, telomeres reach a critical length and chromosomes become uncapped, leading a permanent cell cycle arrest (termed replicative senescence) or apoptosis (programmed cell death) or a DNA damage response (DDR) that results in chromosomal end-to-end fusions [reviewed by Tümpel & Rudolph, 2012].

The progressive loss of telomeric DNA in human somatic (stem) cells is believed to act as a tumor suppressor mechanism, unfortunately, some cells can ignore or bypass the "telomere" checkpoint, because their DNA damage responses are defective, and continue to grow despite the presence of dysfunctional telomeres. In fact, telomerase expression was found to be a hallmark of human cancer: telomerase expression or re-expression and activity can be detected in 90% of tumor samples [Shay & Roninson, 2004]. Telomere loss may act as a tumor suppressor mechanism and also promote tumor growth, then showing a dual role for telomerase in aging and cancer.

2.2. Telomere diseases, dyskeratosis congenita (DC)

This loss of cell viability associated with telomere shortening is thought to contribute not only to the onset of degenerative diseases that occur during human aging, but also to several age associated diseases such as cancer, coronary artery disease, and heart failure [Donate & Blasco, 2011; Ogami *et al.*, 2004; Starr, 2007], (**Fig. 8**).

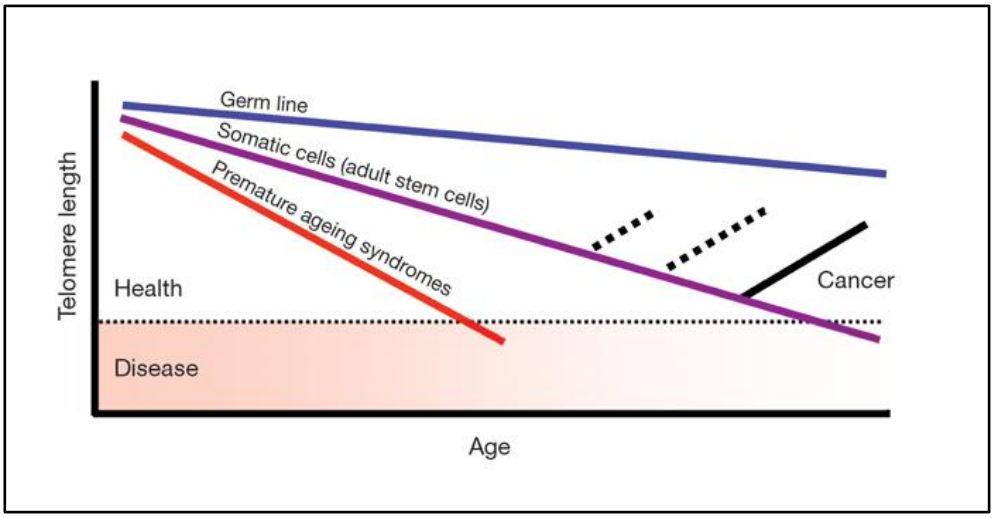


Figure 8: Telomeres undergo characteristic length changes over time in normal somatic and germ line cells, and in premature aging syndromes. Normal somatic cells, including adult stem cells, suffer progressive telomere attrition coupled to cell division or to increasing age of the organism. This attrition has been proposed to contribute to multiple age-related pathologies. In germline cells, telomere shortening is attenuated owing to high levels of telomerase activity. By contrast, telomere shortening is accelerated in several human premature aging syndromes, and patients with dyskeratosis congenita and aplastic anemia show decreased telomerase activity and shortened telomeres owing to mutations in the *TERC* and *TERT* telomerase genes. Psychosocial and environmental factors such as perceived stress, social status, smoking and obesity have also been shown to accelerate telomere attrition. In contrast to normal somatic cells, most immortalized cultures cell lines and more than 95% of human tumors aberrantly activate telomerase to achieve immortal growth. Although telomerase activity has been shown to be rate-limiting for mouse aging and lifespan, it is unknown whether increased telomerase activity will be able to extend the lifespan of organisms. *Adapted from Finkel et al. (2007).*

The first disease associated with mutations in human telomerase was identified in patients afflicted with a rare, inherited multisystem disorder called dyskeratosis congenital (DC). The prevalence is approximately 1 in 100,000 individuals, with death occurring at a median age of 16. The clinical manifestations of DC generally appear during childhood and include a monocutaneous triad of abnormal skin pigmentation, nail dystrophy and oral leukoplakia. The symptoms are accompanied by a spectrum of

other somatic abnormalities such as developmental delay, premature hair loss and bone marrow failure. Bone marrow failure is the principal cause of premature mortality, followed by pulmonary disease and cancer [Mason & Bessler, 2011; Dokal, 2011].

Most clinical presentations of DC are associated with an impaired proliferative capacity of tissues. In addition, the number of hematopoietic progenitor cells is decreased in DC patients. There is evidence that all DC patients have some defects in telomere biology, and that those defects affect the renewing capabilities of hematopoietic stem cells [Drummond *et al.*, 2007]. Furthermore, all DC causal mutations identified to date are found in telomerase components or in telomere stabilizing components [reviewed by Mason & Bessler, 2011 and Dokal, 2011]. Three modes of inheritance of DC have been identified: X-linked recessive, autosomal dominant-DC and autosomal recessive-DC. In its X-linked form, DC is caused by mutations in the RNA-binding protein dyskerin, a telomerase holoenzyme component critical for stabilizing *TR* [Heiss *et al*., 1998]. Autosomal dominant forms of DC are caused by mutations in the genes encoding TERT, *TR* and TINF2, clearly showing that the disease is caused by a failure of telomerase [Armanios *et al.*, 2005; Vulliamy *et al.*, 2001b; Savage *et al.*, 2008]. Autosomal recessive forms of DC are caused by mutations in the genes encoding the proteins NOP10, NHP2 and TCAB1 [Walne *et al*., 2007, Vullinamy *et al.*, 2008; Zhong *et al.*, 2011],

Regardless of the pattern of genetic inheritance, all patients with DC have very short telomeres, implying a common pathway underlying the mechanism of this disease [Mitchell *et al.*, 1999; Vulliamy *et al*., 2001a, 2004; Armanios *et al*., 2005]. When patients with mutations in the *DKC1* gene (encoding dyskerin) are categorized by disease severity, those with the most severe phenotypes have shorter telomeres than those with the mildest phenotypes (age over 15 years with no coexistent aplastic anemia) [Vulliamy *et al.*, 2006]. Using the telomere flow-FISH assay and a cut off of total LTL below the first percentile, DC patients could be distinguished from their unaffected relatives with 100% sensitivity and 90% specificity [Alter *et al*., 2007]. However, due to the variability of the symptoms of each of the mutations, not everything can be explained by telomere shortening [Vulliamy *et al*., 2011]. Interestingly, DC families with mutations in either *TERT* or *TR* genes demonstrate genetic anticipation with a worsening of disease severity and an earlier onset of symptoms with successive generations [Vulliamy *et al*., 2004; Armanios *et al*., 2005]. The onset and severity of disease correlates with progressive telomere shortening in

later generations. Siblings that do not inherit the mutated *TR* gene do not have symptoms. Even though these siblings inherit short telomeres from the affected parent, the non-mutated telomerase preferentially acts on the shortest telomeres to normalize their lengths [Goldman *et al*., 2005]. Thus, DC patients have to inherit both short telomeres and have a mutation in one of the components of telomerase in order to show genetic anticipation.

2.3. Telomeres and telomerase in aging

Organismal aging is characterized by the declining ability to respond to stress, increasing homeostatic imbalance and increased risk of disease, that eventually results in mortality. Such functional decline can result from the loss or diminished function of postmitotic cells or from failure to replace such cells by a functional decline in the ability of cells to sustain replication and cell divisions [Aubert & Lansdorp, 2008].

One of the central mechanisms responsible for the aging of cells is the shortening of telomeres, as telomere attrition has been shown to contribute to a persistent DDR, which contributes to p53 activation and cellular responses to stress during replicative senescence, leading to the irreversible loss of division potential of somatic cells where telomerase is not expressed. Telomerase expression, robust in pluripotent stem cells and early stages of embryonic development, is however restricted to stem cell compartments in the context of the adult organism [Blasco, 2005].

Adult stem cells reside at specific compartments within tissues, which are enriched in cells with the longest telomeres [Flores, *et al.*, 2008], then with sufficient telomere reserve in young or adult organisms to efficiently repopulate tissues and repair lesions. In old organisms, telomeres of adult stem cells may be too short [Flores *et al.*, 2008], and critically short telomeres are recognized as DNA damage. This triggers a complex signaling cascade with several steps: (i) activation of DDR proteins (53BP1, NBS1 and MDC1); (ii) activation of ATM and ATR kinases in senescent cells; (iii) activation of CHK1 and CHK2 kinases; (iv) phosphorylation and activation of several cell cycle proteins; and (v) the p53-mediated DNA damage signalling response impairs stem cell mobilization. These changes can induce a transient proliferation arrest allowing cells to repair their damage or lead to apoptosis or senescence if the DNA damage persists and exceeds a certain threshold [Kuilman *et al.*, 2010].

DNA damage can be caused by either exogenous or endogenous sources from the organism's own metabolism, which generates reactive oxygen species (ROS). Damage to telomeric DNA by ROS produced by either dysfunctional mitocondria [Harman, 1988; Wallace, 2005] or by signalling pathways predispose cells to apoptosis or senescence. DNA damage signals originating from telomeres could be replication-independent and the sensitivity of cells to DNA damage could increase as the overall telomere length declines. Then, telomeres are important targets for stress and this has important consequences for the ageing process [Aubert & Lansdorp, 2008].

In addition to a decline in stem cell function and accumulation of DNA, there are several others mechanisms that influence the aging process, as accumulation of protein damage, alterations in gene expression, checkpoint responses, then damage to multiple cellular constituents accounts for aging process [Kirkwood, 2005], and several biomarkers as senescence associated β-galactosidase (SA-β-gal), lipofuscin [Porta, 2002; Terman & Brunk, 1998] and others stress-associated markers are used for assessing signs of aging and/ or oxidative stress.

The relevance of telomere shortening induced by dysfunctional telomerase to physiological aging is suggested by the phenotype of the telomerase deficient mouse models [Blasco *et al.*, 1997; Lee *et al.*, 1998]. Early generations of *TR* deficient mice ($mTR^{-/-}$) do not show abnormalities presumably because laboratory mice have a much longer telomere length (8 to 10 times) than humans (5–15 kb). However, late generations of $mTR^{-/-}$ mice have defects in cell viability of highly proliferative tissues. They have a shorter lifespan compared with wild-type mice and show hair loss or early graying of hair, decreased capacity for wound healing and a slight increased incidence of cancer as a consequence of chromosomal instability but, however, resistance to induction of skin cancer [González-Suárez *et al*., 2000]. Similarly, after successive generations of TERT-deficient ($mTERT^{-/-}$) mice, telomere shortening causes a decrease in life expectancy and the regenerative capacity [Strong *et al*., 2011]. However, the telomerase deficient mouse models do not reproduce the symptom that is the leading cause of death in DC patients, the bone marrow failure, as they have longer telomeres [Zijlmans *et al.*, 1997] and show telomerase activity in most tissues [Prowse & Greider, 1995; Martin-Rivera *et al.*, 1998]. Although there are two mouse models showing bone marrow failure, like the one combining mutations in *TR* and in one of the telomere binding proteins (POT1) [Hockemeyer *et al*., 2008], and the one lacking TRF1 [Beier *et al*., 2012], that help to clarify the mechanism by which bone marrow failure occurs, to

date, has not been described any mutation neither in POT1 neither in TRF1 in the DC patients. For these reasons, an animal model complementary to the mouse which is capable to reproduce the symptoms of human telomere diseases would be extremely useful to study the telomere biology.

In summary, shortening of telomeres associated with organismal aging, is sufficient to impair stem cells mobilization and tissue regeneration and it is proposed to be a key determinant of organismal longevity, then telomere length measurement may be useful to monitorize the healthspan of the cells in aging.

3. Alternative lengthening of telomere (ALT)

In most of tumor cells, telomere maintenance is achieved by re-expression of telomerase. Interestingly, tumors have been described where telomerase could not be detected. Further studies described that homologous recombination constitutes an alternative method (**Fig. 9**) to maintain telomere DNA in human tumors and tumor-derived cell lines [Bryan *et al*., 1997; reviewed by Draskovic & Lodoño-Vallejo, 2013]. While the bulk of cancer and immortalized cells utilize telomerase re-expression to maintain telomere length, about 10-15% of tumors described operate using the ALT mechanism [Durant, 2012].

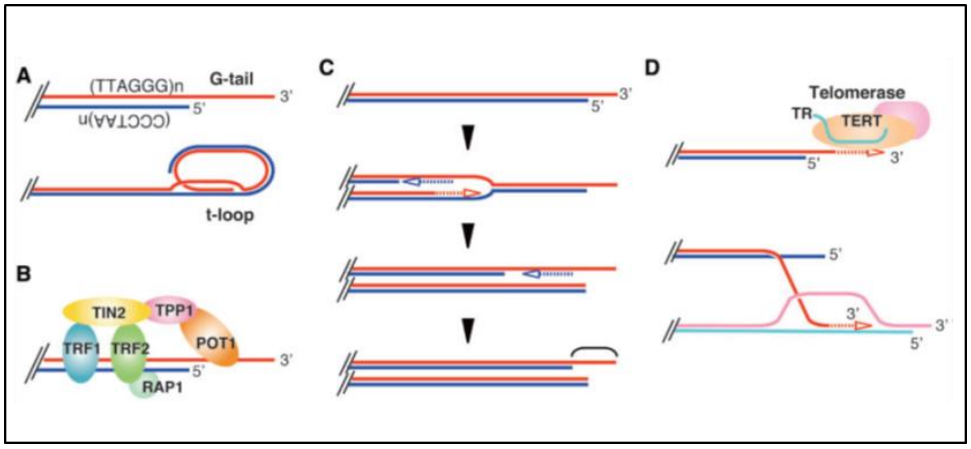


Figure 9: Telomere structure and lengthening. (A) Vertebrate telomere DNA. (B) Shelterin complex in human cells. (C) End-replication problem. The G-strand (red) is completely replicated to the end. However, the C-strand (blue) of telomere DNA is synthesized as a lagging strand and thus the copy of the very end cannot be accomplished. This causes the gradual shortening of telomere DNA through rounds of cell divisions. (D) Two types of telomere lengthening mechanism. The G-strand is primarily elongated in the telomerase pathway (top). Telomerase-independent pathway (bottom) is generally recombination-mediated. *Adapted from A. Nabetani and F. IshikawaJ. Biochem. 2011*.

Moreover, the first evidence that a homologous recombination mechanism contributes to telomere length maintenance was discovered in telomerase-defective strains from *Saccharomyces cerevisiae* [Lundblad & Blackburn, 1993]. Telomere recombination also occurs in primary cells from mice initiating by short telomeres, even in the presence of telomerase [Morrish & Greider, 2009] as described in the yeast *Kluyveromyces lactis* [McEachern & Iyer, 2001], suggesting that the initiation of telomere recombination is more likely due to the disruption of the capping structure at

short telomeres, and not the loss of telomerase. Another interesting data, it has been showed that telomere maintenance by telomerase and by recombination can coexist in human cells [Cerone *et al*., 2001].

A combination of markers of ALT have been described and include a heterogeneous terminal telomeric DNA profile, the presence of extrachromosomal telomeric circles, which are similar in size to telomeric t-loops and have been proposed to result from homologous recombination events (rolling-circle replication) at the telomeres (**Fig. 10**), and the presence of novel nuclear structures, the ALT-associated PML (Promyelocytic Leukemia) bodies known as APBs which contain: PML protein, (TTAGGG)*n* telomere DNA, telomere binding proteins (TRF1, TRF2, hRAP1, TIN2), and the DNA repair and recombination MRE11/RAD50/NBS1 (MRN) protein complex [Yeager *et al.*, 1999; Grobelny, 2000; Wu, 2000]. Telomere sister chromatid exchanges (T-SCEs) have also been detected in human ALT tumors [Londoño-Vallejo *et al*., 2004].

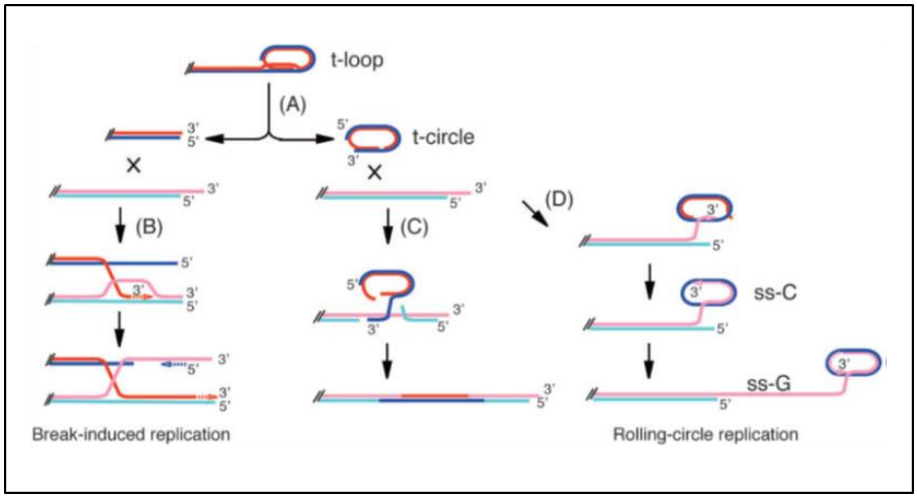


Figure 10: Telomere DNA metabolism in ALT cells. Four types of recombination events result in particular DNA metabolisms and unique products. (A) Recombination within t-loop causes rapid telomere shortening and t-circle formation. (B) Recombination between telomeres of chromosome ends initiates break-induced replication. (C) Transfer of a strand of t-circle into telomeres at chromosomal DNA initiates elongation of the repetitive DNA. (D) Invasion of t-circle by G-tail triggers rolling-circle replication. *Adapted from A. Nabetani and F. IshikawaJ. Biochem. 2011*.

Further studies oriented to look for ALT targeted tumor therapy showed that depletion of NBS1 inhibited the formation of t-circles [Compton *et al.,* 2007] and also resulted in inhibition of ALT-mediated telomere maintenance [Zhong *et al.,* 2007]. The recent report of Flynn *et al.,* (2015) shows that chromatin-remodeling protein ATRX loss compromises cell-cycle regulation of the telomeric noncoding RNA TERRA and leads to persistent association of replication protein A (RPA) with telomeres after DNA replication, creating a recombinogenic nucleoprotein structure and that inhibition of the protein kinase ATR, a critical regulator of recombination recruited by RPA, disrupts ALT and triggers chromosome fragmentation and apoptosis in ALT cells.

4. The zebrafish as a vertebrate model

Zebrafish (*Danio rerio*) is a tropical freshwater teleost fish belonging to the Cyprinidae family, order Cypriniformes. Since it was first used in a scientific laboratory 30 years ago, its popularity in biomedical research has significantly increased due to their unquestionable advantages respect other vertebrate models: (1) As a vertebrate, zebrafish shares considerable genetic sequence similarity with humans. (2) High fecundity and large production of embryos (around 200 eggs/female/week) makes phenotype-based forward genetics doable [Patton & Zon, 2001]. (3) Low maintenance cost, small space needed and easy to transfer among different labs by transporting their eggs. (4) A variety of methods have been developed to manipulate gene function: transient gene knock down by using morpholinos (chemically-modified antisense oligonucleotides that transiently knockdown the gene expression by preventing the protein formation) [Ekker, 2004], transient protein overexpression by injecting mRNA or plasmids, chemical mutagenesis [Rohner *et al.*, 2011], conditional transgene expression, generation of stable transgenic lines and targeted mutagenesis by using zinc finger nucleases (ZFNs) [Meng *et al.*, 2008; Leong *et al.*, 2011] and a transposon strategy [Kawakami, 2004]. (5) Small molecules screening [Murphey & Zon, 2006], conditional targeted cell ablation [Curado *et al.*, 2007], *in vivo* cell physiology and imaging [Detrich, 2008]. (5) Embryos are transparent and their develop following fertilization is external and fast, from embryo to larva in just three days, and to adult in three months (**Fig. 11**).

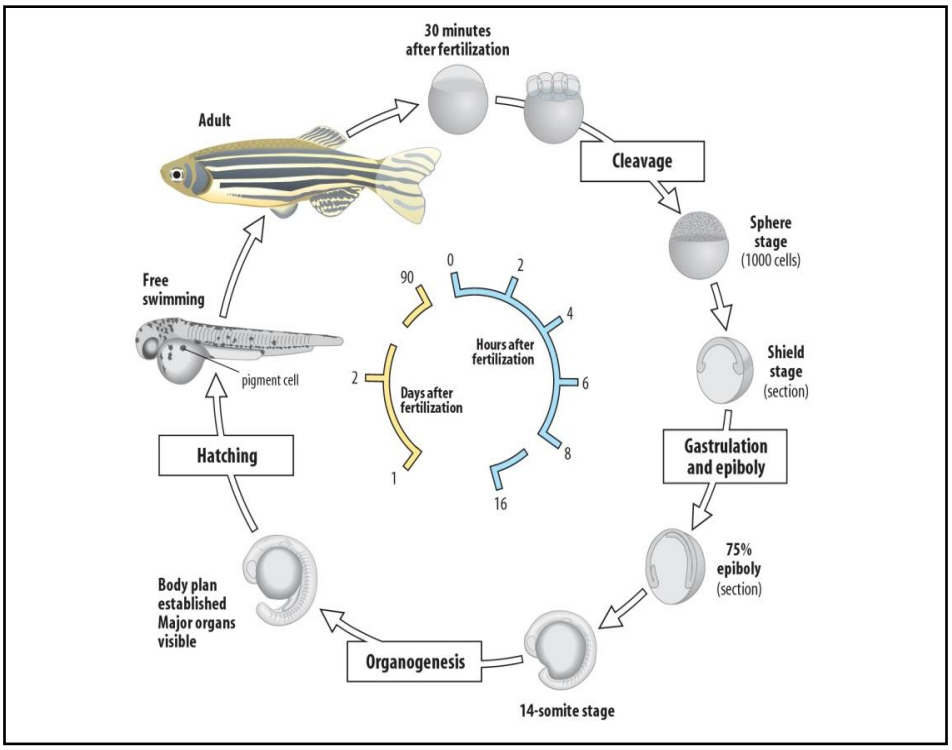


Figure 11: Schematic representation of zebrafish development from embryo to adulthood, highlighting both the speed of the development and the embryo transparency. *Adapted from the website www.daniorerio.com*

The ease of accessibility and transparency of the embryo allows the effects of genetic manipulations to be analyzed at a cellular level of resolution unprecedented in a vertebrate model system.

All these advantages have lead to the increased interest of scientists using zebrafish as an animal model for the study of both, basic and applied science in the last years and, nowadays, zebrafish has been proposed as an excellent vertebrate model for the study of the immune system [Renshaw & Trede, 2012], hematopoiesis [Martin *et al.*, 2011], vascular development [Isogai *et al.*, 2009; Quaife *et al.*, 2012; Gore *et al.*, 2012], neurogenesis [Schmidt *et al.*, 2013] and cancer research [Mione & Trede, 2010], among others.

4.1. Telomeres and telomerase in zebrafish

The zebrafish, *Danio rerio*, has 25 chromosomes with the vertebrate TTAGGG telomere sequence resulting in 100 or 200 telomeres per cell. Telomere length varies among species, with an average length 10-15 kilo base pairs (kb) in humans and a noticeably greater length in mice (20-150 kb) of the common laboratory species *Mus musculus*, The telomere length in zebrafish was determined to 15-20 kb which are closer to the human telomere length than the mouse laboratory mice.

The coding sequence of zebrafish telomerase reverse transcriptase (zfTERT) was revealed and cloned, and the expression of telomerase at mRNA, protein, and functional levels was assayed several years ago [Lau *et al*., 2008]. Comparing the *tert* genes, proteins and the reverse transcriptase (RT) domain in fish (medaka, fugu and zebrafish) and mammals (mouse, human) evolutionary conservation among vertebrate TERT was revealed [Au *et al.*, 2009] (**Fig. 12**).

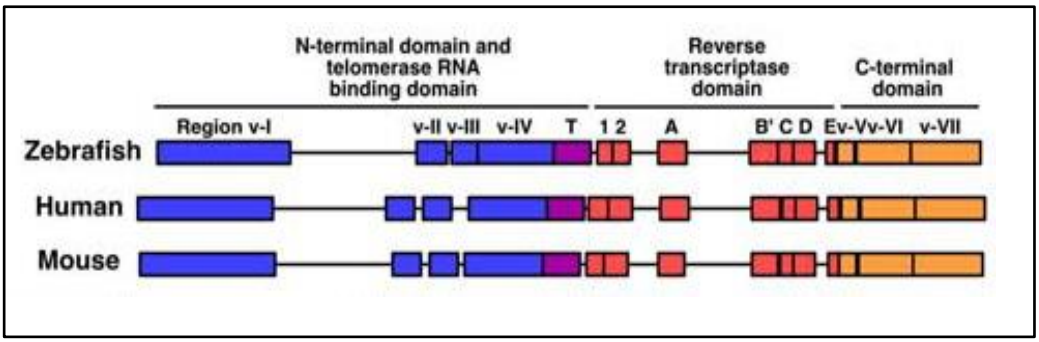


Figure. 12. Comparison of Tert gene domains between zebrafish, human and mouse.
Amino acid sequence comparison and determination of conserved regions between the zebrafish, human and mouse. The alignment outputs are displayed by the boxshading and amino acids that are identical in all three species. Comparisons between the amino acid sequences in the TR binding regions v-I, v-II, v-III, v-IV in blue, telomerase specific motif T in purple, the reverse transcriptase regions 1, 2, A, B', C, D and E in red and the C-terminal regions v-V, v-VI and v-VII are shown by yellow boxes. (Adapted from Imamura, 2008).

In relation to the zebrafish telomerase RNA component (zf*TR*), the bioinformatic identification, secondary structure comparison, and functional analysis of the smallest known vertebrate *TR*s from five teleost fishes, including zebrafish, showed the conservation of the structure and function of teleost *TR*, supporting the use of zebrafish as a model organism for the study of telomerase biology [Xie *et al.*, 2008].

Telomerase activity and expression was detected in all zebrafish tissues, and telomerase activity is maintained throughout development and adulthood. Zebrafish also showed tissue specific differences in telomere length and variations of the telomere length between individuals as observed for mouse and humans. Since then, the zebrafish has been considered as a potential model for aging, cancer, and regeneration studies.

4.2. Zebrafish as an aging and regeneration model

Longevity studies on outbred zebrafish found a mean lifespan to be about 42 months with the longest living individuals surviving for 66 months. Interestingly, different wild-type strains showed different maximum lifespans [Gerhard *et al.,* 2002].

A study on several tissues from marine species including zebrafish found that they have functional telomerase activity and telomere lengths similar to that found in human cells and tissues [McChesney *et al.,* 2005]. The authors also suggested the interest to study the mechanisms of telomere dysfunction during aging of fish in a single generation, which differs greatly from the multiple generational experiments necessary when using inbred strains of mice. A study using a small teleost fish *Oryzias latipes* showed that telomeres shorten during aging despite the fact that a considerable amount of telomerase activity is ubiquitously detected throughout the life of the fish [Hatakeyama *et al.,* 2008]. However, other longitudinal study on zebrafish reported that telomeres do not appreciably shorten throughout the lifespan of the fish in any studied organ [Lund *et al.,* 2009].

The zebrafish like many other teleosts undergoes gradual senescence with similarities to mammalian senescence, showing a slow decline in physiological functions and systems. Senescence-associated β-galactosidase (SA-β-gal), activity was detected in skin and oxidized protein accumulation in muscle from aging zebrafish [Kishi *et al.,* 2003]. Moreover, several mutants were identified using aging biomarkers as SA-β-gal and ROS metabolics, [Kishi *et al.,* 2009].

In addition, the zebrafish show a clear decline in cognitive performance with advanced age [Yu *et al.,* 2006]. Together, the different factors contributing to zebrafish organismal aging may be resumed in figure **13.**

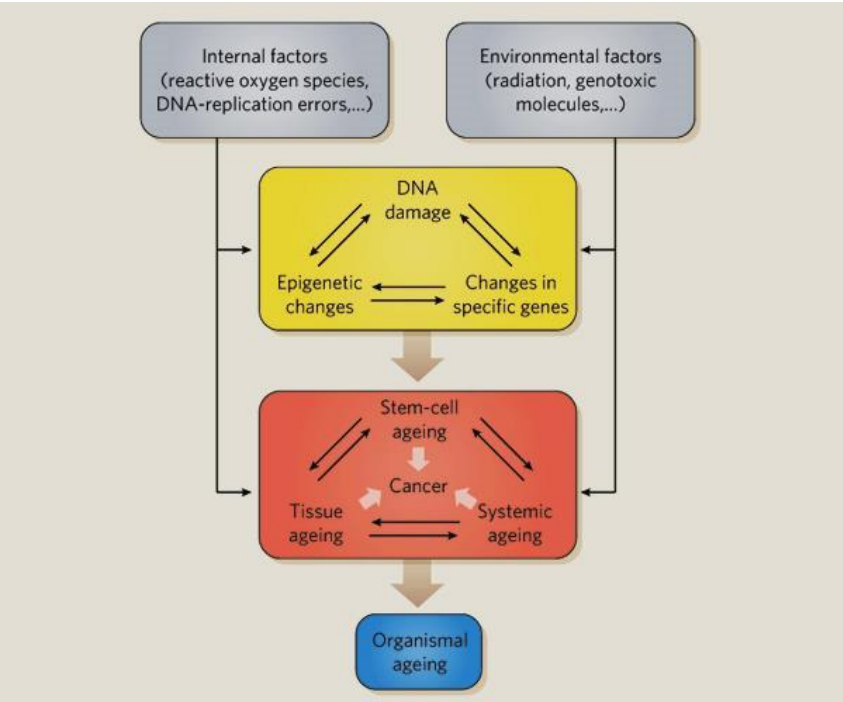


Figure 13. **Factors and networks influencing ageing.** As stem cells are essential for tissue homeostasis and repair throughout life, several groups have explored what factors influence alterations in their function with age. These studies, together with previous work, suggest that the story is complex, involving interactions between different networks and at several levels. At the genomic level, both internal and environmental factors cause alterations in individual genes, groups of genes through epigenetic changes, and chromosomes, at least some of which arise from direct damage to DNA. At the levels of cells and tissues, functional changes in stem cells and other cells in the tissue influence each other and are, in turn, influenced by systemic changes that may be conveyed from one tissue to another via the circulation. All of these may contribute to the possible development of cancer in tissues throughout the body. The ultimate outcome is organismal ageing. (*Adapted from Brunet & Rando, 2007*).

The previous and sometimes controversial results make necessary further studies to shed light on the telomere and telomerase behaviour during aging process. Thus, the zebrafish have been established as new model complementary to the mouse model to study aging and also telomerase dysfunction diseases.

In contrast to mammals, the zebrafish retains remarkable regenerative abilities in retina, fins, heart, spinal cord and other tissues to later advanced ages. Several studies have been reported: (i) neurons in most brain nuclei grew axons beyond the transection site into the distal spinal cord within 6 weeks [Becker *et al.*, 1997], (ii) a vigorous regeneration gave rise to new fibres after small mechanical lesions of skeletal muscle in *Brachydanio rerio* [Rowlerson *et al.*, 1997], (iii) new nephrons developed in

the weeks after the initial renal injury in adult zebrafish [Reimschussel, 2001], (iv) adult zebrafish fully regenerate hearts within 2 months of 20% ventricular resection [Poss *et al.,* 2002], (v) several works reported fast and complete regrowth of fin tissues after amputation in larval and adult zebrafish [Johnson & Weston, 1995; Poss *et al.,* 2003] (vi) retinal ganglion cells in adult zebrafish can regenerate their axons [Bernhardt *et al.,* 1996].

The regeneration process involves progenitor cells/resident stem cells [Brittijn *et al.,* 2009; Flores *et al.,* 2005]. In mammals, telomerase is expressed in germ cells and in the stem cell compartment of several adult tissues Moreover, the zebrafish has constitutively abundant telomerase activity in somatic tissues from embryos to aged adults [Kishi *et al.,* 2003; McChesney *et al.,* 2005]. Notably, a recent study on various tissues from aquatic species including the zebrafish suggests that telomerase may be important for tissue renewal and regeneration after injury rather than for overall organism longevity [Elmore *et al.,* 2008]. It has been proposed that telomerase may be important for tissue regeneration after injury as many marine animals have the capacity to regenerate, the constant expression of telomerase activity may be critical for cells to maintain telomere homeostasis during the regenerative process, as has been reported in a study that concluded the telomere length was unaffected after fin-clipping experiments [Lund *et al.,* 2009].

In fact, phenotypes associated with premature loss of tissue regeneration, including the skin (hair loss, hair greying, decreased wound healing) are found in mice deficient for telomerase [González-Suárez *et al.,* 2000; 2001; Cayuela *et al.,* 2005].

Together, these previous advances and the multiple advantages of the zebrafish make this an interesting and promising model to decipher the role of telomerase in regeneration.

.

Objectives

The specific objectives of the present work are:

1. Characterization of telomere length, telomerase expression and activity in zebrafish (*Danio rerio*) throughout life.

2. Characterization of the *tert*-deficient zebrafish line.

3. Study of telomere length, telomerase expression and activity in zebrafish (*Danio rerio*) during the caudal fin regeneration process with and without telomerase.

Chapter I:

Characterization of telomere and telomerase during aging and regeneration in zebrafish

Abstract

Telomere length and telomerase activity are important factors in the pathobiology of human diseases. Age-related diseases and premature aging syndromes are characterized by short telomeres, which can compromise cell viability, whereas tumour cells can prevent telomere loss by aberrantly upregulating telomerase. The zebrafish (*Danio rerio*) offers multiple experimental manipulation advantages over other vertebrate models and, therefore, it has been recently considered as a potential model for aging, cancer, and regeneration studies. However, it has only partially been exploited to shed light on these fundamental biological processes. The aim of this study was, therefore, to investigate telomere length and telomerase expression and activity in different strains of zebrafish obtained from different stock centres to determine whether they undergo any changes during aging and regeneration. We found that although both telomerase expression and telomere length increased from embryo to adulthood stages, they drastically declined in aged fish despite telomerase activity was detected in different tissues of old fish. In addition, we observed a weaker upregulation of telomerase expression in regenerating fins of old fish, which well correlates with their impaired regeneration capacity. Strikingly, telomeres were elongated or maintained during the fin regeneration process at all ages and after repeated amputations, likely to support high cell proliferation rates. We conclude that the expression of telomerase and telomere length are closely related during the entire life cycle of the fish and that these two parameters can be used as biomarkers of aging in zebrafish. Our results also reveal a direct relationship between the expression of telomerase, telomere length and the efficiency of tissue regeneration.

1. Introduction

During the last decade, the research on the processes of human aging and tumour formation has grown considerably in order to prevent or halt the progression of aging and cure age-associated diseases and cancer. Compared with other models, the zebrafish offers multiple experimental manipulation advantages and has recently been considered as a potential model for aging, cancer, and regeneration study [Lieschke & Currie, 2007; Feitsma & Cuppen, 2008; Gerhard & Cheng, 2002; Gerhard, 2007; Kishi, *et al*., 2003].

It is well established that the accumulation of cellular damage is at the origin of both cancer and aging. One of the best known molecular mechanisms of aging is the progressive attrition of telomeres with age both in human and mice [Harley *et al.*, 1990; Flores, *et al*., 2008]. Recent studies in the mouse model showed that telomerase might have a fundamental role in tumour growth and survival [Blasco, 2005]. Furthermore, short telomeres and defective telomerase activity have been involved in the pathobiology of several age-related diseases and premature aging syndromes, such as dyskeratosis congenita and aplastic anaemia [Mason & Bessler, 2004].

Telomeres are nucleoprotein complexes at the end of the chromosomes [Greider, 1996] which consist of TTAGGG repeats and several associated proteins [Blackburn, 1991]. As conventional DNA-dependent DNA polymerase fails to fully replicate the ends of linear molecules, 50–200 bp of telomeric DNA repeats are lost with every round of cell division. At the cell level, cell cycle arrest, cell senescence or crisis occur when telomeres reach a critical size, compromising cell viability. The telomerase, a reverse transcriptase compensates for the telomere loss in those cell types where it is expressed [Greider & Blackburn, 1985, 1987, 1989; Chan & Blackburn, 2002], but its level of activity in most adult tissues is not sufficient to counteract telomere shortening with aging [Collins & Mitchell, 2002]. In human, telomerase expression is principally restricted to highly proliferating cells (germ cells and progenitor/stem cells) in adults, whereas telomerase activity is expressed during human embryonic development [Broccoli *et al.*, 1995; Counter *et al*., 1995; Wright *et al*., 1996] and it is highly detectable in immortalized cells and many tumour cell tissues [Kim *et al*., 1994; Hiyama *et al*., 1995; Hiyama *et al*., 1995].

Although human organs have a limited ability to heal and regenerate damaged or lost tissue, the zebrafish retains remarkable regenerative abilities in retina, fins, heart, spinal cord and other regenerate damaged or lost tissue, the zebrafish retains remarkable

regenerative abilities in retina, fins, heart, spinal cord and other tissues to later advanced ages [Becker *et al*., 1997; Poss *et al.*, 2002; 2003; Reimschuessel, 2001; Rowlerson *et al*., 1997]. Moreover, the zebrafish has constitutively abundant telomerase activity in somatic tissues from embryos to aged adults [Kishi *et al*., 2003; McChesney *et al.*, 2005]. Notably, a recent study on various tissues from aquatic species including the zebrafish suggests that telomerase may be important for tissue renewal and regeneration after injury rather than for overall organism longevity [Elmore *et al*., 2008]. Despite this substantial advance, the advantages of this vertebrate model to decipher the role of telomerase in regeneration, aging and cancer have only been partially exploited. Therefore, the aim of this study was to investigate telomere length and telomerase expression and activity from different strains of *Danio rerio* obtained from different stock centres and to determine whether it undergoes any changes during aging. We found that the expression of telomerase and the telomere length are closely related during the entire life cycle of the fish and that these two parameters can be used as biomarkers of aging in zebrafish. Our results also reveal a direct relationship between the expression of telomerase, telomere length and the efficiency of tissue regeneration.

2. Materials and Methods

2.1. Ethics statement

The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University Hospital ‘‘Virgen de la Arrixaca’’ (Spain) for the use of laboratory animals.

2.2. Maintenance of zebrafish

Zebrafish (*Danio rerio*), AB (from the Zebrafish International Resource Centre (ZIRC), WIK (from ZIRC and Tübingen Zebrafish Stock Centre), TE and TL (Tübingen Zebrafish Stock Centre), were maintained in recirculating tanks following instructions from ‘‘The zebrafish handbook: a laboratory use of zebrafish, *Brachydanio rerio*’’. Adult fish were maintained at 26ºC, with a light:dark cycle of 14:10 hours and were fed twice daily, once with dry flake food (PRODAC) and once with live arthemia (MC 450, IVE AQUACULTURE). Zebrafish embryos were maintained in egg water at 28.5ºC and were fed at 5days with NOVO TOM and with live arthemia at 11 days of life.

The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and they were approved by the Bioethical Committee of the University Hospital Virgen de la Arrixaca (Spain) under approval number (PI06/FIS0369/040706).

2.3. The analysis of zebrafish telomerase expression

Total RNA was extracted from whole zebrafish embryos, whole zebrafish adults at several ages and different zebrafish tissues at several ages with TRIzol Reagent (Invitrogen), following the manufacturer’s instructions and treated with DNase I, Amplification grade (1 unit/mg RNA, Invitrogen). The SuperScript III RNase H2 ReverseTranscriptase (Invitrogen) was used to synthesize first strand cDNA with oligo-dT18 primer from 1 µg of total RNA at 50ºC for 60 min.

Real-time PCR was performed with an ABI PRISM 7700 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1

min at 60ºC, and finally 15 s at 95ºC, 1 min 60ºC, and 15 s at 95ºC. For each mRNA, gene expression was corrected by the rRNA11S content in each sample. The primers used were ZfTERT F2: 59-CGGTATGACGGCCYATCACT-39 and ZfTERT R1:59-TAAACGGCCTCCACAGAGTT- 39 for zebrafish TERT, and F:59-ACAGAAATGCCCCTTCACTG-39 and R:59-GCCTCTTCTCAAGGTTG- 39 for 11S rRNA.

2.4. Cell isolation

Zebrafish of different genotypes and stages were anesthetized with 0.05% benzocaine, briefly rinsed in 0.5% chilled bleach, crushed and incubated in PBS supplemented with antibiotics for 30 min., centrifuged (600 g, 5 min.), incubated in Trypsin (0.5 mg/ml)/EDTA (0.1 mg/ml) in PBS for 1 min., centrifuged (600 g, 5 min.) and then incubated in Colagenase (0.5 mg/ml) in RPMI medium supplemented with $CaCl_2$ $2H_20$ (0.7 mg/ml) for 30 min. The cell suspensions were obtained by pipetting, smashing and finally filtering the digested tissues through a 100 mm mesh, washed and resuspended in PBS.

2.5. TRF analysis

Telomere length assay was modified from Blasco et al., 1997. Cells were isolated and embedded in agarose plugs following instructions from the supplier (CHEF agarose plung kit from BioRad). Cells or DNA embedded in agarose plugs was digested with MboI and electrophoresed through 0.5% agarose gels in 0.5X TBE. Separation was for 6 hr at 120 V. The gel was blotted and probed with a 1.6 kb fragment containing the sequence (TTAGGG)n. Southern blot was hybridized to radioactively labelled probe at 65ºC in 0.01 g m^{-1} BSA, 200 mM sodium phosphate, 15% formamide, 1 mM EDTA, 7% sodium dodecyl sulphate (SDS). Filter (Hybond N+ Amersham Pharmacia Biotech) was washed three times in 0.2 X SSC, 0.1% sodium dodecyl sulphate at 65ºC for 30 min.

The telomeric specific probe was labelled with [a-32P]-dCTP using Ready-to-Go DNA labelling beads according to the supplier’s instructions (GE Healthcare).

2.6. Interphase Q-FISH

Q-FISH on interphases was performed as described in Canela *et al.,* 2007. Cell suspensions were obtained as described before. Cy3 and DAPI images were captured at 100x magnification using a Nikon Digital Camera DXM 1200C on a Nikon Direct Eclipse fluorescence microscope. Telomere fluorescence signals were quantified using the TFL-TELO program (from Peter Lansdorp, Vancouver, Canada).

Telomere fluorescence values were converted into kb by external calibration with the HeLa L cell line with known telomere length of 23.82 kb and with the L5178Y-S, L5178Y-R lymphocyte cell lines with known telomere lengths of 10.2 and 79.7 kb, respectively.

2.7. Flow-FISH

One million of cells from each fin sample obtained as described before were washed in 2 ml PBS supplemented with 0.1% BSA. Each sample was divided in two replicate tubes: one pellet was resuspended in 500 ml hybridization buffer and another in hybridization buffer without FITC-labeled telomeric PNA probe as negative control. Samples were then denatured for 10 minutes at 80ºC under continuous shaking and hybridized for 2 h in the dark at room temperature. After that, the cells were washed twice in a washing solution (70% deionized Formamide, 10 mM Tris, 0.1% bovine serum albumin (BSA) and 0.1% Tween-20 in dH_2O, pH 7.2). The cells were then centrifuged, resuspended in 500 ml of propidium iodide solution, incubated 2 h at room temperature, stored at 4ºC and analyzed by flow cytometry within the following 48 h.

2.8. Telomerase activity assays

The TRAPezeH XL Telomerase Detection Kit (Millipore, Cat.#S7707) was used to qualitatively measure the telomerase activity of whole zebrafish embryos extracts. The protein extracts were obtained according to the manufacturer's instructions. Human carcinoma cells (included in the telomerase detection kit) were used as a positive control. As a specific negative control, the higher protein concentration assayed of every sample extract was incubated with 1 mg of RNAse A (QIAGEN) at 37ºC for 20 min.

To quantitatively determine the telomerase activity of both whole zebrafish larvae and organ extracts from four fishes per group of 6, 12 and 30 months old, a real-time quantitative TRAP (Q-TRAP) analysis was performed as described by Herbert et al., (31). The protein extracts were obtained as described by the authors. For making the standard curve, a 1:10 dilution series of telomerase-positive simple (human carcinoma cells) was used. To quantify the telomerase activity, PCR amplification was performed as indicated by the authors. After PCR, real-time data were collected and converted into Relative Telomerase Activity units performing the calculation: RTA of sample =10 (Ct sample-Yint)/slope. The standard curve obtained was: y=23.2295x+23.802.

2.9. Tail regeneration assay

Zebrafish from AB strain at various stages (1.5, 3, 6, 18 and 24 months old) were anesthetized with 0.05% benzocaine. The fin tissue was removed within approximately 2 mm of the base of the caudal peduncle using a razor blade. The fish were allowed to recover and the animals were returned to recirculating wáter heated to 30ºC for the duration of the experiment. Each fish was tracked individually to calculate regeneration progress over time. Zebrafish fins were imaged before amputation and again on day 3, 5, 8 and 14 postamputation. Percent fin regeneration was determined based on the area of regrowth divided by the original fin area ± standard error.

To study the telomere length in caudal fin regeneration by flow-FISH assay, we obtained, by excision, caudal fin tissue (fin clip 1) from AB strain zebrafish at various life stages (3.5, 12, 18, 24 and 33 month-old) as described previously. A second and third excision of fin tissues were repeated with 5 days intervals to obtain regenerated tissues (fin clip2 and fin clip 3, respectively).

2.10. Statistical analysis

Data were analyzed by ANOVA and a Tukey multiple range test to determine differences between groups. The differences between two samples were analyzed by Student's *t* test.

3. Results

3.1. Telomerase expression increase during development and decrease with aging

We first analyzed the expression of TERT in four different zebrafish genotypes at larval and juvenile stages and also in several organs throughout life (**Fig. 1**). The results showed a significant increase of telomerase expression between the larval and the juvenile stage of zebrafish (**Fig. 1A**) in the four analyzed genetic backgrounds. In addition, telomerase expression also increased during the developmental stages in all organs, but drastically declined in older fish with the exception of the muscle (**Fig. 1B**).

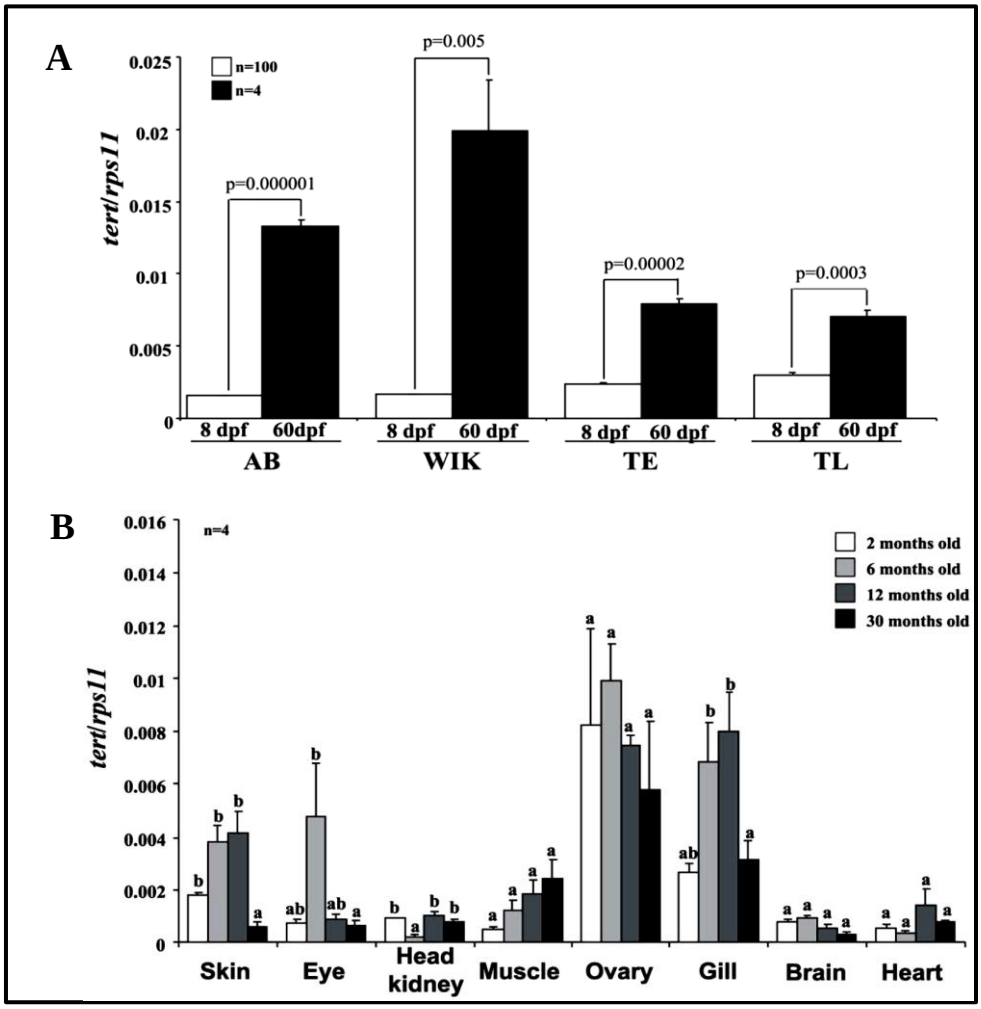
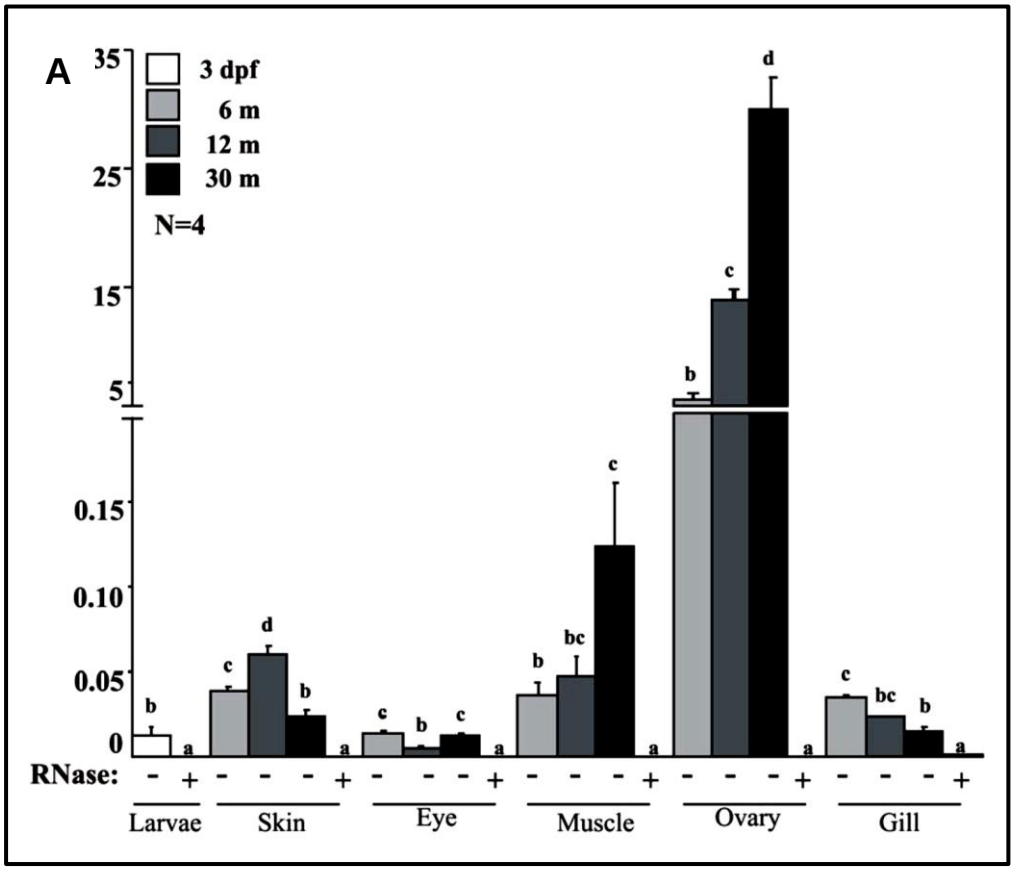


Figure 1. Dynamic of TERT gene expression in the zebrafish. The mRNA levels of tert gene were determined by real-time RT-PCR in larval and juvenile stages of the indicated genotypes (**A**) and in different tissues of 2–30 month-old fish of the AB genotype. Gene expression is normalized against rps11. Each bar represents the mean ± S.E. from 100 pooled animals for larvae and 4 individual fish for all the rest (**A, B**) and triplicate samples.

3.2. Telomerase activity is detected in zebrafish of all ages

Because telomerase activity was regulated at different levels, we examined the correlation between zebrafish TERT transcript levels and telomerase activity, assayed by conventional Telomerase Repeat Amplification Protocol (TRAP) and Q-TRAP (Quantitative), which has been reported to be a rapid and accurate assay for the quantification of telomerase activity [Wege *et al.*, 2003; Herbert *et al.*, 2006].

Telomerase activity was detected in larvae as well as in different tissues from different age fish, including 30-month-old fish (**Fig. 2**). To confirm the specificity of the TRAP assay in zebrafish samples, we pretreated the protein extracts with RNase. This treatment abolished telomerase activity and served as a negative control. We obtained a good correlation between telomerase expression and telomerase activity in most samples (**Figs. 1B** and **2A**). Notably, we found very high telomerase expression and activity in ovary, even though very old female (30 months old) are not fertile. The muscle showed increased telomerase activity during aging. However, we found a significant decrease in telomerase activity during aging (between 12 and 30 months) in gill and skin, and an invariable activity in the eye.



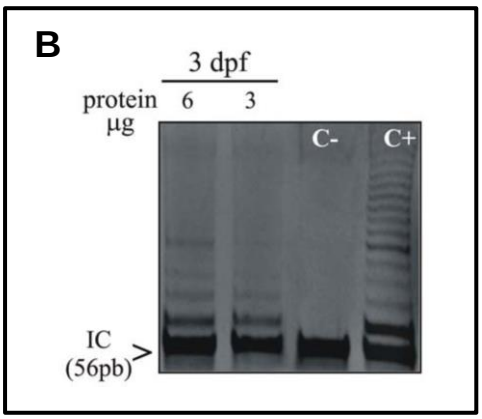


Figure 2. **Very old fish have telomerase activity.** Telomerase activity was measured quantitatively and qualitatively in whole zebrafish embryos (3 days post-fertilization, dpf, n = 100) and in several organs from adults with different ages (6, 12 and 30 months old, n = 4). **A**, Q-TRAP assay using 1 mg of protein extract. Results are expressed as the mean value ± S.E. from triplicate samples relative to telomerase-positive cells. Different letters denote statistically significant differences between different ages of each sample according to a Tukey test. **B**, TRAP assay using protein extract from whole zebrafish embryos. A ladder of bands indicates the presence of telomerase activity. The lowest band (56 pb) is the internal control (IC). Lanes C- and C+ correspond to telomerase- negative and positive controls, respectively. In all cases, to confirm the specificity of the assay, a negative control is included for each sample, treated with 1 mg of RNAse at 37ºC for 20 min. The Q-TRAP assay was also performed using 0.1 mg of protein extract and the same relative results were obtained (data not shown).

3.3. Telomere length and telomere shortening in zebrafish

In normal somatic cells, telomeres shorten with each cell division. Telomerase activity is thought to be required for telomere length maintenance in mammals, as it is in yeast. Telomerase deficient mice show telomere shortening [Blasco *et al.*, 1997]. At present, the actual length of zebrafish telomeres and how telomere length changes with the age of this species are both unknown.

To determine telomere zebrafish length and if telomere shortening occurs even though telomerase activity is present in somatic tissues throughout their lives, we used both the TRF-Southern blot and the Q-FISH techniques. TRF analysis revealed that telomere length in adult zebrafish is around 16 kb ± 3.5 Kb (**Fig. 3**), as reported by others [Kishi *et al.*, 2003; McChesney, *et al.*, 2005; Elmore *et al.*, 2008; Lund *et al.*, 2009]. TL zebrafish background showed shorter telomere that the other background at all studied ages (**Fig. 3**). However, we also found that telomere length increased during development until a critical time (around 18 months of age) when it started to decrease (**Figs. 3C–D**).

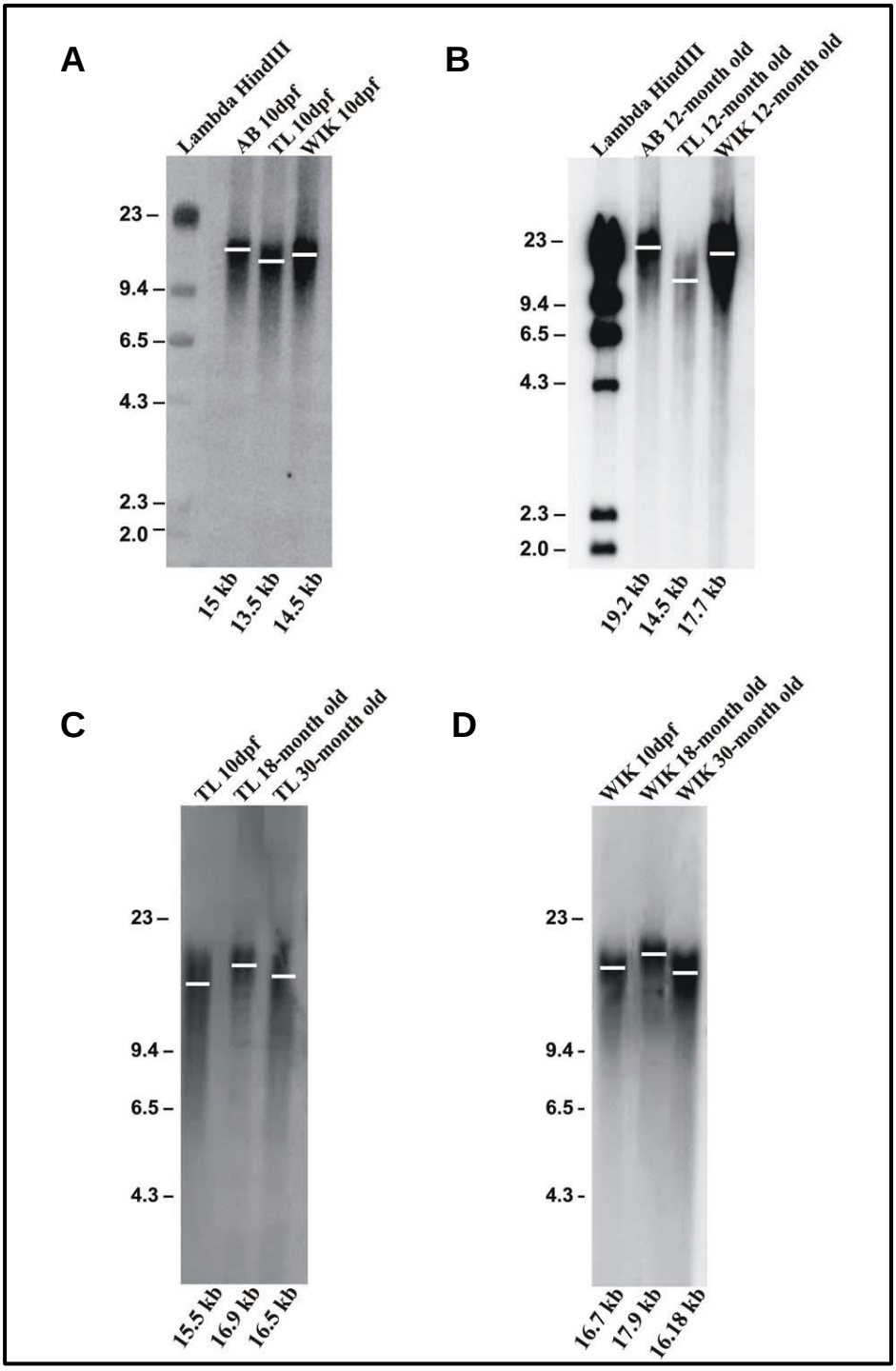


Figure 3. Dynamic of telomere length in zebrafish assayed by TRF. A representative TRF shows telomere length of different zebrafish background from larvae (**A**) and adults (**B**), and in TL (**C**) and WIK (**D**) zebrafish genotypes throughout their life cycles. Telomere length (Kb) is calculated using a quantitative algorithm involving the signal intensity of each telomere smear and the migration of the lambda HindIII ladder (lane 1). 3–5 independent TRF experiments were perform. We used a zebrafish embryos (10 dpf, n = 100) and adult fish for each strain with different ages (12, 18 and 30 months old, n = 3–4).

To further confirm the results obtained using the TRF assay, we analyzed telomere length variation throughout the life of the zebrafish using the more sensitive and specific Q-FISH method. The low detection limit of Q-FISH (,0.1 kb of telomere repeats) allows quantification of very short telomeres (1–3), and the use of a fluorescent peptide nucleic acid (PNA) probe against telomeric repeats facilitates the specific labelling of telomeres at the single cell level [Lansdorp *et al.,* 1996; Poon *et al.,* 1999; Zijlmans *et al.,* 1997]. In mammals, a recent study showed that mean telomere length values obtained using the conventional Q-FISH analysis correlated excellently with telomere length values obtained using Q-FISH in interphase nuclei [Canela *et al*., 2007]. We used QFISH in interphase nuclei to determine the mean telomere length values of the whole organism in three zebrafish genotypes (AB, WIK and TL) at different ages. These results allowed us to generate telomere length frequency histograms and determinate the percentage of cells with very short telomeres (<1000 a.u.f.) and very long telomeres (>3000 a.u.f.). Our results were consistent with those obtained previously by TRF analysis (**Fig. 3**). In the case of the AB genotype, the telomere length showed a statistically significant increase between the larvae stage at 8 days post fertilization (1338.70 a.u.f. ± 45.14) and the juvenile stage at 2 months of life (2104.75 a.u.f. ± 58.08). After that, the telomere length of the young adult zebrafish at 7 months of life showed a higher mean value (2337.65 a.u.f. ± 109.57), which remained unchanged at the adult age of 18 months of life (2327.59 a.u.f. ± 85.09). Finally, we observed a very significant decrease (1754.37 a.u.f. ± 58.47) in 24 month-old fish. (**Fig. 4A**) Telomeres lengthening during development as well as its shortening in old fish were very well illustrated by the telomere length frequency histograms (Fig. 4B). In the three genotypes, the percentage of very long telomeres (.3000 a.u.f.) showed its highest value at the adult age (18 month-old) and fell drastically at old age (24 month-old). Thus, we found 32.20% versus 7.86% in AB, 29.26% versus 11.67% in WIK, and 30.77% versus 18.90% in TL. As shown in **Fig. 4A** and **4B**, both average telomere length and telomere length frequency histograms were very similar in the different zebrafish genotypes. However, the TL background always showed an average telomere length shorter than the other strains. In general, zebrafish has a telomere length in the range of 12.13 kb ± 0.41 to22.28 kb ± 0.04.

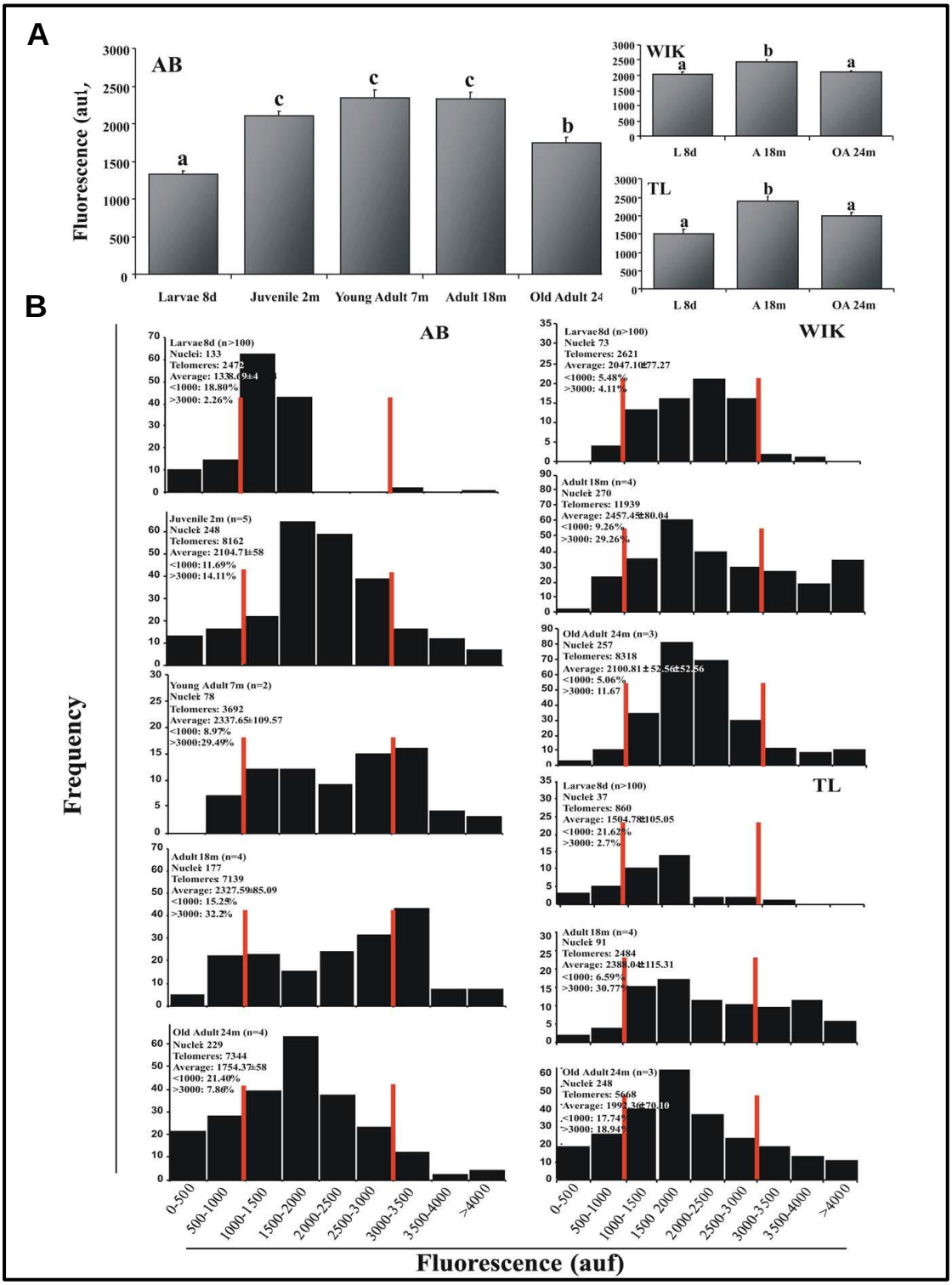


Figure 4. Dynamic of telomere length assayed by Q-FISH. A, Graph showing the mean telomeric fluorescence values. Data are mean values ± S.E. and statistical significance was assessed using the Tukey test (p,0.05). **B**, Histograms showing telomere fluorescence frequencies. The red lines demarcate both the shortest (,1000 auf) and the longest telomere percentage (.3000 auf) clearly illustrating telomere lengthening throughout the life cycle to adulthood and shortening in old age.

3.4. Impaired caudal fin regeneration with aging

The zebrafish, as most aquatic animals, has a high capacity for tissue regeneration and the importance of telomerase upregulation during the repair of injured tissues has already been tested in other fish species [Elmore *et al*., 2008]. To establish this relationship in zebrafish, we performed a caudal fin regeneration assay to assess the regeneration process at the different zebrafish ages. Our results (**Fig. 5A**) showed that old adult zebrafish have a lower growth rate, reaching 50% of their caudal fin regeneration 12 days post-amputation (dpa) and did not display normal caudal fin regeneration (**Fig. 5B**).

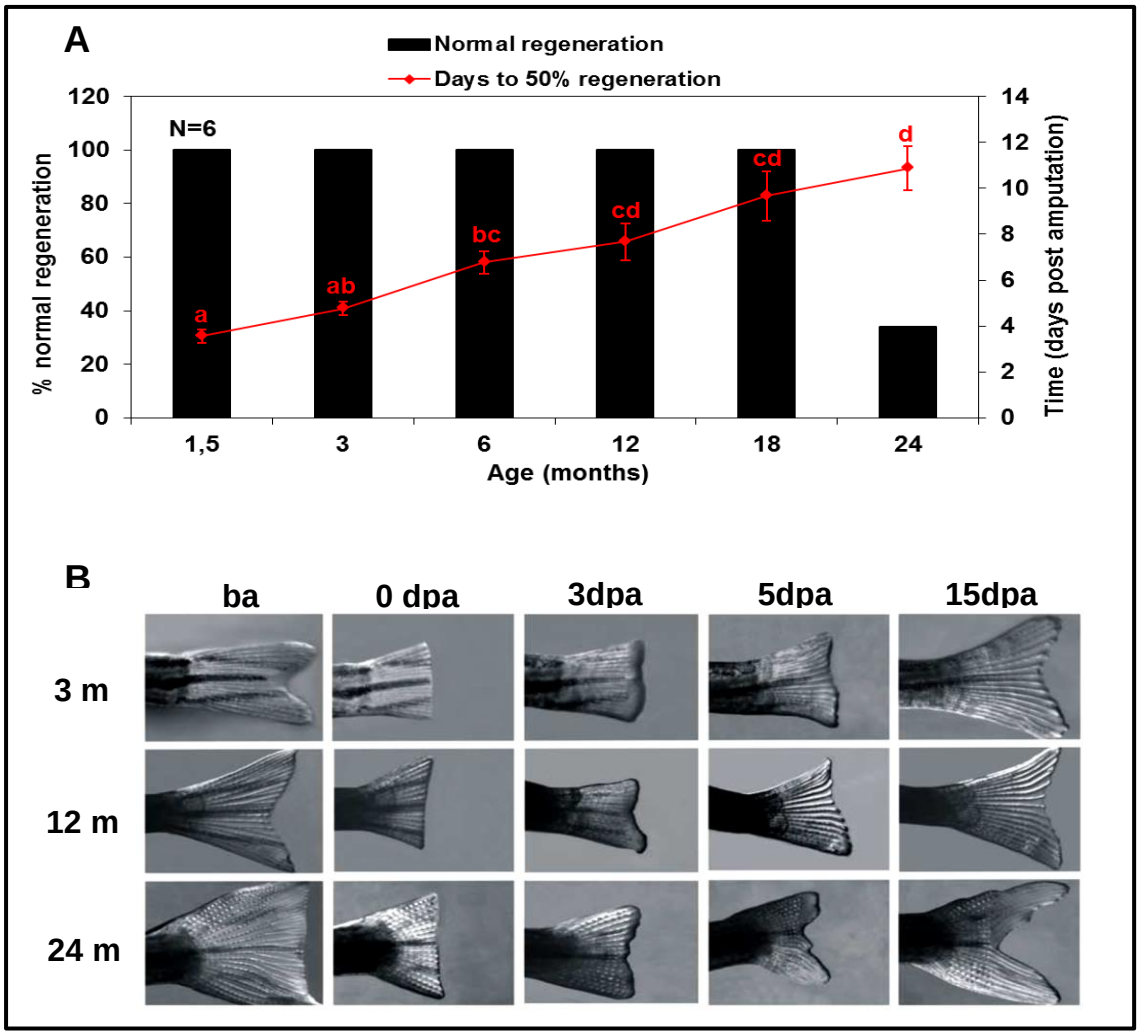


Figure 5. Old fish show impaired regeneration response. A, Percent fin regeneration was determined based on the area regrowth divided by the original fin area, n = 6 (ba: before amputation, dpa: days post-amputation). Black bars show normal caudal fin regeneration and red line indicate the days to reach 50% of the regeneration. **B**, Representative image of the fin regeneration assay.

These results suggest a direct correlation between the lower expression of telomerase and the impaired caudal fin regeneration in older fish. We analyzed the telomerase expression from caudal fin tissues of 3, 12 and 24 month-old zebrafish before and after amputation, by real-time RT-PCR. We observed a significant decrease of telomerase expression with aging (**Fig. 6A**), upregulation in 3 month old zebrafish (58% of upregulation compared with basal expression) and caused weak increased telomerase expression in 24 month old fish (18% of upregulation) at 5dpa (**Fig. 6B**).

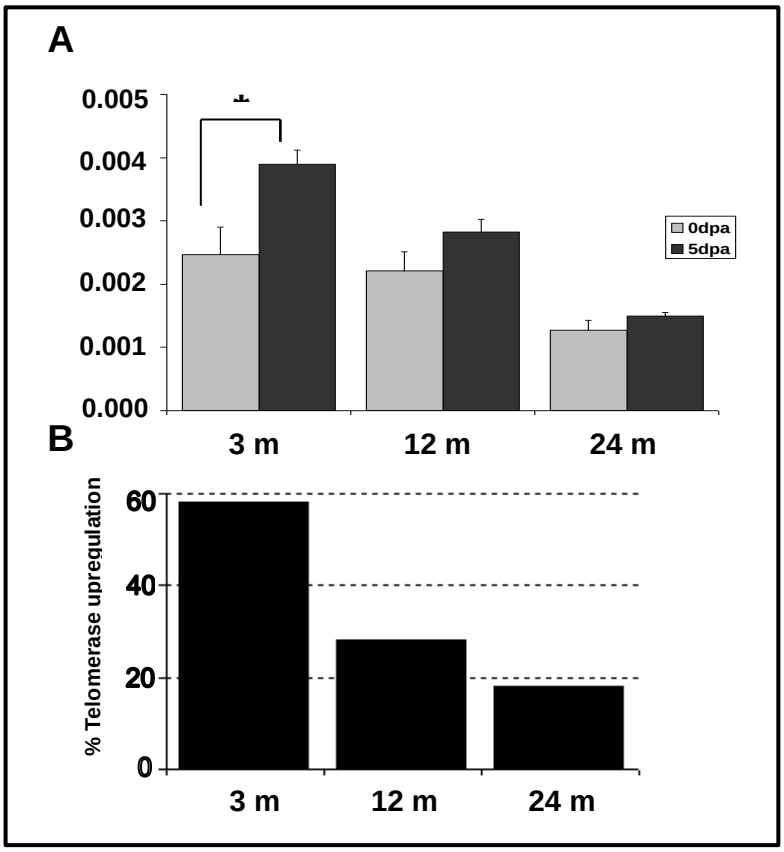


Figure 6. Analysis of TERT expression in regenerating fin. A, At 0 and 5 dpa, assayed by real time RT-PCR (n=6). Gene expression is normalized against rps11. Data are mean values ± S.E. **B,** Percentage of telomerase upregulation at 5 dpa.

The incomplete and deficient fin regeneration in old zebrafish might be correlated with the decreased telomerase expression and telomere length observed at this stage of life. In order to establish whether the upregulation of telomerase expression during the regeneration process has any physiological consequence, we measured telomere length in caudal fin tissues at different fish ages before and after the regeneration process. We found using flow cytometry-fluorescence in situ hybridization (Flow-FISH: **Fig. 7A–B**)

that the telomere length of cells from the caudal fin increased during development until a critical age of 18 months, when it gradually decreased. While shortest telomeres were elongated during the life cycle, the percentage of longest telomeres increased until 18 months (**Fig. 7B**). These results are in agreement with our TRF and Q-FISH data using whole organisms in three zebrafish genotypes (**Figs. 3** and **4**).

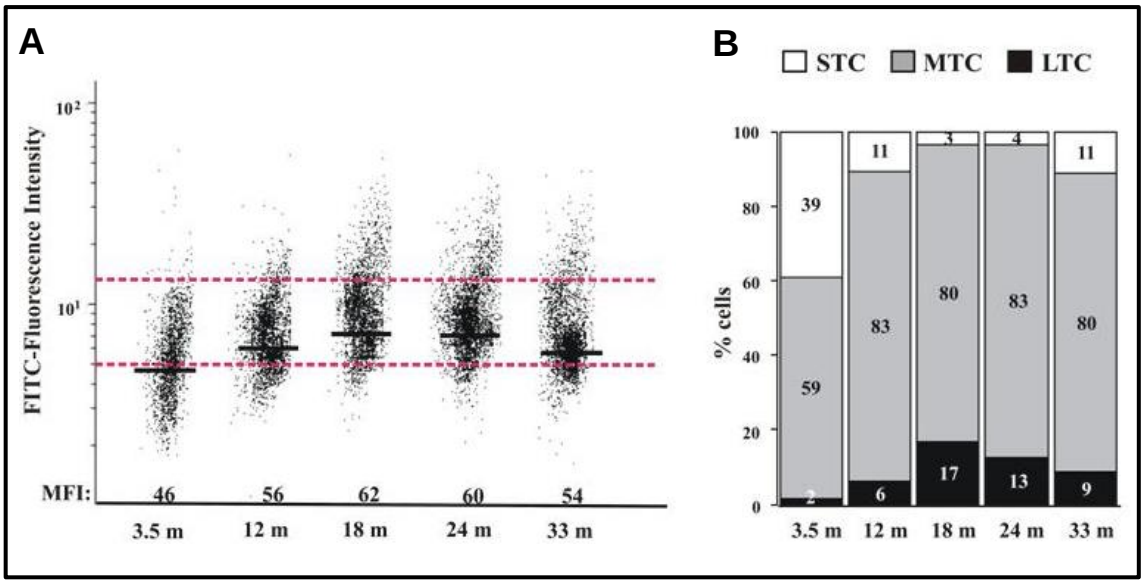


Figure 7. Dynamic of telomere length in zebrafish caudal fin assayed by Flow-FISH. A, Representation of the zebrafish caudal fin cells distribution (clip 1) according to their telomere length., Medium Fluorescence Intensity (MFI) is indicated for each age, (n = 5). The same trend was observed in the three independent experiments. **B**, Graphic representation of the percentage of cells with long telomere (LTC), medium telomere (MTC), and short telomere (STC), delimited by dotted red lines, from clip1 at different ages

We next sought to determine the effects of several amputations in telomere behaviour, when the caudal fin was amputated (clip one) and allowed to regenerate for 5 days (clip two, **Fig. 8**).

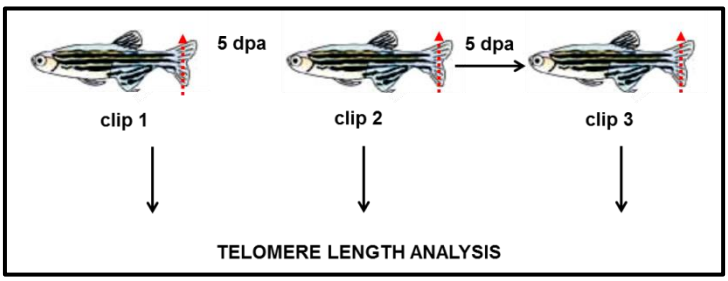


Figure 8. Experimental design of the Flow-FISH assay. Clip 1 (1st fin excision), clip 2 (2nd excision) and clip 3 (3rd excision).

We found that the average telomere length increased at all ages, as indicated in **Figure 9** by the mean fluorescent intensity (MFI). Strikingly, while, very young fish (3.5 month-old) were able to further increase their telomere length after a third amputation, older fish showed weak telomere attrition but were able to maintain their original telomere length. The inefficient activation of telomerase expression in oldest fish might be responsible for this behaviour. We also observed that short telomeres decreased, whereas long telomeres increased, after the second clip. In the third clip, however, the percentage of cells with short telomeres increased again in older fish. Collectively, these results suggested that the upregulation of telomerase expression was sufficient to increase telomere length during the rapid cellular proliferation associated with the regeneration process, but after reclipping telomere length was not maintained, except in very young fish (3 month-old) that show a robust telomerase induction.

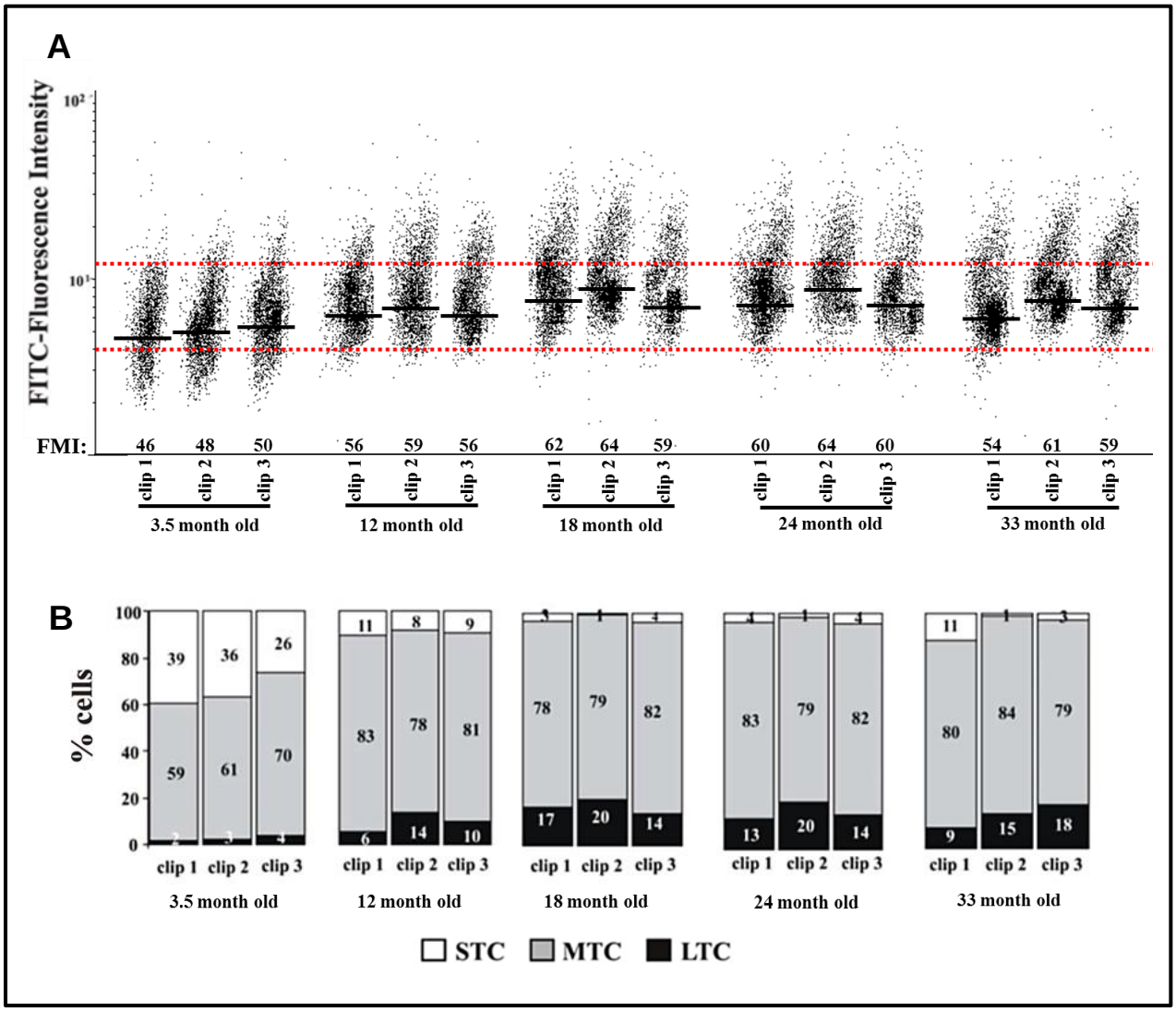


Figure 9. Behaviour of telomere length during fin regeneration by Flow-FISH assay. A, Representation of the zebrafish caudal fin cells distribution (clip 1, clip2 and clip3) according to their telomere length. MFI is indicated for each age, (n = 5). The same trend was observed in the three independent experiments. **B**, Graphic representation of the percentage of cells with long telomere (LTC), medium telomere (MTC), and short telomere (STC), delimited by dotted red lines, from clip1, clip 2 and clip 3, at different age.

4. Discussion

Although, it has been extensively shown that telomeres and telomerase are involved in mammalian aging and cancer promotion and that the zebrafish is now widely used as a good model organism for assessing these complex processes [Gerhard & Cheng, 2002; Gerhard, 2007; Kishi *et al.*, 2009] so far there have only been a few incomplete studies about telomeres and telomerase biology in this species. The zebrafish, with a life-span of 3 years, is the only vertebrate system in which telomerase function can be studied in a high-throughput manner. We used this species to perform an exhaustive study of telomerase expression and telomere length among a broad range of tissues throughout its life.

A high level of *TERT* gene expression has been reported in almost all zebrafish tissues in contrast to what happens in the corresponding mammalian tissues [Lau *et al.*, 2008]. Our results show that telomerase is expressed in all tissues tested at different stages of life (larvae, juvenile, adult and old fish). However, the expression of *TERT* mRNA drastically decreased in all tissue examined of old fish (more than two years old), with the exception of the muscle. These results are in line with published data in which continuously proliferating myocytes have also been observed although there was no lipofucsine granule accumulation in the muscle of zebrafish with advancing age [Kishi, 2004]. These data correlated well with the presence of telomerase activity in all tissues examined of adult fish of different ages and with the significant decrease of telomerase activity in old zebrafish samples, with the exception of muscle and ovary samples.

In all the analyzed strains, we observed an increased telomere length from larvae to adult fish and a significant telomere shortening in aged fish. Our results are not in agreement with recently published data [Lund *et al*., 2009] in which, using a TRF assay, the authors conclude that telomeres do not shorten with age. These discrepancies might be explained by the methodology used. It is well-known that the TRF technique is not sensitive enough for detecting changes in telomere length and it is difficult to detect short telomeres, something that is crucial in these aging studies [Canela *et al*., 2007].

Therefore, we combined this technique with the most accurate Q-FISH technique. Moreover, all our studies have been performed using whole organisms from various strains obtained from different stock centres in order to avoid a particular telomere length associated with a strain and/or to a given stock centre. These results were also

confirmed using a specific tissue, such as the caudal fin, where we observed by Flow-FISH that telomere length increased until 18 month old and gradually decreased after this age. Overall, our results showed that the three wild type strains analyzed showed slightly different telomere lengths. TL is the genetic background showing the shortest telomeres and such differences may have an impact on cancer susceptibility and aging. In fact, we have observed that the life-span of the TL background is less than 3.5 years, while the AB and WIK strains have longer life-spans in our zebrafish facility. Further studies should be performed to establish whether these differences are important in aging and cancer processes.

In this study, we have also established that there is a direct relation between the levels of telomerase expression, telomerase activity and telomere length in zebrafish. Haploinsufficiency for *TERT* leads to premature telomere shortening in human and causes the aging disease known as dyskeratosis congenita [Armanios *et al*., 2005; Du *et al*., 2007]. This means that telomerase levels control telomere length in human and in zebrafish, and therefore, high *TERT* expression might prevent telomere erosion and delay senescence in adult animals.

In contrast to mammals, lower vertebrates have a remarkable capacity to regenerate complex structures after damage, including heart, spinal cord, retina and fins. This process involves progenitor cells/resident stem cells [Brittijn *et al*., 2009; Flores *et al*., 2005]. In mammals, telomerase is expressed in germ cells and in the stem cell compartment of several adult tissues. It has been proposed that telomerase may be important for tissue regeneration after injury. In fact, phenotypes associated with premature loss of tissue regeneration, including the skin (hair loss, hair greying, decreased wound healing) are found in mice deficient for telomerase [González-Suárez *et al*., 2000; 2001; Cayuela *et al*., 2005]. Our results showed that the zebrafish was able to regenerate the amputated fin at all ages but the fish with the lowest level of telomerase expression; i.e. older fish, had severely impaired fin regeneration. Indeed, the efficiency of regeneration showed a direct correspondence with telomerase expression (3 days/young fish versus 12 days/old fish). In fact, we have observed an increase in *TERT* expression in caudal fin, when amputated in young (3 month-old) and old fish (24 month-old), but only significant in young fish (58% of upregulation). Therefore, young fish responded better than old fish after injury. Importantly, the upregulation of telomerase expression was correlated with telomere length behaviour after reclipping, as the different age groups responded to the injury lengthening their

telomeres. Our data were consistent with the idea that telomeres would need to be maintained during increased cell proliferation associated with tissue renewal. Although the strong upregulation of telomerase expression observed in 3 month old fish did not correlate with a strong telomere length increase, this group was the only one able to elongate their telomeres after consecutive amputations. However, fish older than 3 months, although showing an increase in telomere length after a second clip, are not able to maintain this elongation after a prolonged injury (clip 3). This behaviour might be related to the inefficient activation of telomerase expression in fish older than 3 months. Curiously, whereas the oldest fish had a similar telomeric response to adult fish, they showed incomplete and deficient fin regeneration. However, regeneration is a complex process in which many genes/factor are involved. Therefore, further studies are required to clarify the role of telomerase and telomere length in regeneration. Further gain- and loss-of-function experiments for two telomerase components will shed light on the role of telomerase in regeneration.

Another important insight of our study is the declined telomerase expression, telomere length and regeneration capacity as biomarkers for aging in zebrafish. Until now, SA β-Gal activity, melatonin deficiency and cognitive function were all used as aging biomarkers [Kishi *et al*., 2008; Yu *et al*., 2006; Tsai *et al*., 2007]. These results indicate that telomere length, telomerase expression and regeneration capacity are highly dependent on zebrafish age and, therefore, they are useful for evaluating the aging process of zebrafish.

Further studies are necessary to establish if all the cells express telomerase or only a specific population. A transgenic zebrafish expressing a reporter driven by *TERT* promoter might be useful for identifying in vivo cells with high/low telomerase activity, i.e. progenitor- stem cells/aging cells. Although there are obvious differences between human and zebrafish, such as the high expression of *TERT* alongside its life-span, both species show declined telomere expression and telomere length with aging. We therefore propose that the zebrafish can be used to identify genes and drugs that affect the ability to restore aging phenotypes using telomere length or telomerase expression, which have been identified as good aging biomarkers in this study.

Chapter II:

Characterization of the telomerase-deficient zebrafish line

Abstract

The study of telomere biology is crucial to the understanding of aging and cancer. In the pursuit of greater knowledge in the field of human telomere biology, the mouse has been used extensively as a model. However, there are fundamental differences between mouse and human cells. Therefore, additional models are required. In light of this, we have characterized telomerase-deficient zebrafish (*Danio rerio*) as the second vertebrate model for human telomerase-driven diseases. We found that telomerase-deficient zebrafish show p53-dependent premature aging and reduced lifespan in the first generation, as occurs in humans but not in mice, probably reflecting the similar telomere length in fish and humans. Among these aging symptoms, spinal curvature, liver and retina degeneration, and infertility were the most remarkable. Although the second-generation embryos died in early developmental stages, restoration of telomerase activity rescued telomere length and survival, indicating that telomerase dosage is crucial. Importantly, this model also reproduces the disease anticipation observed in humans with dyskeratosis congenita (DC). Thus, telomerase haploinsufficiency leads to anticipation phenomenon in longevity, which is related to telomere shortening and, specifically, with the proportion of short telomeres. Furthermore, p53 was induced by telomere attrition, leading to growth arrest and apoptosis. Importantly, genetic inhibition of p53 rescued the adverse effects of telomere loss, indicating that the molecular mechanisms induced by telomere shortening are conserved from fish to mammals. The partial rescue of telomere length and longevity by restoration of telomerase activity, together with the feasibility of the zebrafish for high-throughput chemical screening, both point to the usefulness of this model for the discovery of new drugs able to reactivate telomerase in individuals with DC.

1. Introduction

In most organisms telomeres are composed of simple repetitive sequences whose length is maintained by the telomerase ribonucleoprotein. Telomerase contains two essential components: the telomerase reverse transcriptase (TERT) and telomerase RNA (TR) which provide the template for the reverse transcription of new telomere DNA by TERT [Blackburn, 1991]. Loss of telomerase function results in progressive telomere shortening and chromosomal instability which ends up having implications for aging and cancer ([Blasco *et al*., 1997]. Therefore, the study of telomere biology is critical to the understanding of these two processes [Blasco, 2005]. The mouse has been used extensively as a model for human telomere biology [Goytisolo & Blasco, 2002]. However, there are strong differences between mouse and human telomere length [Autexier, 2008]. Therefore, other models have been characterized to clarify the role played by telomerase in aging and cancer, such as *Saccharomyces cerevisiae* [Cohn & Blackburn, 1995], *Caenorhabditis elegans* [Wicky *et al*., 1996], *Arabidopsis thaliana* [Fitzgerald *et al*., 1999], *Gallus gallus* [Swanberg *et al*., 2010] and *Danio rerio* [Anchelin *et al.*, 2011]. The zebrafish (*Danio rerio*) has the potential to emerge as a key vertebrate model in this regard. In fact, during the last decade, several studies about the role of TERT complex in aging, cancer and regeneration have been reported [Imamura *et al*., 2008]. The zebrafish TERT and TR components have been cloned and characterized [Lau *et al*., 2008]. In terms of telomere length, zebrafish telomeres (15-20 kb) are relatively similar to human telomeres (10-15 kb). Although telomerase is constitutively active in multiple organs, unlike the situation in the corresponding mammalian tissues, the expression of TERT mRNA, telomerase activity and telomere length all decreased drastically in almost all the fish tissues older than 18 months of age that we examined, indicating that they are useful markers for evaluating the aging process in zebrafish [Anchelin *et al.*, 2011].

The use of morpholino-based gene knockdown in zebrafish allows for the modelling of the role of telomerase and telomeres in premature aging syndromes, such as dyskeratosis congenita, a human disease characterized by shortened telomeres [Calado & Young, 2009; Kirwan & Dokal, 2009]. Thus, TERT or dyskerin knockdown results in embryonic hematopoietic defects at the onset of circulation characterized by the impaired differentiation of blood cells and their eventual apoptosis [Kishi *et al*., 2008; Zhang *et al*., 2012], as occurs in human dyskeratosis congenita and hypochromic anaemia. Furthermore, a number of zebrafish mutants have now been developed as

models for human telomeric diseases. For example, the mutation in Nap10, one of the known H/AXA RNP complex genes with mutations linked to dyskeratosis congenita [Pereboom *et al*., 2011], or the mutation in the telomeric repeat binding factor 2 (TRF2) [Kishi *et al*., 2008] have both shown premature aging phenotypes. Therefore, the zebrafish is a powerful model which offers a unique opportunity to contribute to the advancement in biological and behavioural gerontology. The availability of mutant genotypes with identified aging phenotypes, in combination with a wealth of information about zebrafish development and genetics as well as the existence of multiple mutant and transgenic lines, should significantly facilitate the use of this outstanding vertebrate model in deciphering the mechanisms of aging, and in developing preventive and therapeutic strategies to prolong the productive lifespan ('healthspan') in humans.

We have thus initiated the characterization of the first zebrafish model of the *tert* gene. Using an identified and publicly available mutant line that carries a mutation in the telomerase gene that was generated by ENU mutagenesis at the Sanger Institute Zebrafish Mutant Project (termed hu3430), we have defined the behavioural, morphological, functional and histopathological features of *tert* mutant zebrafish. The first generation of mutant animals showed patent reduction of telomere length, premature aging, decreased fertility and a shorter lifespan than wild-type zebrafish. These results suggest that telomerase function is critical for organ homeostasis in zebrafish, as occurs in the mouse [Blasco *et al*., 1997; Chiang *et al*., 2004; Yuan *et al*., 1999], but only in the first generation. In fact, these mutants can only be bred for one generation due to their reduced fitness levels. The second generation showed a high percentage of abnormal phenotypes and embryonic lethality, which correlated with drastic telomere shortening and genomic instability. Notably, telomere attrition triggered p53-dependent apoptosis in early embryonic stages and p53 deficiency rescued the adverse effects of telomere loss, as reported in the mouse [González-Suárez *et al*., 2000; Leri *et al*., 2003]. Therefore, this zebrafish model provides a new platform for examining novel TERT functions in the aging process and discovering drugs for the treatment of premature human aging disorders [Zon & Peterson, 2005].

2. Materials and Methods

2.1. Maintenance of zebrafish

Wild-type AB zebrafish (*Danio rerio*) were obtained from the Zebrafish International Resource Center (ZIRC). The *tert* mutant line (allele hu3430) was obtained from the Sanger Institute and the *p53*mutant line *zdf1* (P53M214K) [Berghmans et al., 2005] was kindly provided by Leonard I. Zon (HSCRB, Harvard University, Cambridge, MA). Zebrafish were maintained in recirculating tanks following instructions from '*The zebrafish book*' (Westerfield, 2000). Adult fish were maintained at 26°C, with a 14:10 hour light:dark cycle and were fed twice daily, once with dry flake food (PRODAC) and once with live artemia (MC 450, INVEAQUACULTURE). Zebrafish embryos were maintained in egg water at 28.5°C and were fed at 5 days with NOVOTOM and with live artemia at 11 days of life.

The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and were approved by the Bioethical Committee of the University Hospital Virgen de la Arrixaca (Spain) under approval number PI06/FIS0369/040706.

2.2. Cell isolation

Zebrafish were anesthetized at different stages with 0.05% benzocaine, briefly rinsed in 0.5% chilled bleach, crushed and incubated in phosphate buffered saline (PBS) supplemented with antibiotics for 30 minutes, centrifuged (600 ***g***, 5 minutes), incubated in trypsin (0.5 mg/ml)/EDTA (0.1 mg/ml) in PBS for 1 minute, centrifuged (600 ***g***, 5 minutes) and then incubated in collagenase (0.5 mg/ml) in RPMI medium supplemented with $CaCl_2$ $2H_2O$ (0.7 mg/ml) for 30 minutes. The cell suspensions were obtained by pipetting, smashing and finally filtering the digested tissues through a 100 μm mesh, washed and resuspended in PBS.

2.3. Telomerase activity assay

A real-time quantitative TRAP (Q-TRAP) analysis was performed as described by Herbert et al. [Herbert *et al*., 2006]. The protein extracts were obtained as described by the authors. To quantify the telomerase activity, PCR amplification was performed as

indicated by the authors. We performed the standard curve as described in Anchelin *et al.*, 2011. After PCR, realtime data were collected and converted into relative telomerase activity (RTA) units performing the calculation: RTA of sample=10 (Ct sample-γint)/slope. The standard curve obtained was: y=–3.2295x+23.802.

2.4. qPCR analysis

Total RNA was extracted from whole zebrafish larvae and different zebrafish tissues at several ages using the TRIzol Reagent (Invitrogen), following the manufacturer's instructions, and was treated with DNase I Amplification grade (1 unit/µg RNA, Invitrogen). The SuperScript III RNase H-Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligodT18 primer from 1 µg of total RNA at 50°C for 60 minutes.

Real-time PCR was performed with an ABI PRISM 7700 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 minutes at 95°C, followed by 40 cycles for 15 seconds at 95°C, for 1 minute at 60°C, and finally for 15 seconds at 95°C, for 1 minute at 60°C, and 15 seconds at 95°C. For each mRNA, geneexpression was corrected by the ribosomal protein S11 (*rps11*) content in each sample. The primers used were TERT-F2: 5_- CGGTATGACGGCCTATCACT-3_ and TERT-R1: 5_-TAAACG - GCCTCCACAGAGTT-3_ for zebrafish *tert*, P53-F: 5_-GATA - GCCTAGTGCGAGCACAC-3_ and P53-RWT: 5_-AGCTGCATGGGGGGGAT- 3_ for zebrafish *p53*, and F: 5_-ACAGAAAT -GCCCCTTCACTG-3_ and R: 5_-GCCTCTTCTCAAAACG - GTTG-3_ for *rps11*.

2.5. Histological analysis

Several adult tissue samples (eye, liver and testicle) were fixed in 4% buffered formalin (Panreac Quimica) for 24 hours, processed and paraffin-embedded. Histological hematoxylin-eosin (H&E) and periodic acid Schiff's (PAS) staining were carried out in 4-µm sections using standard protocols. Stained histological sections were examined using conventional light microscopy at 200X, 400X and 630X.

TUNEL assay was carried out on zebrafish testis tissue from $tert^{+/+}$ and $tert^{-/-}$, using a DeadEndTM Colorimetric Apoptosis Detection System (Promega) following the

manufacturer'sinstructions. Stained histological sections were examined by conventional light microscopy at 400X.

2.6. Metaphase spreads

Methods were adapted from Lee and Smith [Lee & Smith, 2004]. At 23 hpf, zebrafish embryos previously dechorionated by pronase treatment were treated with colchicine (4 mg/ml) for 8 hours. Icecold 0.9. PBS with 10% fetal bovine serum was added and embryos filtered subsequently through a 100 µm and 40 µm mesh. Cell suspensions were then centrifuged at 250 ***g*** for 10 minutes at 4°C. The supernatant was decanted and cells were incubated for 25 minutes at 28.5°C in a hypotonic solution (1.1% sodium citrate, 4 mg/ml colchicine). Cells were centrifuged at 450 ***g*** for 10 minutes at 4°C and ice-cold Carnoy's methanol: glacial acetic acid fixative (3:1, v:v) was added. This last step was repeated, and chromosomes were then dropped onto microscope slides and allowed to dry overnight at 37°C.

2.7. Q-FISH

Q-FISH on interphasic and metaphasic cells was performed as described in Canela *et al.* [Canela *et al.*, 2007]. Cy3 and DAPI images were captured with 100. and 60. objectives, respectively, using a Nikon Digital Camera DXM 1200C on a Nikon Direct Eclipse fluorescence microscope. Telomere fluorescence signals were quantified using the TFL-TELO program (from Peter Lansdorp, Vancouver, Canada).

2.8. Flow-FISH

One million cells from each sample was divided into two replicate tubes: one pellet was resuspended in a 500 µl hybridization buffer and another in a hybridization buffer without an FITC-labeled telomeric peptide nucleic acid (PNA) probe, as a negative control. Samples were then denatured for 10 minutes at 80°C under continuous shaking and hybridized for 2 hours in the dark at room temperature. After that, the cells were washed twice in a washing solution (70% deionized formamide, 10 mM Tris pH 7.2, 0.1% BSA, 0.1% Tween-20 in dH_2O). The cells were then centrifuged at 600 ***g***, resuspended in 500 µl of propidium iodide solution, incubated for 2 hours at room

temperature, stored at 4°C and analyzed by flow cytometry within the following 48 hours.

2.9. Morpholino microinjection

Specific morpholinos (MOs) (Gene Tools) were resuspended in nuclease-free water to 1mM, mixed in microinjection buffer (0.5x Tango buffer and 0.05 % phenol red solution) and microinjected into the yolk sac of one- to eight-cell-stage embryos using a microinjector Narishige IM300 (0.5-1 nL per embryo).

2.10. Apoptosis assay in zebrafish

We performed a TUNEL assay on 48-hpf larvae from the *tert*$^{+/+}$and *tert*$^{-/-}$ genotype, the latter being a normal phenotype (group I) and a defective phenotype (mild: group II, and severe and very severe: group III). We used the ApopTag Red *In Situ* Apoptosis detection kit (S7165-Millipore) following the manufacturer's instructions. Apoptotic cells were counted using ImageJ software.

2.11. Quantitative analysis and statistics

Data processing and statistical analyses were performed using Microsoft Excel and Graph Pad Prism version 5.01, which were used to generate each of the graphs shown in the figures, performing statistical tests where appropriate. Data were analyzed using ANOVA and a Tukey multiple range test to determine differences between groups. The differences between two samples were analyzed using Student's *t*-test. The comparison of survival between mutant fish and their wild-type controls shown in the Kaplan-Meier survival curves was performed using the Log Rank test.

3. Results

3.1. Telomerase deficiency results in a lack of telomerase activity and telomere shortening

We obtained a publicly available mutant line in the *tert* gene generated by ENU mutagenesis at the Sanger Institute (hu3430). This mutant line has a nonsense mutation (T>A) that results in a premature stop codon and a truncated protein with only the first 156 of the 1,088 amino acids and an absence of the RNA-binding and reverse transcriptase domains (**Fig. 1**).

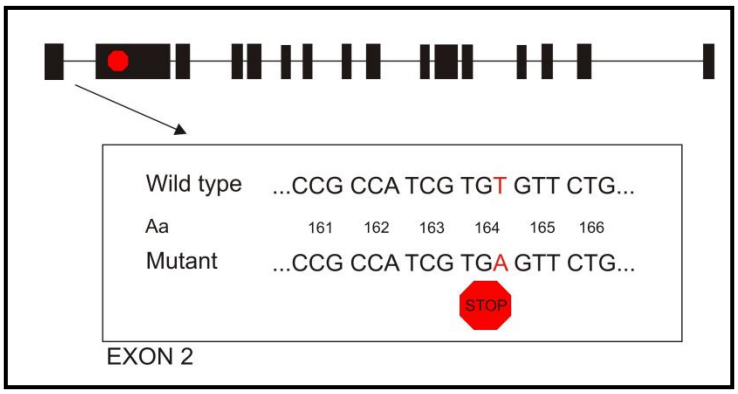


Figure 1. Schematic representation of the telomerase gene mutation.

As expected, this line did not have any telomerase activity (**Fig. 2**).

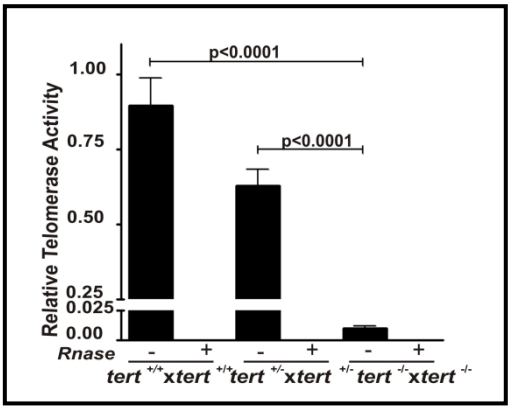


Figure 2. Telomerase activity was measured quantitatively in whole zebrafish embryos (3 days post-fertilization, dpf, n=100) by Q-TRAP using 0.1 mg of protein extract. Results are expressed as the mean value ± S.E. from triplicate samples relative to telomerase-positive cells. Statistical significance was assessed using Student's t-test ($p<0.05$). To confirm the specificity of the assay, a negative control is included for each sample, treated with 1 µg of RNAse at 37 ºC for 20 min.

Although *tert* gene expression was not affected in larvae, the lack of telomerase activity may have been responsible for the induction of tert expression in adult tissues, such as the skeletal muscle (**Fig. 3**).

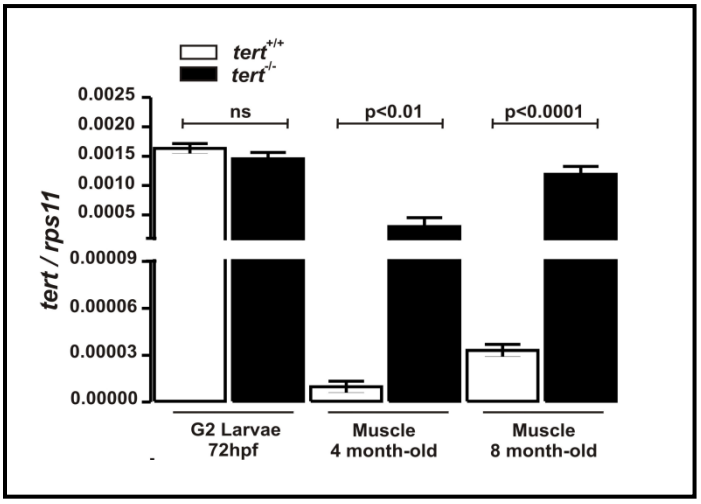


Figure 3. The mRNA levels of *tert* gene were determined by real-time RTPCR in larvae and adult muscle tissue of the indicated genotypes. Gene expression is normalized against *rps11*. Each bar represents the mean ±S.E. from 100 pooled animals for larvae and three individual fish for adult tissue and triplicate samples.

In addition, a shorter telomere length was observed in 3 month-old *tert*$^{-/-}$ juveniles (**Fig. 4**), as assayed by Flow-FISH.

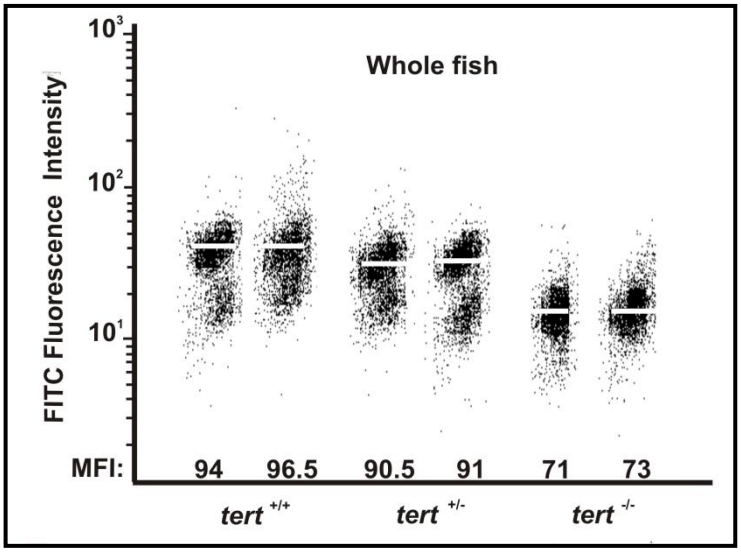


Figure 4. Representation of 3 month-old zebrafish cell distribution according to telomere length. Medium Fluorescence Intensity (MFI) is indicated for each genetic background. The same trend was observed in the three independent experiments.

Because telomere length has been proposed as a good aging biomarker in the zebrafish model [Anchelin *et al.*, 2011], we measured the mean telomere length of cells from whole mutant fish and several of their organs at three different stages, namely young adult (4 months old), adult (8 or 12 month-old) and older adult (18 or 24 month-old), in order to determine the relationship between the aging process and telomere length in the absence of telomerase activity. The results show that telomere length of wild type zebrafish increased between juvenile and the young adult stage followed by a decrease at the older adult stage (**Fig. 5**), as already reported (Anchelin *et al.*, 2011). In contrast, *tert*$^{-/-}$ specimens showed a telomeric length much shorter than their wild type siblings but, surprisingly, they were able to maintain telomere length throughout their life (**Fig. 5**).

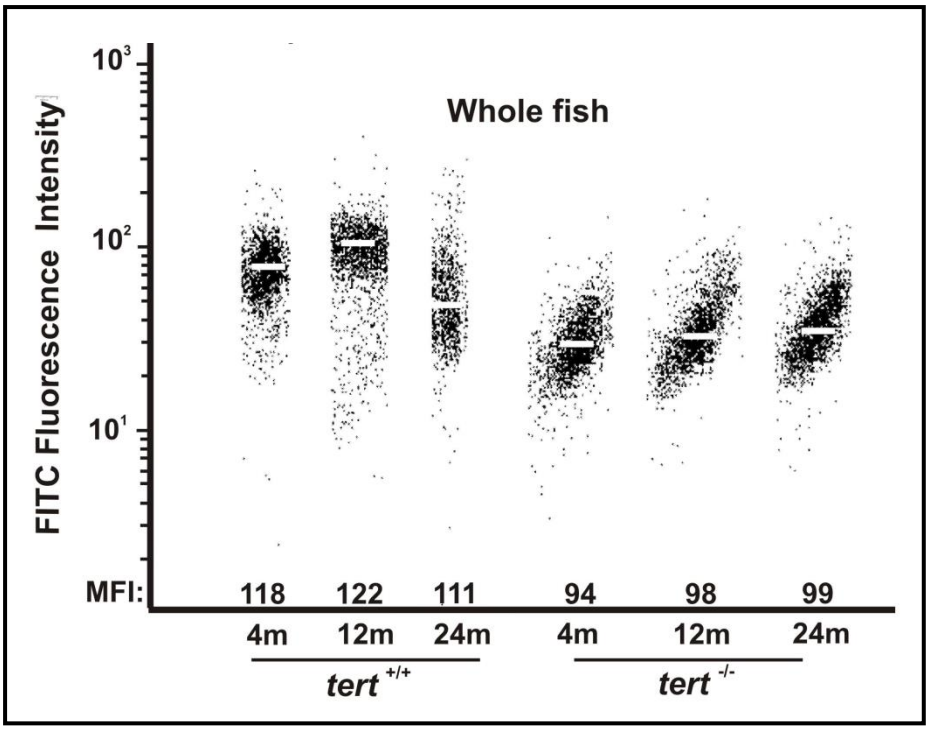


Figure 5. Representation of wild type and *tert* mutant zebrafish cell distribution throughout their life according to their telomere length. Medium Fluorescence Intensity (MFI) is indicated for each genetic background. The same trend was observed in the three independent experiments.

We further confirmed this observation in specific tissues, such as those of the muscle and the kidney. Thus, the telomere length of muscle cells slightly decreased throughout the zebrafish lifespan, while kidney cells maintained their telomere length (**Fig. 6**),

indicating that reduction or maintenance of telomere length is tissue specific in the context of telomerase deficiency in zebrafish [Lee *et al*., 1998].

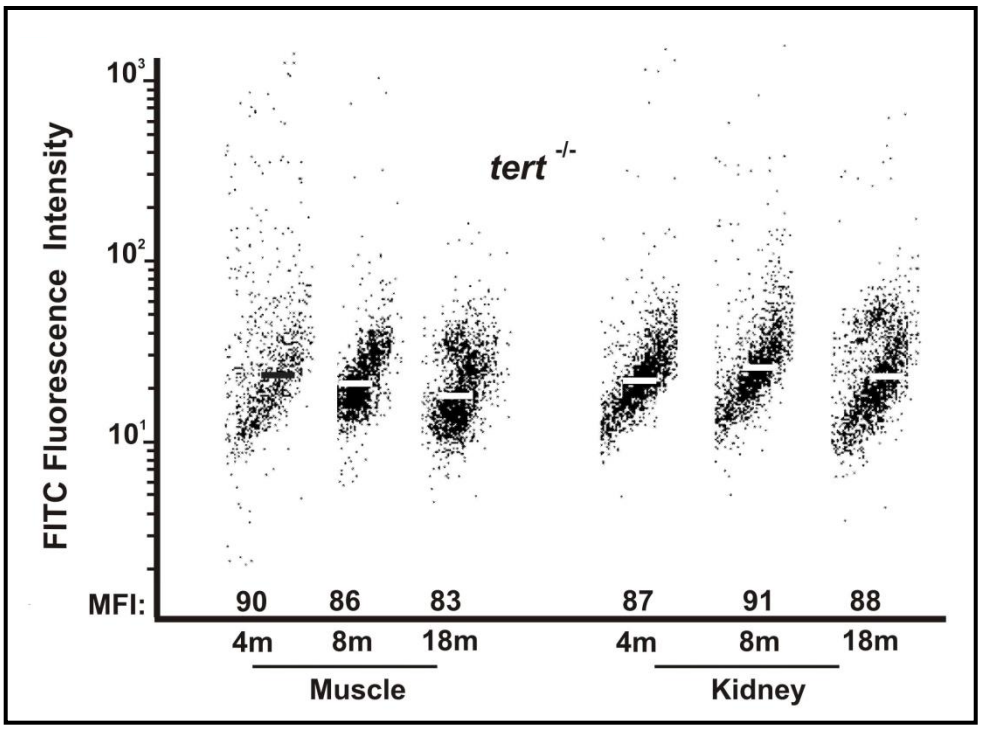


Figure 6. Representation of zebrafish muscle and kidney cell distribution throughout life according to their telomere length. Medium Fluorescence Intensity (MFI) is indicated for each genetic background (m=month). The same trend was observed in the three independent experiments.

3.2. Telomerase deficiency results in a reduced lifespan and premature aging

Outbred zebrafish have a mean lifespan of 42 months and exhibit a gradual senescence similar to humans [Gerhard & Cheng, 2002; Gerhard *et al*., 2002]. In order to assess the role of telomerase during the aging process, we used *tert*$^{-/-}$, *tert*$^{+/+}$ and *tert*$^{+/-}$ specimens from the same genetic background and they were maintained in the same laboratory conditions. We examined fish survival during 110 weeks and obtained Kaplan-Meier survival curves for the three genotypes and found that *tert*$^{-/-}$ zebrafish showed a reduced median lifespan (67.57 weeks) compared with *tert*$^{+/-}$ (105.43 weeks) and *tert*$^{+/+}$ (>110 weeks) (**Fig. 7**).

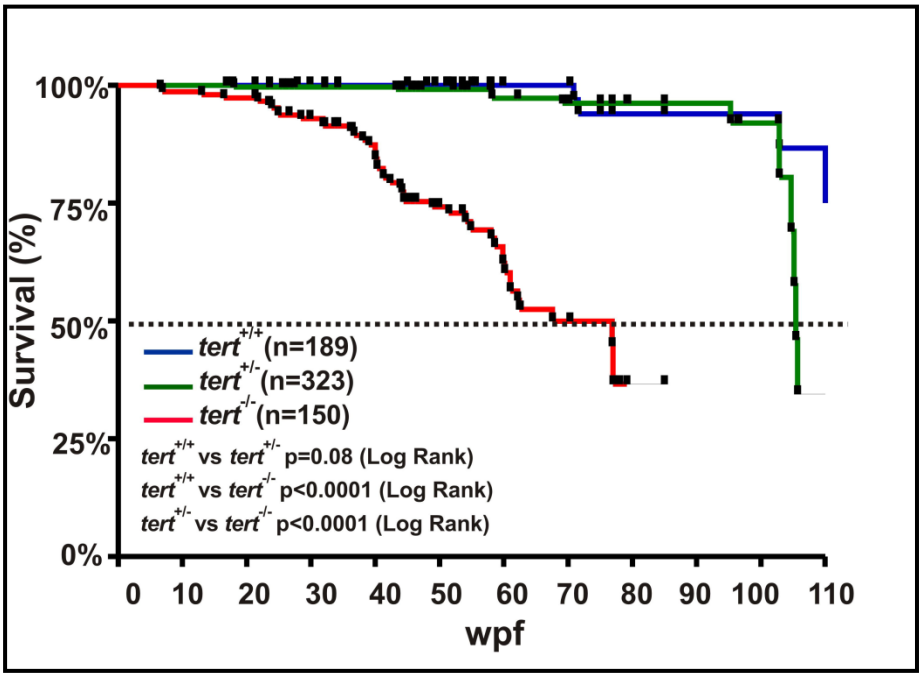


Figure 7. Kaplan-Meier representation of the survival of three genetic backgrounds. Post-hatching time is shown in weeks. A dashed line indicates 50% survival. The Log Rank test was used for statistical analysis.

Older zebrafish show spinal curvature due to muscle degeneration and various degrees of curvature can be observed [Gerhard & Cheng, 2002; Gerhard *et al*., 2002]. We noted that while the *tert* $^{+/+}$ zebrafish did not show spinal curvature before 24 months, *tert*$^{-/-}$ and *tert*$^{+/-}$ zebrafish started to show this sign of aging from 5 and 11 months of age, respectively (**Fig. 8**).

Figure 8. Representative image of a *tert* $^{+/+}$ and a *tert* $^{-/-}$ zebrafish at the same age.

Moreover, 10% of *tert*$^{-/-}$ zebrafish from 3- to 12- month-old manifested spinal curvature compared to 1.2% of *tert*$^{+/-}$ zebrafish and 0% of *tert*$^{+/+}$ fish. Histopathological examination of liver sections from *tert*$^{+/+}$ and *tert*$^{+/-}$ genotypes stained with H&E or PAS revealed no obvious microscopic alterations (**Fig. 9**). In contrast, liver sections from *tert*$^{-/-}$ specimens showed cytoplasmic vacuolization of hepatocytes coinciding with PAS positive areas (**Fig. 9**), suggesting the cytosolic accumulation of lipofuscin, an aging biomarker [Kishi *et al*., 2009], in hepatocytes. Similarly, histopathological examination of the retina from *tert*$^{+/+}$ and *tert*$^{+/-}$ zebrafish genotypes did not reveal any microscopic alterations (**Fig. 9**), while retina from *tert*$^{-/-}$ zebrafish genotype showed a clear atrophy of the layer of rod and cones, which had a disorganized appearance, and a reduction in the thickness of the pigment epithelium, suggesting retinal cell degeneration (**Fig. 9**).

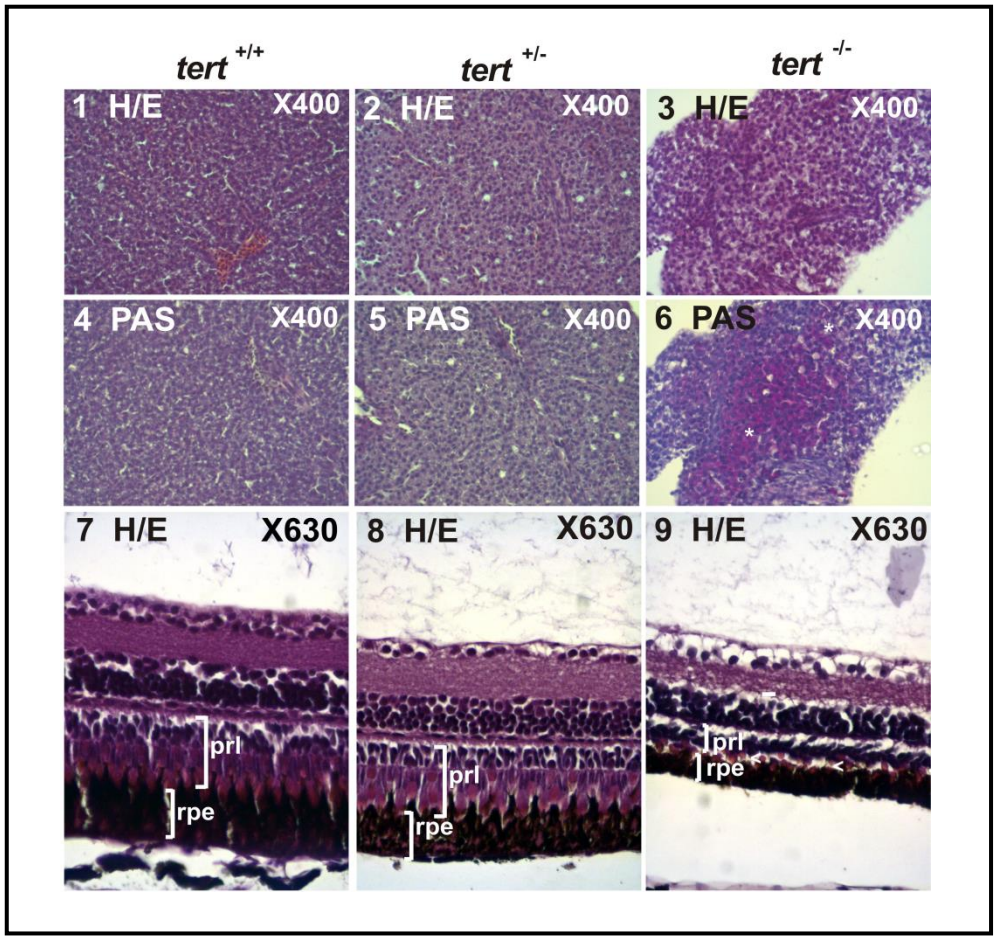


Figure 9. Liver sections stained with H&E showed cytoplasmic vacuolization of hepatocytes (3) and were PAS positive (6, white asterisks). H&E stained eye sections revealed a decreased thickness of both retinal pigmented epithelium (rpe) and the photoreceptor layer (prl) which had a disorganized appearance (9, white arrows). Representative images are from at least three fish per genotype.

By collecting data for two years, we observed a premature infertility in *tert*$^{-/-}$ zebrafish and, therefore, we investigated possible differences in the reproductive capacity of the *tert* mutant zebrafish. As regards clutch size and viability, the three genotypes showed a similar behaviour in the 4 to 10 month-old period, but after that *tert*$^{-/-}$ female zebrafish showed a reduction in the number of eggs per spawn, precisely when *tert*$^{+/+}$ and *tert*$^{+/-}$ females increased their reproductive performance. A Flow-FISH assay performed on testis cells from *tert*$^{+/+}$ zebrafish showed a net constant increase of their telomere length throughout their lifespan, while the telomere length of testis cells from *tert*$^{-/-}$ zebrafish suffered an abrupt decline at the young adult stage (8 month-old) (**Fig. 10**), which might be associated with premature infertility.

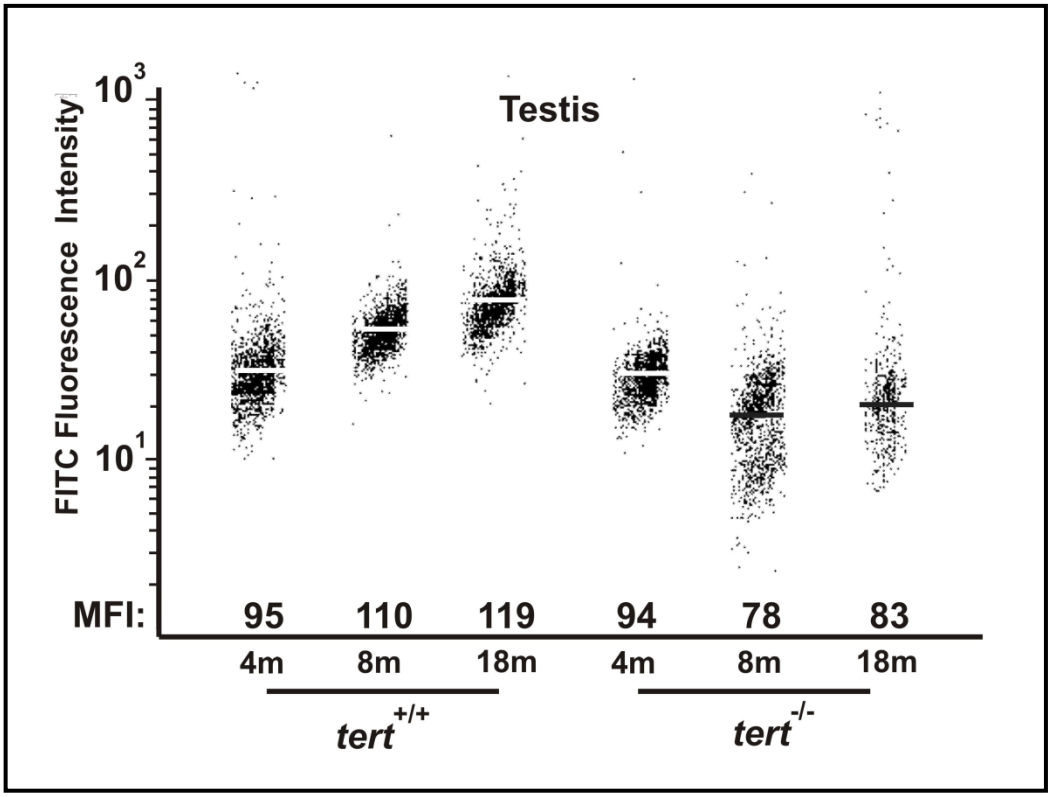


Figure 10. Representation of testis cell distribution from the wild-type and *tert* mutant genotype according to their telomere length. Medium Fluorescence Intensity (MFI) is indicated for each genetic background. The same trend was observed in the three independent experiments.

The histological analysis of telomerase-deficient testes and their wild-type siblings at three different stages further confirmed this result. Both 4 month-old specimens and testis tissue sections from *tert*$^{+/+}$ (**Fig. 11, 1**) and *tert*$^{-/-}$ (**Fig. 11, 2**) showed similar microscopic morphology with the structure of seminiferous tubules and apparently

normal connective stroma. At an age of 8 months, *tert*$^{+/+}$ presented the same morphology as at 4 months of age (**Fig. 11, 3**), while atrophic seminiferous tubules of *tert*$^{-/-}$ testis tissue were apparent (**Fig. 11, 4**). Finally at 18 months, while the *tert*$^{+/+}$ zebrafish preserved the normal testicular architecture (**Fig. 11, 5**), a complete atrophy of both tubules and stroma and the absence of spermatozoa were observed in the testes of *tert*$^{-/-}$ zebrafish (**Fig. 11, 6**). TUNEL assay confirmed the presence of apoptotic cells in the seminiferous tubules of telomerase-deficient testis, being more evident at 8 months of age (**Fig. 6E**). These results correlate well with the decrease in telomere length and suggest a direct link between telomerase function, fertility and aging.

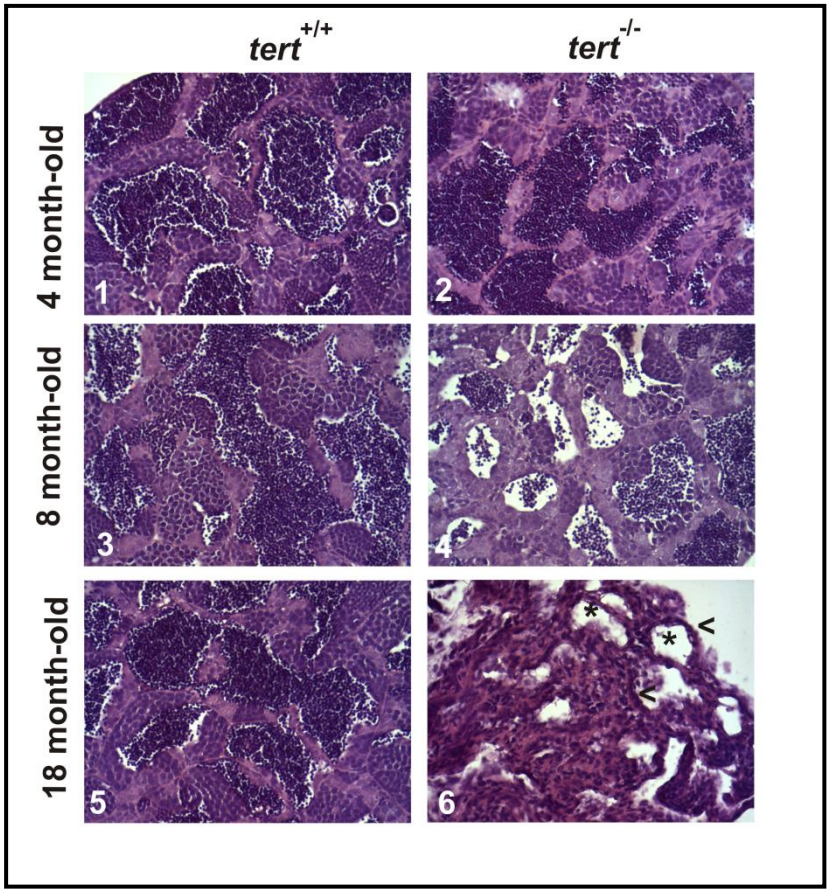


Figure 11. Representative image of zebrafish testis (n=3) from *tert* $^{+/+}$ and *tert* $^{-/-}$ background at 4, 8 and 18 month-old (X200).

3.3. Telomerase-deficient zebrafish can be bred for only one generation

The deletion of either mouse telomerase component, mTERT or mTR, did not show any phenotype in the first few generations when telomeres were long [Chiang *et al*., 2004; Lee *et al*., 1998]. A similar phenotypic delay in the response to telomerase loss has been described in *Caenorhabditis elegans* and *Arabidopsis thaliana* TERT mutants, again with the first generation of telomerase-null mutants being phenotypically normal [Cheung *et al*., 2006; Fitzgerald *et al*., 1999]. However, all these models develop abnormalities and chromosomal instability after several generations.

We wanted to check whether successive generations would show a more apparent phenotype. At 1 day-post fertilization (1 dpf), only 32% of larva survival was found in the second generation (G2) of telomerase-deficient zebrafish larvae obtained from *tert*$^{-/-}$ parental mating compared with 95% survival of wild-type larvae (**Fig. 12**).

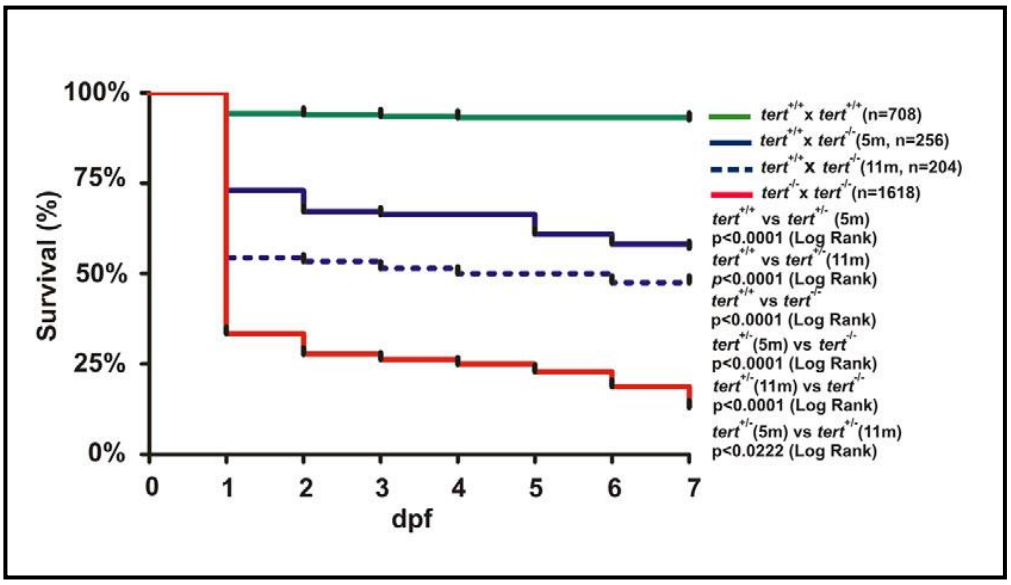


Figure 12. Larval survival curve (Kaplan-Meier representation) of four different genetic backgrounds. *n*, number of zebrafish per genotype. Statistical significance was assessed using the Log Rank test.

Although most G2 *tert*$^{-/-}$ progeny died before 10 dpf, two larvae survived until 25 dpf (**Fig. 7C**). Curiously, we observed that *tert* mutant zebrafish eggs and their corresponding dechorionated embryos were smaller than *tert*$^{+/+}$ zebrafish eggs and 24 hour-post fertilization (hpf) embryos (**Fig. 13**), as found in late generations of telomerase-deficient mice, which have fewer functional stem cells within tissues [Flores & Blasco, 2009; Flores *et al*., 2005].

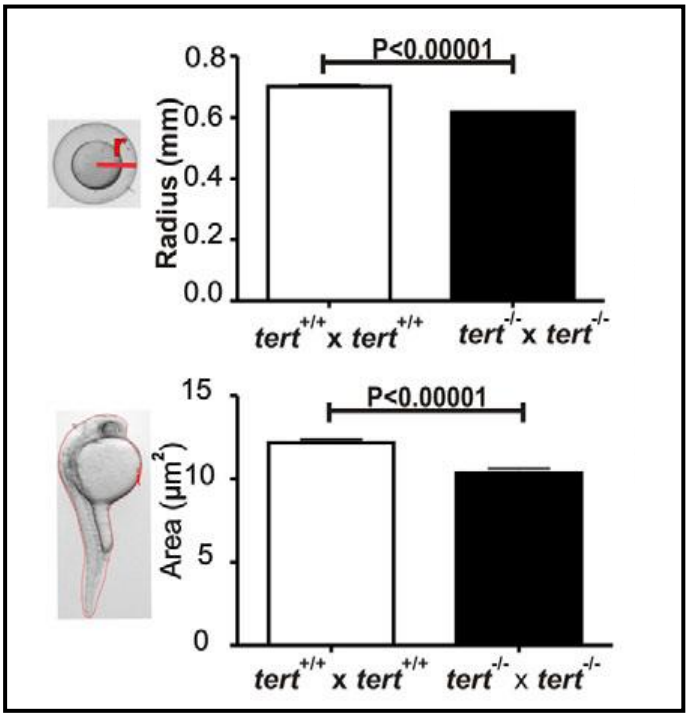


Figure 13. *tert* mutant zebrafish eggs and their corresponding dechorionated embryos had a smaller size than their wild-type siblings at 24 hpf ($n \geq 50$)

Importantly, the larvae obtained from *tert*$^{+/+}$ x *tert*$^{-/-}$ parental mating showed increased survival at 1 dpf, indicating that the reintroduction of one wild-type *tert* allele was able to rescue the viability of the offspring. However, the recovery was not complete, which suggested haploinsufficiency of the *tert* gene. We also observed that the survival of larvae obtained by breeding young (5 month-old) *tert*$^{-/-}$ mutant males with *tert*$^{+/+}$ females was significantly higher (73% vs. 54% survival) than when the males were older (11 month-old) (**Fig.12**).

Telomerase-deficient mice showed telomere shortening in successive generations [Blasco *et al*., 1997]. Therefore, we wanted to determine the telomere length of G2 *tert*$^{-/-}$ progeny. We used Q-FISH in interphasic nuclei to measure the mean telomere length

of 24 hpf larvae. Compared with the *tert*$^{+/+}$ progeny (1750.58 a.u.f. ± 30.90), larvae obtained from *tert*$^{-/-}$ parental mating showed a significant telomere shortening (1390.68 a.u.f. ± 26.33) (**Fig.14A**). The telomere length frequency histograms, where the percentage of very short (<1400 a.u.f.) and very long telomeres (>2000 a.u.f.) is shown, revealing that the G2 *tert*$^{-/-}$ progeny had a higher percentage (58.73%) of very short telomeres compared to 15.13% of the *tert*$^{+/+}$ genotype (**Fig. 14B**).

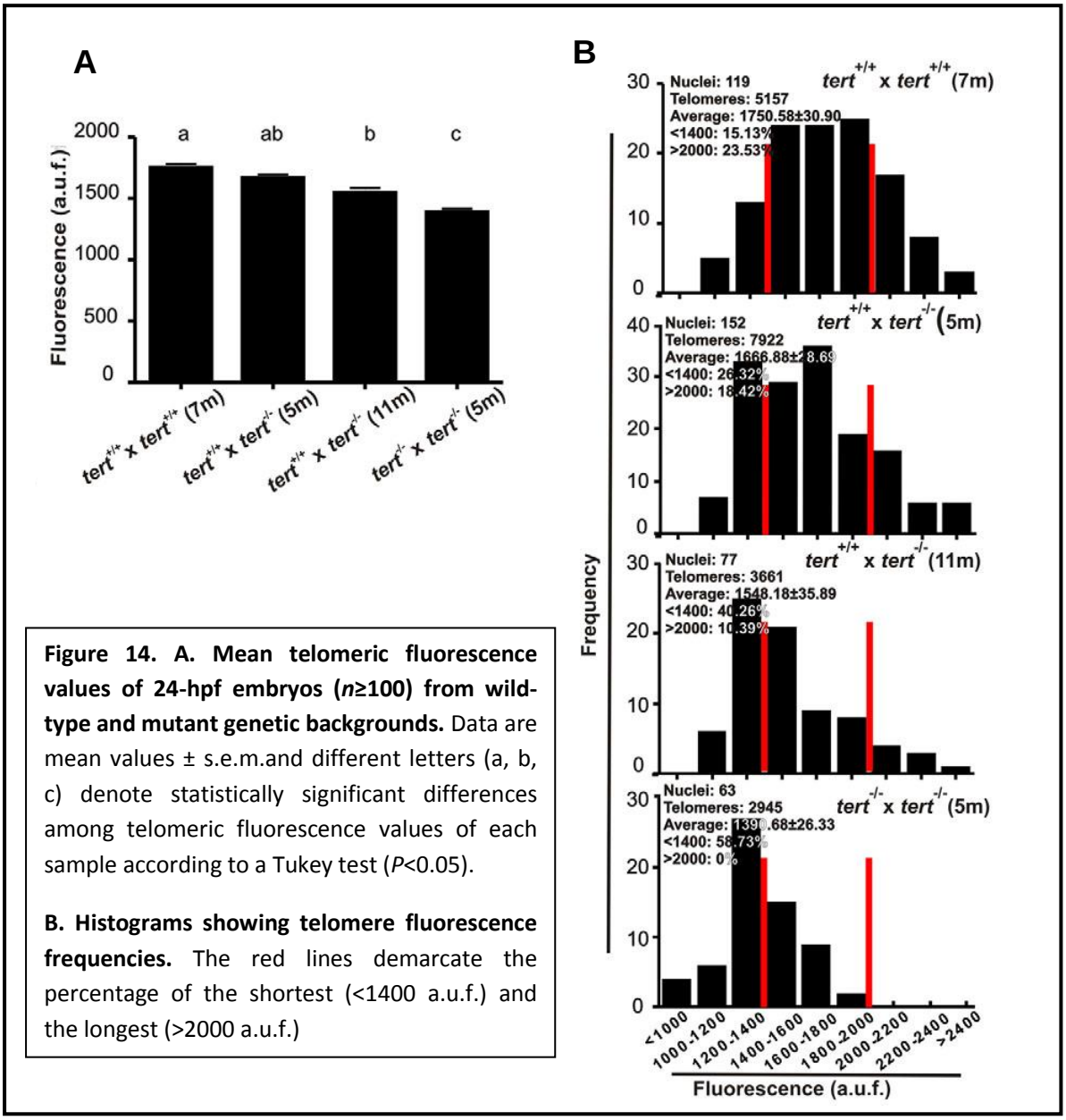


Figure 14. A. Mean telomeric fluorescence values of 24-hpf embryos (*n*≥100) from wild-type and mutant genetic backgrounds. Data are mean values ± s.e.m.and different letters (a, b, c) denote statistically significant differences among telomeric fluorescence values of each sample according to a Tukey test (*P*<0.05).

B. Histograms showing telomere fluorescence frequencies. The red lines demarcate the percentage of the shortest (<1400 a.u.f.) and the longest (>2000 a.u.f.)

As we observed a better survival rate in the offspring obtained from 5- rather than 11 month-old *tert*$^{-/-}$ mutant males outcrossed with *tert*$^{+/+}$ females, we examined whether the telomere length was also rescued in both progenies. It was found that telomere length was shorter for the progeny of the older telomerase-deficient males (1548.18 ± 35.89 a.u.f. vs. 1666.88 ± 28.69) (**Fig. 14A**). In addition, the telomere length frequency histogram revealed 40.26% of very short telomeres for the progeny of 11 month-old male progenitors vs. 26.32 % in the case of 5 month-old males (**Fig. 14B**).

We next studied the gross morphology of 24-72 hpf embryos obtained from *tert*$^{-/-}$ x *tert*$^{-/-}$ and *tert*$^{+/+}$ x *tert*$^{-/-}$ parental mating and observed 3 different phenotypes, while embryos obtained from *tert*$^{+/+}$ x *tert*$^{+/+}$ and *tert*$^{+/-}$ x *tert*$^{+/-}$ parental mating showed a normal phenotype and development (**Fig. 15**). Embryos belonging to group I showed an apparently normal phenotype; embryos in group II had mild defects, such as a bent caudal fin; and embryos from group III showed serious developmental defects, such as a bent caudal fin, pericardial edema and general malformations.

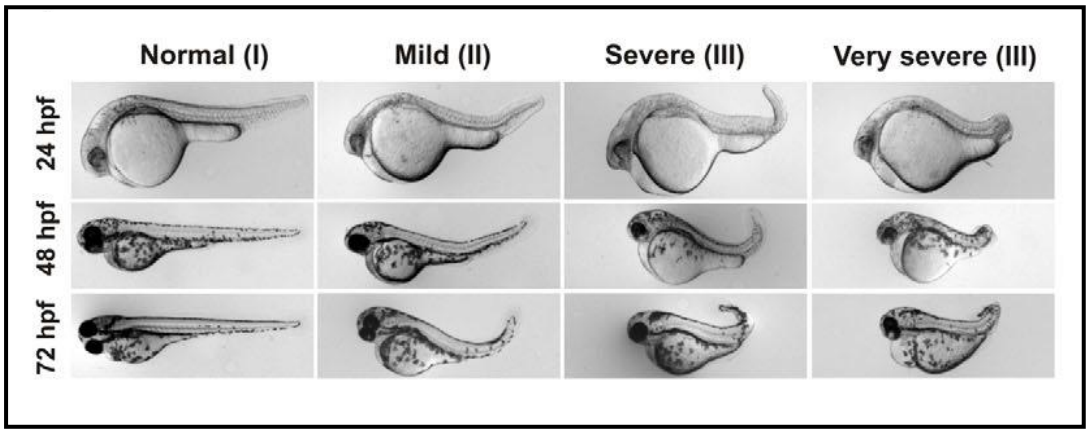


Figure 15. Representative image of the three groups of larvae resulting from *tert*$^{-/-}$ × *tert*$^{-/-}$ and *tert*$^{+/+}$ × *tert*$^{-/-}$ parental matings. The phenotypes were scored as wild type (I), mildly affected (II) and severely affected (III), as indicated in the Materials and Methods.

We also found that the G2 *tert*$^{+/-}$ progeny (obtained from *tert*$^{+/+}$ x *tert*$^{-/-}$ parental mating) had a high percentage of larvae in group I while the G2 *tert*$^{-/-}$ progeny had a high percentage of larvae belonging to group III, as well as a higher mortality (**Fig. 16**). Collectively, these data further corroborate the rescue of offspring viability due to the restoration of one wild-type *tert* allele.

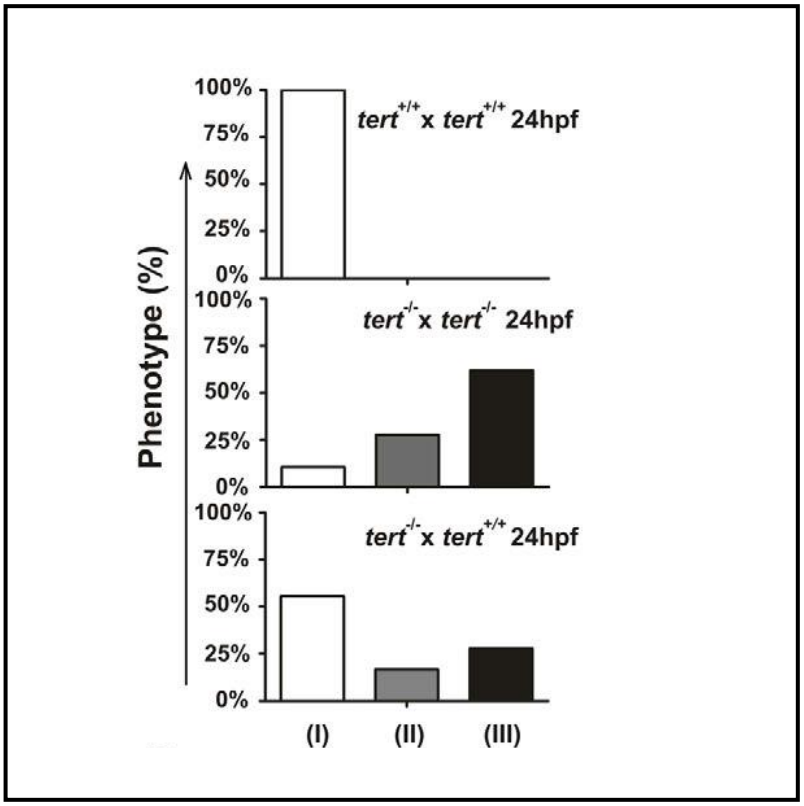


Figure 16. Percentage of larval survival of the three groups observed in Figue 15 at 1 dpf.

We next asked whether telomere length directly correlated with the larval phenotype. To do this, we performed a Q-FISH assay in metaphasic nuclei to compare the telomere length of normal the G2 *tert*$^{-/-}$ progeny (group I) with that of the G2 progeny showing an abnormal phenotype (groups II and III) at 48 hpf. Besides the expected difference between the mean telomere length of cells from *tert*$^{+/+}$ and G2 *tert*$^{-/-}$ embryos, we observed that G2 embryos with an abnormal phenotype (II or III) had significantly shorter mean telomere length than their normal counterparts (I) (**Figs. 17A, B**).

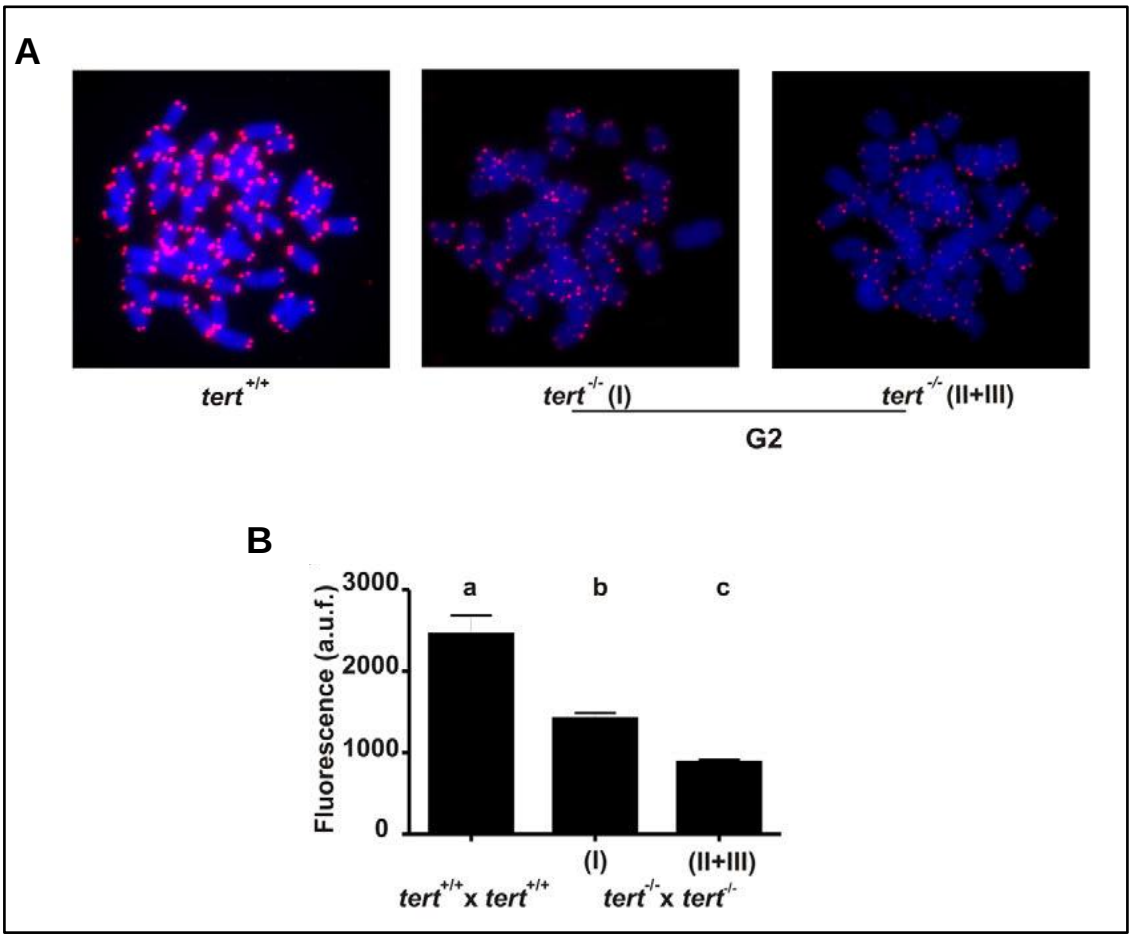
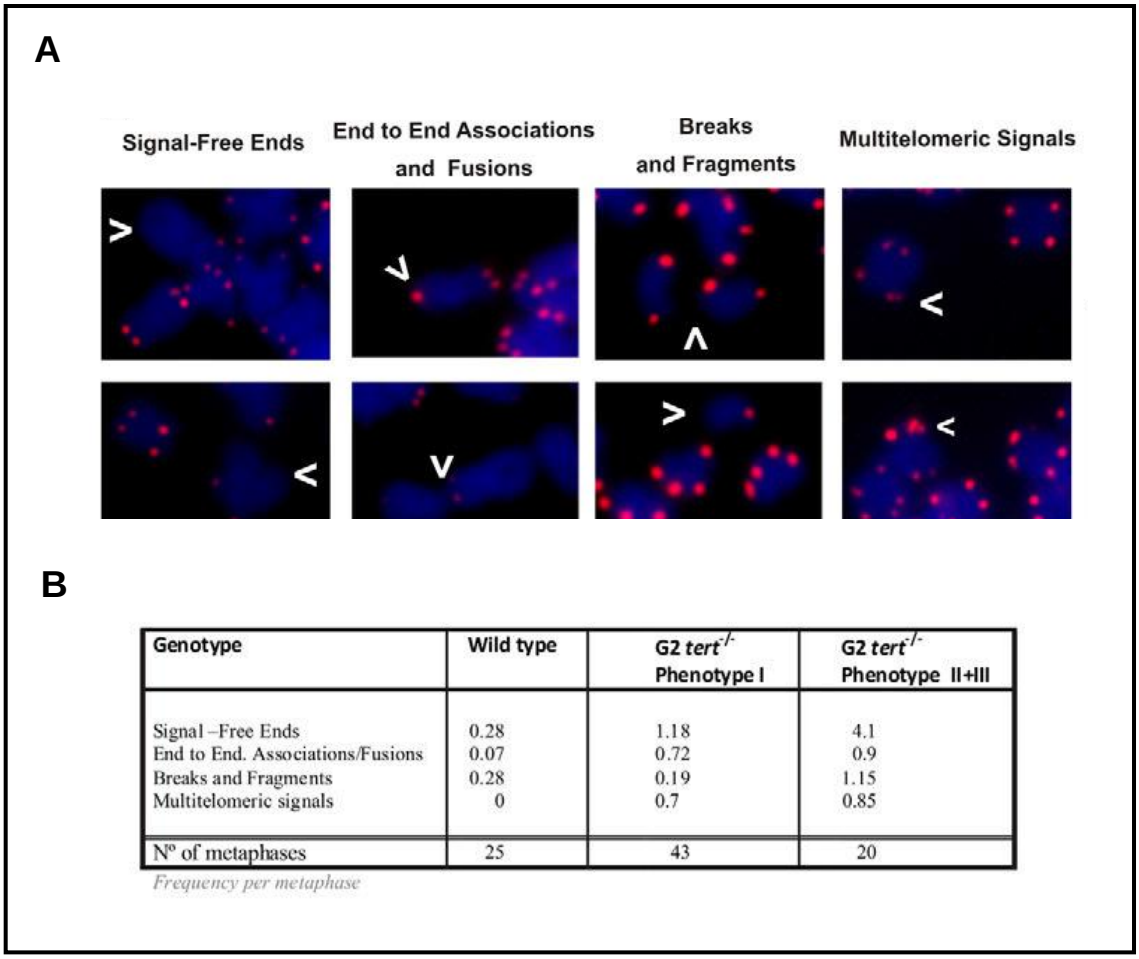


Figure 17. A. Metaphase chromosomes from *tert*$^{+/+}$ and *tert*$^{-/-}$ cells were stained with DAPI, and the telomeres were visualized using FISH analysis. The phenotypes of mutant larvae were scored as indicated above. **B.** Mean telomeric fluorescence values of 24-hpf embryos ($n \geq 100$) from wild-type and mutant genetic backgrounds. Data are mean values ± s.e.m. Different letters (a, b, c) denote statistically significant differences among telomeric fluorescence values of each sample according to a Tukey test ($p<0.05$).

Finally, we carried out a detailed analysis of the chromosomal defects of cells from both the normal and abnormal phenotypes (**Fig. 18B**) and observed an increase in telomere-free chromosome ends, chromosome breakages and chromosomal fragments in metaphasic nuclei from embryos with an abnormal phenotype (**Fig.18A**). Interestingly, G2 *tert*$^{-/-}$ also showed an increased proportion of chromosome ends with multiple telomeric signals (MTS), a type of aberration related to increased telomere fragility [Martinez *et al*., 2009; Sfeir *et al*., 2009]. These chromosomal aberrations could explain the phenotypic differences observed between G2 *tert*$^{-/-}$ normal embryos and those showing severe developmental defects and malformations.



Genotype	Wild type	G2 *tert*$^{-/-}$ Phenotype I	G2 *tert*$^{-/-}$ Phenotype II+III
Signal –Free Ends	0.28	1.18	4.1
End to End. Associations/Fusions	0.07	0.72	0.9
Breaks and Fragments	0.28	0.19	1.15
Multitelomeric signals	0	0.7	0.85
Nº of metaphases	25	43	20

Frequency per metaphase

Figure 18. A. Examples of chromosomal aberrations. **B.** Frequency of chromosomal aberrations in cell from 24-hour larvae. a.u.f., arbitrary units of fluorescence.

3.4. Critically short telomeres activate p53

The high mortality of *tert*$^{-/-}$ G2 progeny drove us to perform a whole-mount TUNEL assay to detect and quantify the presence of apoptotic cells. At 48 hpf, *tert*$^{-/-}$ embryos showed a significantly higher number of apoptotic cells than wild type embryos. More importantly, *tert*-deficient embryos with an abnormal phenotype (II+III) had a much higher number of apoptotic cells than mutant embryos with a normal phenotype (I) (**Figs. 19A,B**).

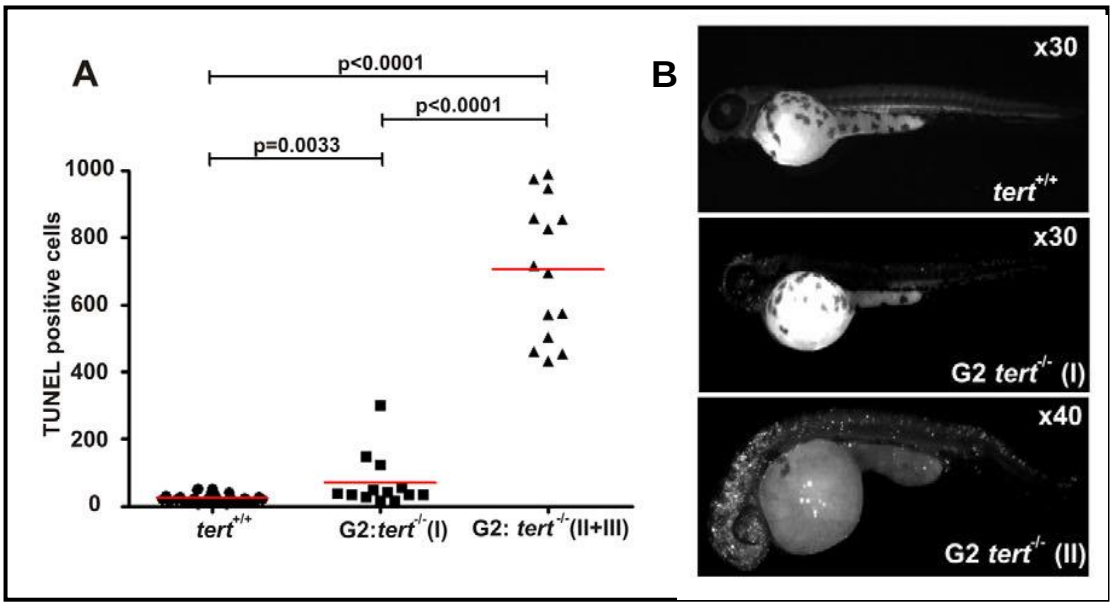


Figure 19. A. Quantification of apoptotic cells based on TUNEL staining (see Materials and Methods). Significance was determined based on Student's *t*-test ($P<0.005$). **B.** Examples of embryos after TUNEL staining.

To test whether the high percentage of apoptotic cells detected in *tert*$^{-/-}$ G2 progeny could be the result of p53 activation through a DNA damage signal, like telomere dysfunction [Maser & DePinho, 2004], we analyzed p53 gene expression using real-time RT-PCR and it was found that there were statistically significant higher mRNA levels of p53 in G2 *tert*$^{-/-}$larvae than in their *tert*$^{+/+}$ siblings at 72 hpf (**Fig. 20**).

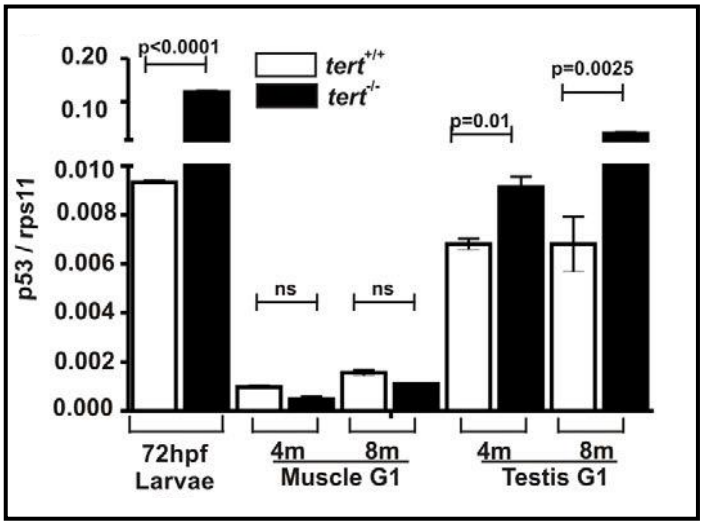


Figure 20. The mRNA levels of the *p53* gene were determined by real-time RT-PCR in 72-hpf larvae, and muscle and testis tissues of adult zebrafish, in the indicated genotypes. Gene expression is normalized against *rps11*. Each bar represents the mean ± s.e.m. from 100 pooled animals for larvae and three individual fish for adult tissue and triplicate samples.

Notably, the transcript levels of p53 were also slightly higher in the testes of 4 month-old G1 *tert*-deficient adults and became especially evident, and statistically significant, in 8 month-old adults, coinciding with the onset of testicular atrophy (**Fig. 11**). These results, together with the decrease in telomere length of testicular cells at the same age (**Fig. 10**), confirm the crucial importance of telomerase in this organ.

To ascertain whether p53 activation was responsible for the induction of apoptosis, developmental malformations and mortality of G2 *tert*$^{-/-}$ larvae; we inhibited p53 expression using a specific morpholino. The results showed that genetic depletion of p53 results in a completely normal development and survival of G2 *tert*$^{-/-}$ embryos (**Fig. 21**).

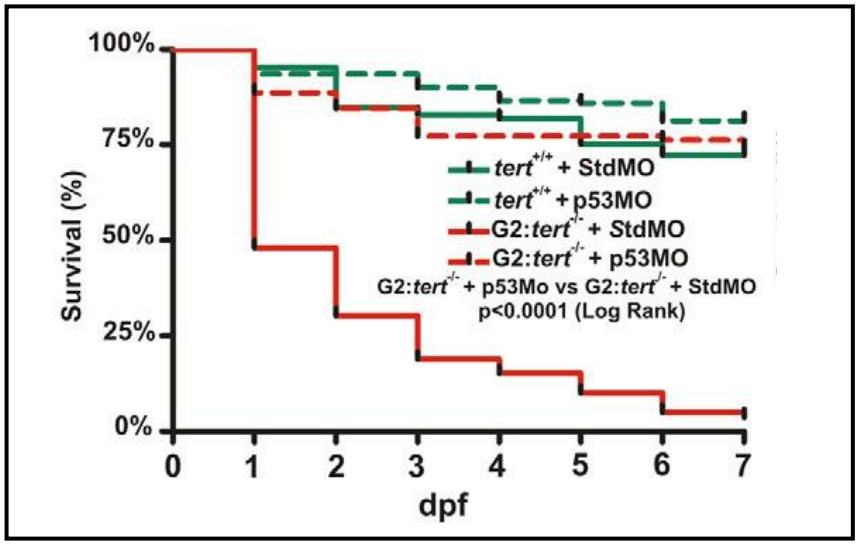


Figure 21. Survival of G2 *tert*$^{-/-}$ larvae microinjected at the one-cell stage with a morpholino against *p53*.

Furthermore, we assessed the effect of the permanent absence of p53 in *tert*-deficient fish by obtaining the G1 of double *p53*$^{-/-}$ and *tert*$^{-/-}$ zebrafish. To this end, we obtained survival curves covering 50 weeks and used *tert*$^{+/+}$ and *p53*$^{-/-}$ zebrafish lines as a reference for normal longevity. Although the G1 *tert*$^{-/-}$ had reduced longevity (p=0.0326), the survival of the G1 *tert*$^{-/-}$; *p53*$^{-/-}$ line showed no significant differences with those of their *tert*$^{+/+}$ or *p53*$^{-/-}$ siblings (**Fig. 22**).

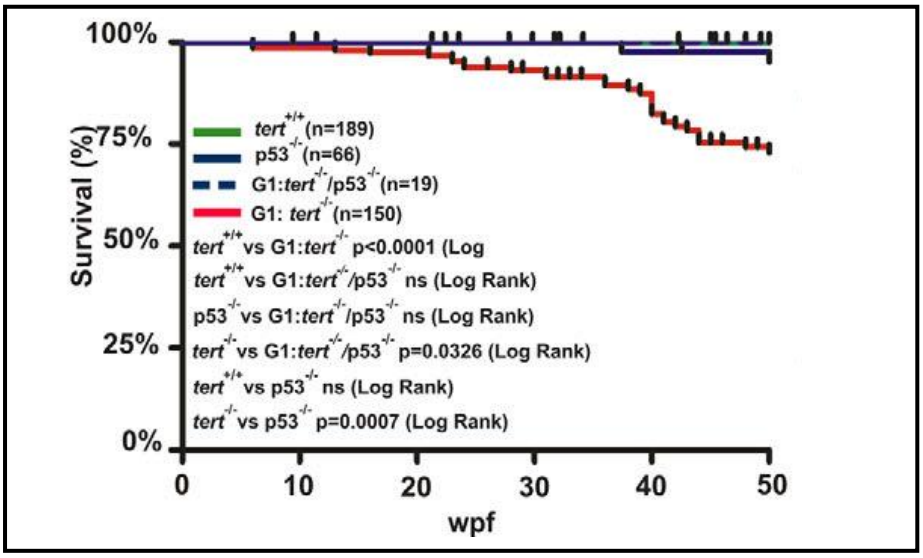


Figure 22. Kaplan-Meier representation of the survival of four genetic backgrounds. Post-hatching time is shown in weeks. The Log Rank test was used for statistical analysis.

Notably, G1 *tert*$^{-/-}$ cells had similar mean telomere signal than their G1 *tert*$^{-/-}$; *p53*$^{-/-}$ siblings (**Figure 23**).

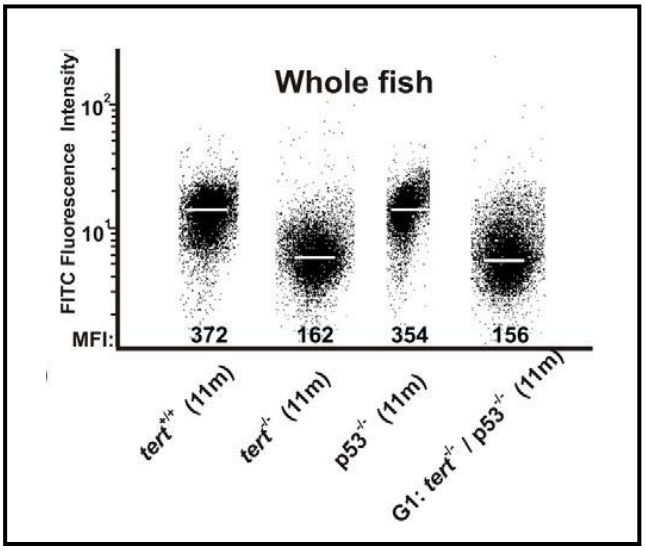


Figure 23. Representation of total cell distribution from the four genotypes at the same age (11 month old) according to their telomere length. Medium fluorescence intensity (MFI) is indicated for each genetic background. The same trend was observed in the three independent experiments.

Furthermore, the histopathological analysis of the male zebrafish testes revealed a normal morphology and the absence of TUNEL+ germ cells (**Fig. 24**), suggesting that p53 senses telomere damage in germ stem/progenitor cell populations leading to massive germ cell apoptosis, and that p53 deficiency did not influence telomere length in *tert*-deficient zebrafish, as occurs in mice [Chin *et al*., 1999].

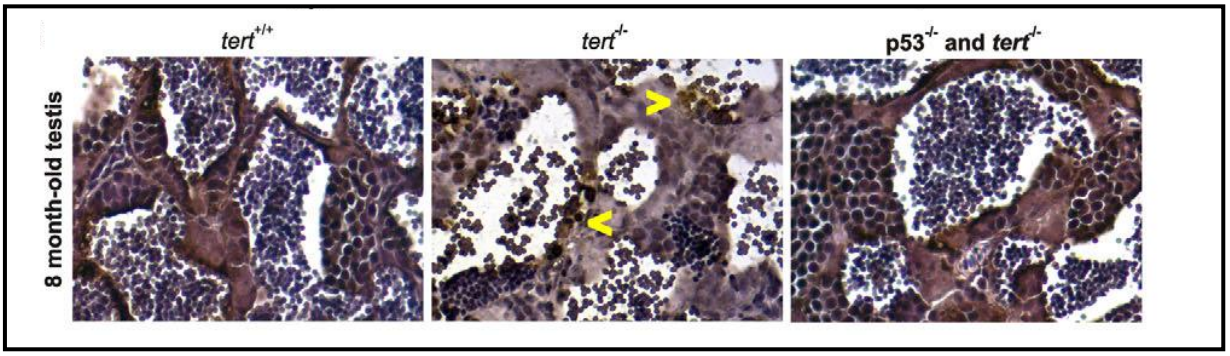


Figure 24. TUNEL assay in zebrafish testis sections from 8-month-old wild-type, *tert* mutant and double-mutant *tert*$^{-/-}$; *p53*$^{-/-}$ zebrafish (400×). Arrowheads indicate apoptotic cells.

As the knockdown of p53 with morpholino was transient (**Fig. 21**), we obtained the G2 *tert*$^{-/-}$; *p53*$^{-/-}$ line to examine whether p53 deficiency was able to rescue the longevity of the G2 *tert*$^{-/-}$ line. **Figure 25A** shows that p53 deficiency partially rescued the viability of the G2 *tert*$^{-/-}$ the first week ($p<0.0001$). However, the telomere length was unaffected (**Fig. 25B**). Notably, although we observed a high percentage of mortality between 10 and 20 dpf of G2 *tert*$^{-/-}$; *p53*$^{-/-}$ line, there were statistically significant differences between the survival of singly and doubly null mutants (**Fig.25C**). Together these data indicate that p53 is able to rescue the survival of *tert*-deficient fish but not telomere length.

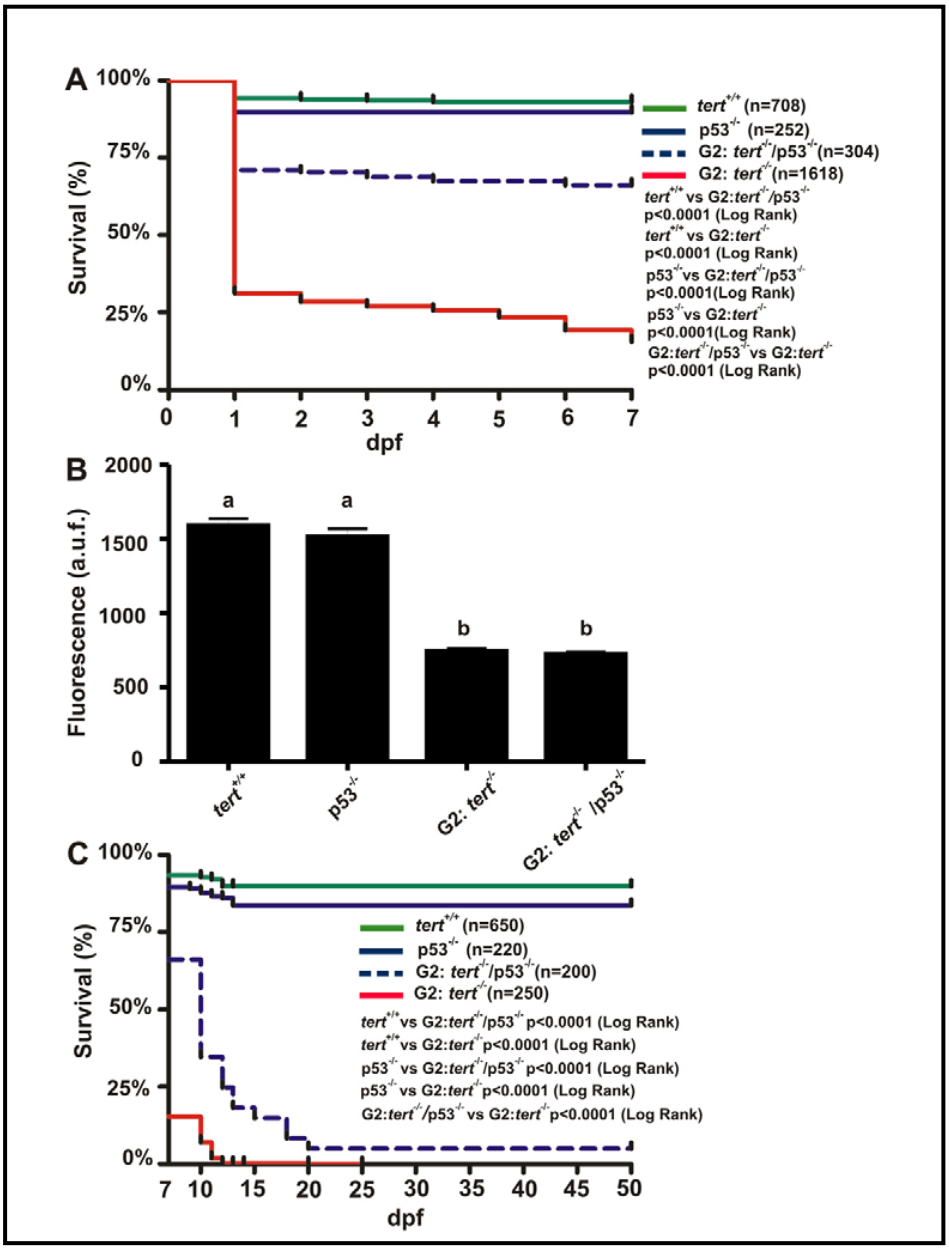


Figure 25. Survival and telomere length of G2 *tert*$^{-/-}$ zebrafish in a *p53*$^{-/-}$ background.
A. Larval survival curve (Kaplan-Meier representation) of four different genetic backgrounds. *n*, number of zebrafish per genotype. Statistical significance was assessed using the Log Rank test. **B.** Mean telomeric fluorescence values of 24-hpf embryos ($n \geq 100$) from wild-type and mutant genetic backgrounds. Data are mean values ± s.e.m. and different letters (a, b) denote statistically significant differences between telomeric fluorescence values of each sample according to a Tukey test $p<0.05$). **C.** Kaplan-Meier representation of the survival of four genetic backgrounds. a.u.f., (arbitrary units of fluorescence).

4. Discussion

The study of telomere and telomerase biology is crucial to the understanding of aging and cancer processes. Although the mouse has been extensively used as a model for these purposes and has been established as a key model to elucidate the role of telomeres and telomerase in aging and cancer, there are fundamental differences between mice and humans. For example, mouse telomere length is much longer than that of humans and the mouse model is not able to completely recapitulate the symptoms of human telomerase deficiency [Autexier, 2008; Mitchell *et al*., 1999]. Therefore, we have characterized a second vertebrate model for studying the role of telomerase and telomeres in aging research. Zebrafish telomeres (15-20 kb) are relatively similar to human ones (10-15 kb) and show a progressive shortening [Anchelin *et al.,* 2011]. The telomerase mutant zebrafish line used in this study was obtained from the Sanger Institute (hu3430 line). This mutation leads to a premature stop codon resulting in a truncated protein removing the RNA-binding and reverse transcriptase domains. As expected, this mutant lacks telomerase activity and shows shorter telomeres than wild-type fish. However, we observed an increased expression of *tert* mRNA, suggesting that the absence of telomerase activity induces the activation of its own promoter to try to compensate for this. This result indicates the existence of regulatory mechanisms of *tert* gene expression that would be worth exploring in future studies.

An increased telomere length from larvae to adult fish stages followed by telomere shortening in aged fish has previously been reported [Anchelin *et al.,* 2011]. These studies have been performed using whole zebrafish specimens from various genetic strains. Unexpectedly, in the present study, the telomeric length in *tert* mutant fish, although shorter than that of the wild type, remained constant or even increased slightly during aging. However, the dynamics of telomere length varied during the zebrafish life cycle in different organs and seems to be tissue specific. Although there was a tendency to a slight decrease in telomere length in the muscle, the decrease in the testis was much more pronounced. In sharpcontrast, kidney cells maintained their telomere length throughoutlife. Although these results might be explained by differences in cell proliferation rates in each tissue [Lee *et al*., 1998], it is tempting to speculate about the involvement of the activation of telomeraseindependent telomere maintenance mechanisms [ALT (alternative lengthening of telomeres)], at least in the highly

proliferative hematopoietic cells of the kidney. Therefore, the *tert*-deficient zebrafish model described in this study might be an excellent model for investigating the role of ALT in telomere maintenance in the absence of telomerase activity.

Telomerase-null mice have no discernible phenotypes in the first generations (G1-G5) because mice have very long telomeres [Blasco *et al*., 1997; Liu *et al*., 2000]. Only when the telomeres become critically short in later generations (G5 and G6) do telomerasevdeficient mice show prominent premature aging symptoms, including impaired spermatogenesis and loss of fertility, bone marrow failure, atrophy of the small intestine, and immunosenescence-related disease [Herrera *et al*., 1999; Lee *et al*., 1998]. However, because humans and zebrafish have a similar telomere length, we were able to observe premature aging symptoms in the G1 of *tert*-deficient zebrafish, with the most apparent phenotype being the sharp decline in the mean life expectancy of *tert* mutant zebrafish. Interestingly, $tert^{+/-}$ zebrafish seemed to have a reduced longevity compared with $tert^{+/+}$. Several signs of aging already described in the zebrafish [Gerhard & Cheng, 2002; Gerhard *et al*., 2002; Kishi *et al*., 2009] were prematurely observed in young (10 month-old) *tert* knockout mutants, such as a higher percentage of $tert^{-/-}$ individuals exhibiting extreme thinness and/or spinal curvature compared with $tert^{+/-}$ and $tert^{+/+}$ siblings (10% versus 1.2% and 0%, respectively), increased lipofucsine accumulation in the liver, and retinal cell degeneration. By contrast, telomere shortening and genome instability in lategeneration telomerase-deficient mice, as well as in humans, is associated with germ cell depletion in the testis [Hemann *et al*., 2001; Lee *et al*., 1998]. Similarly, we also observed the dramatic consequences of telomere shortening in premature testis degeneration and infertility.

In order to study the phenomena of aging earlier in time, we created a G2 of the *tert* mutant and found that almost all G2 embryos/larvae died before the first week of age. These larvae exhibited shorter telomeres (58.73% versus 15.13% in wild type) and were smaller than wild-type larvae, as happens in a percentage of late-generation mouse $TR^{-/-}$ embryos owing to the effect of short telomeres on stem cell functionality [Flores *et al*., 2005; Herrera *et al*., 1999]. Interestingly, the reintroduction of the *tert* gene in *tert*-deficient zebrafish with inherited short telomeres prevented further telomere shortening and organism mortality, as also occurs in mice [Bernardes de Jesus *et al*., 2012; Jaskelioff *et al*., 2011; Samper *et al*., 2001]. This result indicates that a minimum telomere length is necessary to maintain normal tissue homeostasis and, more importantly, that there are therapeutic benefits of new drugs that are able to induce the

expression of the wild-type *TERT* or *TR* allele in heterozygous DC patients. The powerful advantages of the zebrafish for high-throughput drug screening [Zon & Peterson, 2005] could contribute to the identification of such drugs.

It is noteworthy that the telomere length of the offspring obtained from wild-type females and 11 month-old *tert*$^{-/-}$ males was shorter than that of the offspring of wild-type females and 5 month-old *tert*$^{-/-}$ males. Moreover, this correlated with the better survival of the offspring from younger mutant males and, therefore, is indicative of an anticipation phenomenon related with telomere length, which has been described in individuals with DC [Armanios *et al*., 2005; Vulliamy *et al*., 2004]. This anticipation phenomenon could be explained by a haploinsufficiency of the *tert* gene, because the partial telomere length rescue of *tert* heterozygotes was reflected in better organism survival. However, these heterozygotes still had a high proportion of short telomeres compared with the wild type, indicating that telomerase dosage was crucial. Therefore, the *tert*-deficient zebrafish line recapitulates the mechanisms of age in telomerase-deficient mice and individuals with DC [Armanios *et al*., 2005; Knudson *et al*., 2005; Vulliamy *et al*., 2004].

A very recent study has shown that the rate of increase in the percentage of short telomeres, rather than the rate of telomere shortening per month, was a significant predictor of lifespan in both wild-type and telomerase-deficient mice, and those individuals who showed a higher rate of increase in the percentage of short telomeres were also the ones with a shorter lifespan [Vera *et al*., 2012]. Consistent with this idea, the G1 *tert*-deficient zebrafish showed a shorter lifespan than the wild type, and the G2 died prematurely. In addition, the presence of several phenotypes in the G2 of *tert*-deficient zebrafish further supports this notion, because the degree of developmental defects of each phenotype was directly correlated with telomere length and, specifically, with the proportion of short telomeres. Notably, telomere shortening was also accompanied by an increase in the number of signal-free chromosome ends, chromosome fusions and MTSs. Therefore, we speculate that *tert* heterozygous zebrafish with a higher proportion of short telomeres would show an anticipation in aging signals and a shorter longevity. However, further studies are required to characterize *tert* heterozygote zebrafish and to study whether they are able to model the anticipation process observed in individuals with DC.

The pathologies that occur in the telomerase-deficient mouse model are accompanied by a reduction in the proliferative potential due to an activation of p53, which leads to

growth arrest and/or apoptosis in the affected tissues [Leri *et al*., 2003]. In addition, p53 deficiency rescues the adverse effects of telomere loss [Chin *et al*., 1999; Flores & Blasco, 2009]. These results are consistent with the induction of *p53* gene expression in zebrafish tissues with a high proliferation rate, such as those of the testis. In this tissue, *p53* gene expression was higher in 8-month-old *tert* mutant zebrafish compared with their wild-type siblings, coinciding with telomere shortening. Similarly, G2 larvae also showed an activation of *p53* gene expression and an increased number of apoptotic cells in the abnormal phenotypes (groups II and III) compared with the normal one (group I), indicating once again the importance of critically short telomeres and genomic instability. The relevance of p53 in the premature senescence and reduced fertility of G1 and the developmental defects and early mortality of G2 *tert*-deficient larvae was confirmed by the complete rescue of G2 larval survival by transient p53 inactivation and the generation of G1 $tert^{-/-}$; $p53^{-/-}$ double mutants, which showed an absence of morphological alterations and TUNEL+ germ cells in the testes at 8 months of age. Importantly, the G2 $tert^{-/-}$; $p53^{-/-}$ animals had increased longevity, despite the fact that their telomere length did not increase. These results might explain the high percentage of mortality between 10 and 20 dpf of G2 doubly null mutants. Although p53 deficiency was able to initially rescue the adverse effects of telomere loss, sustained cell proliferation in the absence of p53 might result in a progressive telomere shortening and dysfunction that, in turn, would reduce lifespan. Further aging and carcinogenesis studies are required with the G2 doubly null mutants.

To summarize, the telomerase-deficient zebrafish characterized in this study should be considered as a promising model to study telomere-driven aging, because they are able to recapitulate human telomere and telomerase biology. In addition, it is an exceptional vertebrate model for the discovery of new treatments able to temporarily reactivate telomerase expression in individuals with DC.

Chapter III:

Telomere length maintenance during zebrafish caudal fin regeneration

Abstract

Telomeres are essential for chromosome protection and genomic stability, and telomerase function is critical for organ homeostasis. Zebrafish have become a useful vertebrate model for understanding the cellular and molecular mechanisms of regeneration. Regeneration capacity of tissue regeneration in wild-type zebrafish caudal fin is not affected by repetitive amputation but the behavior of telomeres during this process has not been studied yet. In this study, we characterized the regeneration process in a telomerase-deficient zebrafish model and its respective control. Moreover, we studied the regenerative capacity after repetitive amputations and at different ages. Regenerative efficiency decrease with aging in all the genotypes and surprisingly, telomere length is maintained even in the telomerase-deficient genotypes. In addition, we detected telomeric DNA circles in the regenerative cells and we observed a lower regeneration efficiency after inhibition of two genes implicated in ALT pathway during regeneration in adults and larvae. Our results suggest telomere length may be maintained by the regenerative cells through the Recombination-mediated Alternative Lengthening of Telomeres (ALT) pathway, likely to support high cell proliferation rates during the caudal fin regenerative process although further studies are required to completely elucidate this mechanism.

1. Introducion

Tissue regeneration is an evolutionary conserved response to injury [Morrison *et al.*, 2006] and while all animals regenerate some of their tissues by physiological turnover, only few can regenerate appendages. Zebrafish (*Danio rerio*) is one of such organisms, able to regenerate retina, fins, heart, spinal cord and other tissues to later advanced ages [Becker *et al*., 1997; Rowlerson *et al*., 1997; Reimschuessel, 2001; Poss *et al*., 2002, 2003]. Because of its regenerative ability, its simple but relevant anatomy, *in vivo* imaging capability and genetic advantages, zebrafish have become a useful vertebrate model for understanding the cellular and molecular mechanisms of regeneration [Goldsmith & Jobin, 2012]. Caudal fin is the most convenient tissue for regenerative studies due to its easy handling and fast regeneration. Adult zebrafish regenerate their caudal fin within fourteen days after amputation [Poss *et al.*, 2003].

Several groups have investigated cells and genetic signalling pathways regulating the blastema formation [Poleo *et al.*, 2001; Poss *et al.*, 2000, 2002; Lee *et al.*, 2005; Goessling *et al.*, 2009; Chablais & Jazwinska, 2010; Geurtzen *et al.*, 2014; Hirose *et al.*, 2014]. In addition, the regeneration limit of the zebrafish caudal fin was recently investigated [Shao *et al*., 2011; Azevedo *et al*., 2011], however, there are very few studies about the implication of telomeres and telomerase in this process. Moreover, the zebrafish has constitutively abundant telomerase activity in somatic tissues from embryos to aged adults [Kishi *et al*., 2003; McChesney *et al*., 2005]. Notably, a study on various tissues from aquatic species including the zebrafish suggests that telomerase may be important for tissue renewal and regeneration after injury rather than for overall organism longevity [Elmore *et al*., 2008]. Our previous studies about behaviour of telomeres and telomerase during the regeneration process revealed a direct relationship between telomerase expression, telomere length and efficiency of tissue regeneration in wild-type zebrafish caudal fin [Anchelin *et al*., 2011]. On the other hand, our work about characterization of the telomerase-deficient zebrafish suggest that telomerase function is crucial for organ homeostasis in zebrafish [Anchelin *et al*., 2013], as occurs in mouse [Blasco *et al*., 1997; Yuan *et al*., 1999; Chiang *et al*., 2004].

The aim of this study was to further finding out the implication of telomerase during the caudal fin regenerating process, for this, we performed several regeneration assays, through single or multiple injuries at different stages, using $tert^{+/+}$, $tert^{+/-}$ and $tert^{-/-}$ specimens from the same genetic background, maintained in the same laboratory conditions. Our results confirmed the outstanding and almost unlimited caudal fin

regeneration capability of the zebrafish, that only decrease in aged animals, also in the case of the telomerase-deficient genotype. Moreover, the telomere length is maintained even in telomerase-deficient genotypes suggesting the implication of the alternative lengthening of telomeres (ALT) pathway to explain the maintenance of telomere length during this process. We performed various experimental assays based on reported characteristic features of recombination-based replication mechanism to investigate this hypothesis. Together our results indicate that ALT is implicated in the maintenance of telomeres during the caudal fin regeneration process in all genotypes, although the data are statistically more significant in the case of telomerase-deficient fish.

2. Materials and Methods

2.1. Maintenance of zebrafish

Wild-type AB zebrafish (*Danio rerio*) were obtained from the Zebrafish International Resource Centre (ZIRC). The *tert* mutant line (allele hu3430) was obtained from the Sanger Institute. Adult fish were maintained in recirculating tanks following instructions from ‘‘*The zebrafish book*” [Westerfield, 2000]. Adult fish were maintained at 26ºC, with a 14:10 hourlight:dark cycle, and were fed twice daily, once with dry flake food (PRODAC) and once with live artemia (MC 450, INVE AQUACULTURE). Zebrafish embryos were maintained in egg water at 28.5ºC and were fed at 5days with NOVO TOM and with live arthemia at 11 days of life.

The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and were approved by the Bioethical Committee of the University Hospital Virgen de la Arrixaca (Spain) under approval number (PI06/FIS0369/040706).

2.2. Caudal fin regeneration assay in Adult

For the caudal fin regeneration assay, zebrafish from *tert*$^{+/+}$, *tert*$^{+/-}$ and *tert*$^{-/-}$ genotypes were anesthetized with 0.05% benzocaine. The fin tissue was removed within approximately 2mm of the base of the caudal peduncle using a razor blade. For the single amputation assay or the successive amputations assay, zebrafish fins were imaged before and after amputation, as well as different days post-amputation (dpa) following the respective experimental design. The fish were allowed to recover and the animals were returned to recirculating water heated to 32ºC for the duration of the experiment. Each fish was tracked and imaged individually to calculate regeneration progress over time. Percent fin regeneration was determined based on the area of regrowth divided by the original fin area ± standard error.

To study the telomere length in caudal fin regeneration by flow-FISH assay, we obtained by excision and processed as indicated below, the regenerated portion of caudal fin tissue from zebrafish at different clips along the experiment.

2.3. Cell isolation

Regenerated portion of caudal fin tissue obtained from zebrafish at different clips were incubated in PBS, centrifuged (600 g, 5 min.), incubated in Trypsin (0.5 mg/ml)/EDTA (0.1 mg/ml) in PBS for 1 min., centrifuged (600 g, 5 min.) and then incubated in Colagenase (0.5 mg/ml) in RPMI medium supplemented with $CaCl_2$ $2H_20$ (0.7 mg/ml) for 30 min. The cell suspensions were obtained by pipetting, smashing and filtering the digested tissues through a 100 µm mesh and a 70 µm mesh successively, finally washed and resuspended in PBS.

2.4. Flow-FISH

Cells from each fin sample obtained as described before were washed in 2 ml PBS supplemented with 0.1% BSA. Each sample was divided in two replicate tubes: one pellet was resuspended in 500 ml hybridization buffer and another in hybridization buffer without FITC-labeled telomeric PNA probe as negative control. Samples were then denatured for 10 minutes at 80ºc under continuous shaking and hybridized for 2 h in the dark at room temperature. After that, the cells were washed twice in a washing solution (70% deionized Formamide, 10 mM Tris, 0.1% BSA and 0.1% Tween-20 in dH_2O, pH 7.2). The cells were then centrifuged, resuspended in 500 ml of propidium iodide solution, incubated 2 hours at room temperature, stored at 4ºC and analyzed by flow cytometry within the following 48 h.

2.5. CCassay

The assay for the detection of telomeric DNA circles was performed as described [Lau *et al*., 2013]. Zebrafish caudal fin tissues collected from *tert*$^{-/-}$ and *tert*$^{+/+}$ genotypes at 0 hpa, and from the corresponding regenerate portion at 24- and/or 72 hpa were preserved at -80ºC. Genomic DNA (gDNA) was extracted from the fin samples using the "Wizard Genomic DNA Purification kit (Promega). A rolling circle amplification of partially double-stranded C-circles was performed with 0.2µg/µl bovine serum albumin, 0.1% Tween-20, 4µM dithiotreitol (DTT), 1 µM dNTPs, 3.75U ϕ29 polymerase, 1X ϕ29 polymerase buffer and 16 ng of g DNA. It was incubated at 30ºC for 8 h then at

65ºC for 20 min. For each sample, the assay was done with and without ϕ29 polymerase.

After that, the telomeric secuences were detected through real-time PCR using C circles amplification reaction as template. The primers used were:

Telom F: 5'-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3' and Telom R: 5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3'. Finally, a second real-time PCR was performed with the same amplification reactions to detect rps11 as a standard value. The primers used were: rps11 F:5'-ACAGAAATGCCCCTTCACTG-3' and rps11 R: 5'-GCCTCTTCTCAAAACGGTTG-3'. Both qPCRs were performed with and without ϕ29 polymerase. All PCR results were expressed as mean of triplicate reactions ±SEM. Means were compared using unpaired two-tailed Student's t-test.

. 2.6. Morpholino injection and electroporation

Adult *tert*$^{+/+}$, *tert* $^{-/-}$ zebrafish were anesthetized in 0.05% benzocaine prior to the amputation of the distal portion of their caudal fins, proximal to the first lepidotrichial branching point. Following the surgery, the fish were returned to recirculating water heated to 32ºC. Vivo-porter coupled morpholino oligonucleotides (MOs) (Gene Tools) containing a 3' fluorescein tag were used at 1.5mM. The MO sequences were as follows:

Nbs1: 5'-GATTTACACAGAGAAGATTACCTCC-3'

Negative control 5'-CCTCTTACCTCAGTTACAATTTATA-3'

MOs were injected into the regenerating tissue on the dorsal part of each zebrafish tail fin at 48 hours post-amputation (hpa). The other uninjected half was considered as an internal control in order to monitor the normal growth. Immediately after injections, the dorsal half was electropored using a NEPAGENE electroporator, five consecutive 50 msec pulses, at 15 V with a 30 sec pause between pulses, were used. A 3-mm-diameter platinum plate electrode (CUY 650-P3 Tweezers, Protech International) localized the pulses to approximately the dorsal one-half of the fin.

After the procedure, fins were documented at different time points by capturing fluorescent and brightfield signals simultaneously using the standard excitation and emission settings for the fluorophore FITC on a SteReo Lumar V12 stereomicroscope with an AxioCam MR5 (Carl Zeiss). In order to calculate the percentage area of the

outgrowth between the injected and non-injected part, the values were inserted in the following formula : (Exp 3 days-Exp 2 days)/(Cont 3 days-Cont 2 days)*100, where Exp is the area of the outgrowth of the MO-treated regenerate and Cont is the area of the corresponding outgrowth of the uninjected control half.

2.7. Amputation of zebrafish larval fin primordia and chemical exposure by immersion.

48 hours post-fertilization (hpf) and dechorionated zebrafish larvae obtained from *tert* $^{+/-}$ x *tert* $^{+/-}$ breeding crosses were anesthetized in 0.008% tricaine, and placed on an agar plate to amputate the caudal fin primordia with a surgical razor blade just posterior to the notochord. The larvae were individualized in each well of 96- well plates containing ATR Inhibitor IV at 10µm or egg water E3 as negative control. Larval fin were imaged before amputation, after amputation and at 24 hours post-amputation to measure and calculate the regeneration area.

2.8. Genotyping of zebrafish larvae from tert $^{+/-}$ x tert $^{+/-}$ breeding crosses.

Genomic DNA was extracted from individual larvae. The larvae were incubated in alkalyne lysis solution at 95ºC for 1h, chilled on ice, neutralizing solution was added and after centrifugation, the supernadant was used as the DNA template for PCR. The primers used to distinguish *tert* $^{-/-}$ larvae from wild-type and heterozygous larvae were: Zf TERT F: 5'-TGCCGGAGGTCTTGGCG-3' and Zf TERT R: 5'-CGCACACCTGCAGAACA-3' for *tert*$^{+/+}$ genotype, Zf TERT F: 5'-TGCCGGAGGTCTTGGCG-3' and Zf TERT R: 5'- CGCACACCTGCAGAACT-3' for *tert* $^{-/-}$ genotype. The PCR products were examined by gel electrophoresis and red safe staining staining.

3. Results

3.1. Caudal fin regeneration is affected by aging in the three genotypes

It is known that after a single excision of the zebrafish caudal fin, a regenerative process is activated and it takes approximately two weeks to fully regenerate all the tissues and structures that compose a functional fin [Akimenko *et al*., 2003; Poss *et al.*, 2003]. Recent studies show that consecutive repeated amputations of zebrafish caudal fin do not reduce its regeneration capacity [Azevedo *et al*., 2011; Shao *et al*., 2011].

To further finding out the role of telomerase during this regenerating process, we used *tert*$^{+/+}$, *tert*$^{+/-}$ and *tert*$^{-/-}$ specimens from the same genetic background, maintained in the same laboratory conditions to regeneration assays after a single amputation or after repeated amputations.

After a single amputation (**Fig. 1A**) on zebrafish from the three genotypes and at different stages of life, we observed that all the genotypes regenerate. The regeneration curve (**Fig. 1B**) showed that 8 month-old *tert*$^{+/+}$ zebrafish regenerate their entire caudal fin faster than *tert*$^{+/-}$ and *tert*$^{-/-}$ zebrafish, as wild-type fish regenerate at 16 days-post-amputation (dpa), while *tert*$^{+/-}$ and *tert*$^{-/-}$ zebrafish regenerate at 19 and 22 dpa respectively.

In addition, we note that *tert*$^{+/-}$ and *tert*$^{-/-}$ zebrafish need more time to reach 50% of their caudal fin regeneration than wild-type zebrafish at all ages (**Fig. 1C**), and that difference is more significant at 11 months of age, when telomerase-deficient fish can be considered aged [Anchelin *et al*., 2013]. In our previous work, we described that 18 and 24 month-old wild-type zebrafish needed 9 and 11 days respectively to reach 50% of regeneration [Anchelin *et al.,* 2011], then the older zebrafish show a lower growth rate than younger ones.

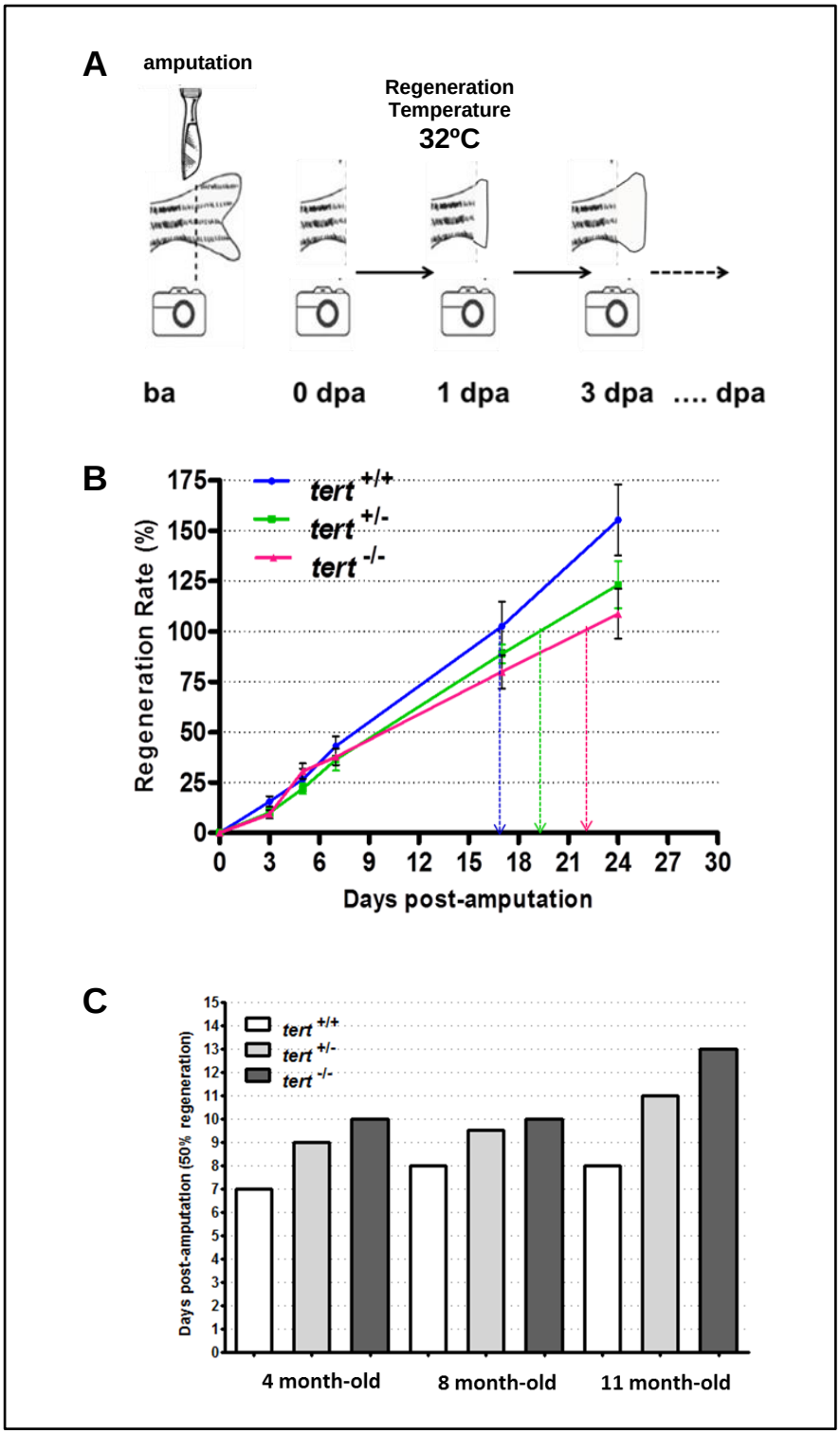


Figure 1. The three genotypes regenerate with different rate. A, Experimental design with a single amputation, at three different stages of life (4, 8, and 11 month-old), n=6. **B,** Caudal fin regeneration curve of 8 month-old zebrafish from the three genotypes. **C,** Days to reach 50% of the caudal fin regeneration at three different ages and from the three genotypes.

We realize a consecutive repeated amputation experiment using young adult fish (4 month-old) and old adult fish (11 month-old) from the three genotypes (**Fig. 2A**). The resulted regeneration curve (**Fig. 2B**) showed that after 11th repeated consecutive amputations, with 7 days intervals, the young zebrafish from the three different genotypes are able to regenerate their caudal fin at a similar regeneration rate. Whereas in the case of the old fish, the *tert*$^{+/-}$ and *tert*$^{-/-}$ zebrafish showed a lower and statistically different regeneration rate than their wild type *tert*$^{+/+}$ counterpart which continues to show a high regeneration rate, although slower than their younger siblings. Regeneration efficiency is affected by aging in all genotypes.

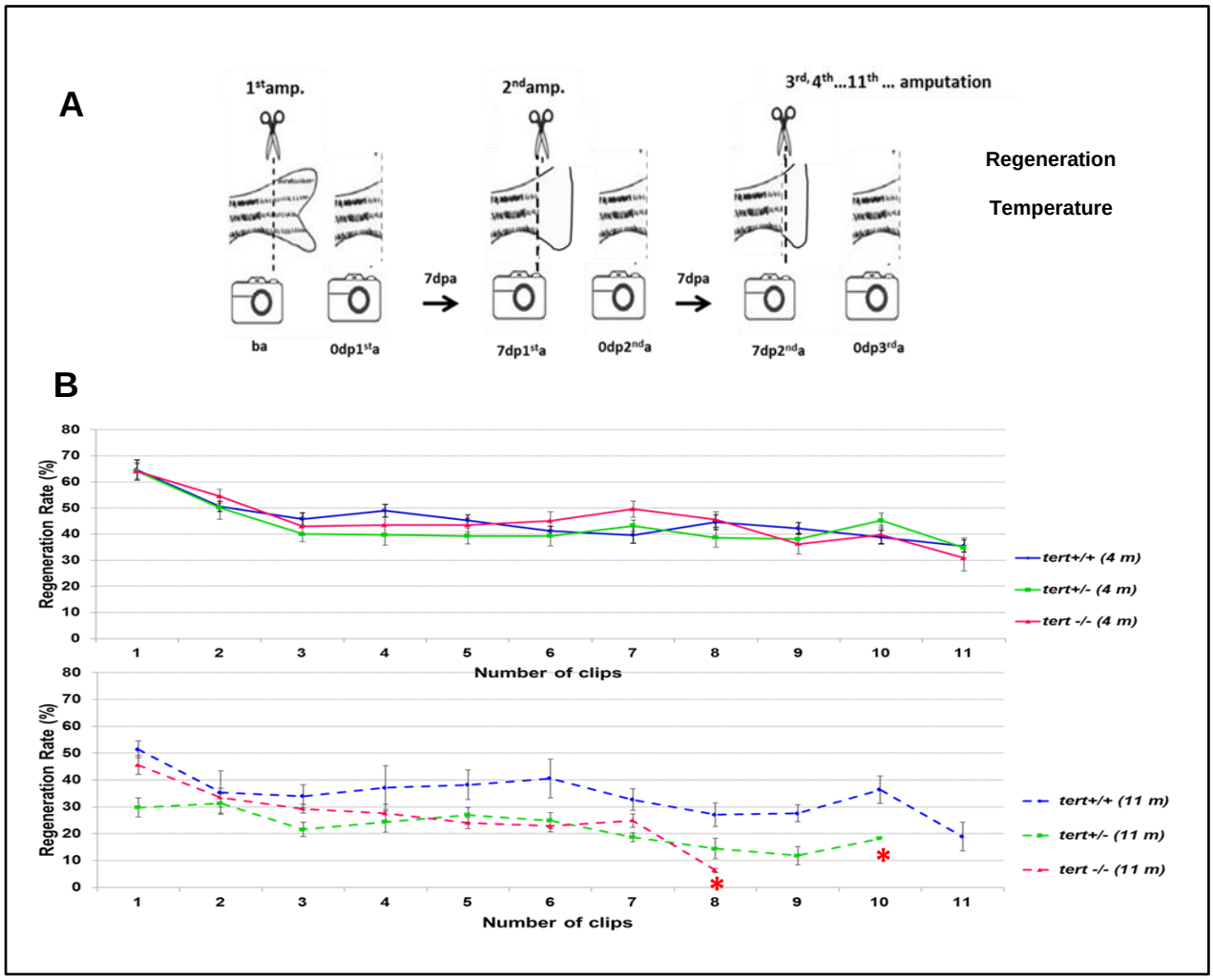


Figure 2. Aging affects the regeneration rate. A, Experimental design with successive excisions, n=6. **B,** Curves of the regeneration rates of the two groups of age from the three genotypes, red asterix means dead fish.

3.2. Telomere length is maintained during the regeneration assay in the three genotypes

To assess the dynamic of telomere length, fin regenerative tissue was collected from each group at different clips along the regeneration assay. We observed by Flow-FISH that telomere length is maintained in the two age groups and even in telomerase-deficient genotypes (**Fig. 3**). We note that young fish from all genotypes have a similar telomere length after the first amputation (clip1), while old *tert*$^{-/-}$ zebrafish show a lower telomere length if compared with the old *tert*$^{+/+}$ and *tert*$^{+/-}$ zebrafish.

These results suggest that telomerase is not essential for the regeneration of the zebrafish caudal fin and lead us to further investigate the mechanism by which these cells are able to maintain their telomere length throughout an assay of repeated amputations which means a continual renewal of tissues with high cell proliferation rates.

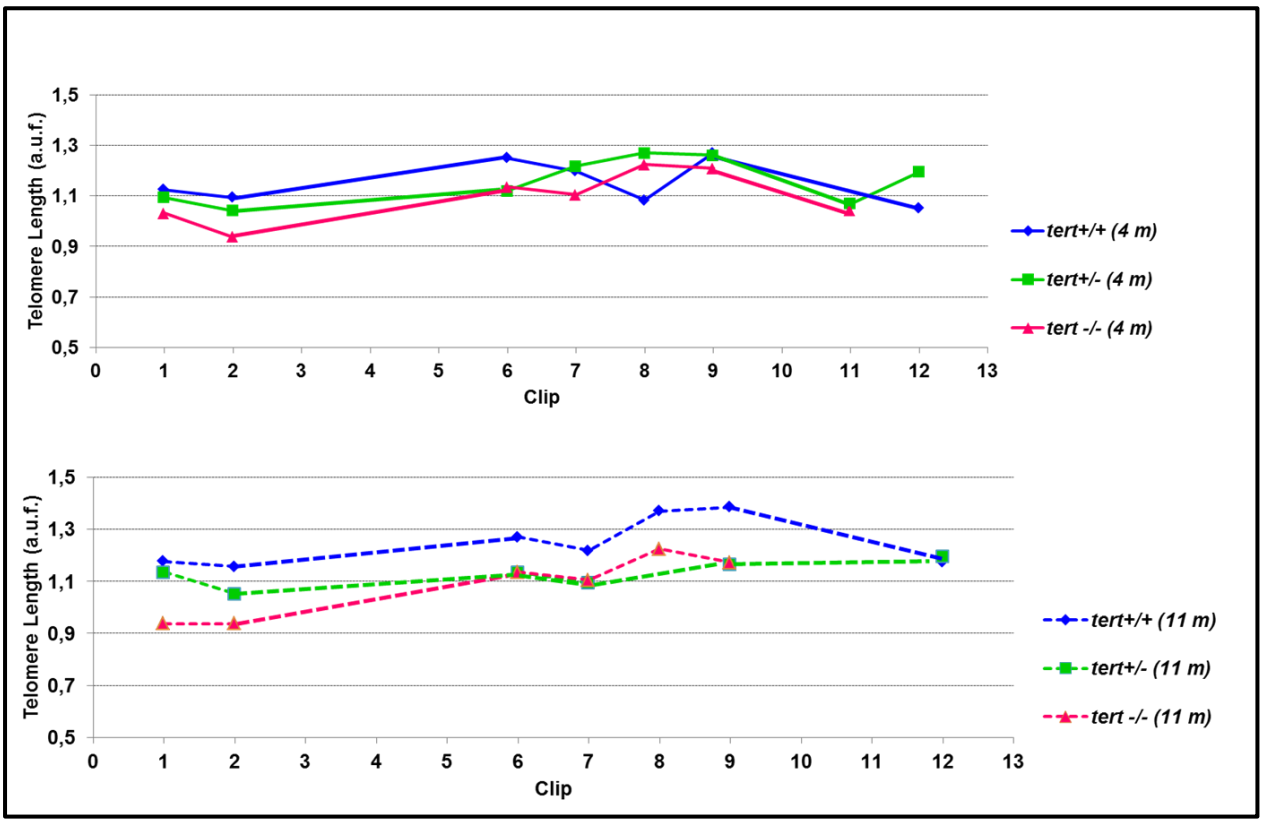


Figure 3. Telomere length is maintained during the regeneration assay. Curves showing the mean telomere length of the cells from fin regenerative tissue of the two groups of age from the three genotypes at different clips.

3.3. ALT is implicated in telomere length maintenance during regeneration process

Numerous studies revealed that in addition to the role of telomerase in maintaining telomere length, homologous recombination constitute an alternative mechanism (ALT) to maintain telomere DNA in telomerase-deficient cells [reviewed by Draskovic & Lodoño-Vallejo, 2013, Conomos *et al.*, 2013].

3.3.1. Detection of telomeric DNA circles

A characteristic of ALT is the presence of abundant extrachromosomal linear and circular telomeric DNA, so we set out to detect and quantify circular telomeric DNA in the cells obtained from the caudal fin regenerative portion using the quantitative and sensitive CCassay [Lau *et al.*, 2013] (**Fig. 4A**). We observed a statistically significant abundance of C circles at 24 hpa in the cells from the telomerase-deficient zebrafish compared with a slight increase in the cells of the wild-type zebrafish (**Fig. 4B**).

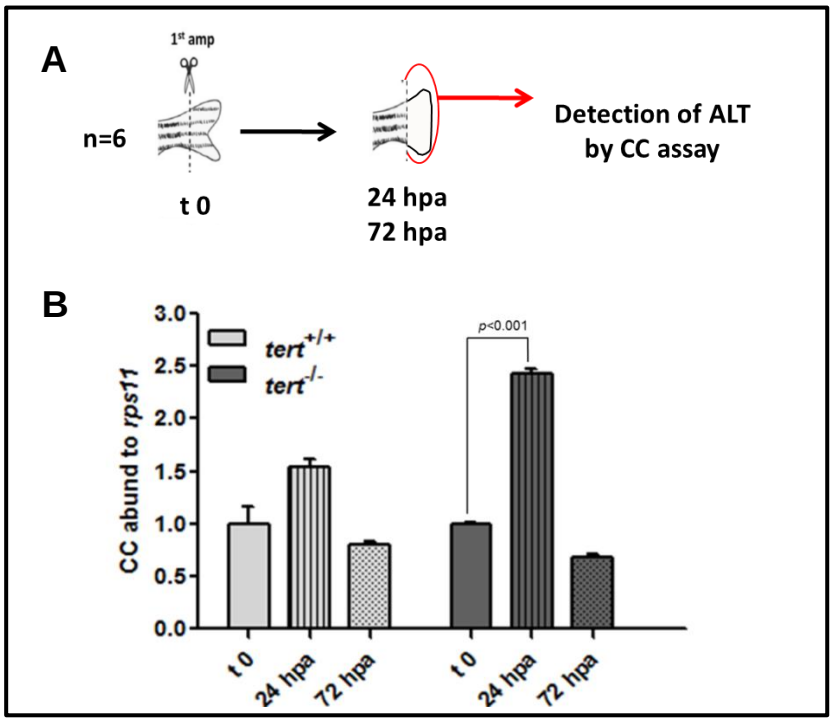


Figure 4. C circles quantification by CC assay in cells from regenerative tissues. A, Experimental design of CC assay . **B,** CCircles abundance in regenerative portion cells from wild-type and telomerase-deficient adult zebrafish. Statistical significance was assessed using the Student's *t*-Test ($p<0.05$).

3.3. 2. Inhibition of *Nbs1* gene affects adult caudal fin regeneration

NBS1 protein belonging to MNR recombination protein complex (Mre11–Rad50–Nbs1) implicated in Homologue Recombination process, is required for both the ALT phenotype and the formation of telomeric circles and its inhibition causes ALT dysfunction [Compton *et al.,* 2007; Zhong *et al.,* 2007]. To further investigate whether telomere length was maintained through ALT mechanism during the regeneration process, we inhibited *Nbs1* expression by transfection of an *in vivo* fluorescent morfolino against *Nbs1* or a standard morpholino as negative control in the 48hpa caudal fin regenerative blastema following by electroporation of the injected area, and we measured the regenerated area 24 hours later to calculate the regeneration area (**Fig. 5A**). Our results showed that inhibition of the *Nbs1* gene caused a regeneration inhibition in both genotypes, although higher in the *tert*$^{-/-}$ zebrafish compared with the *tert*$^{+/+}$ zebrafish (**Fig.5B**).

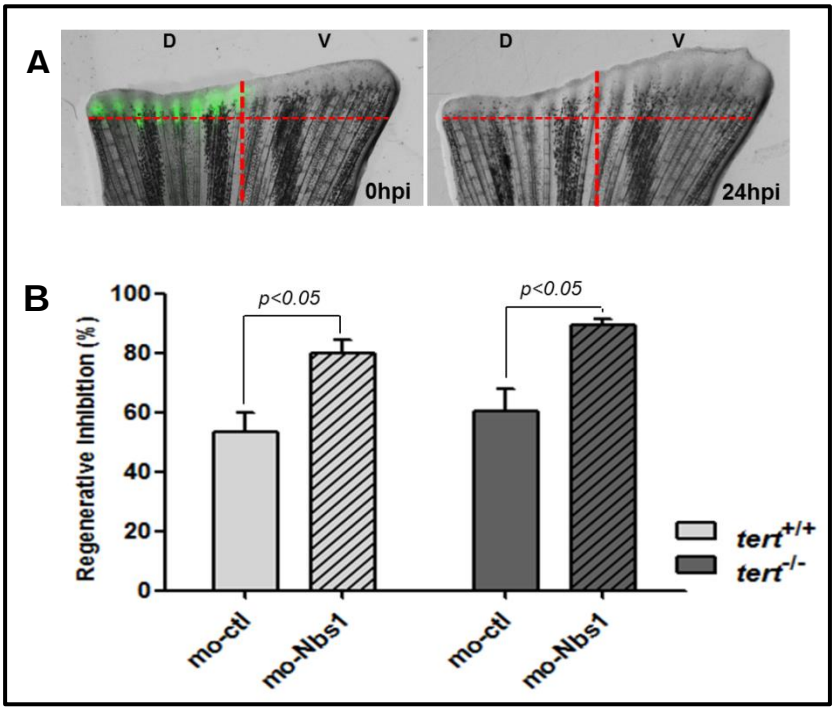


Figure 5. Measurement of regeneration inhibition by *Nbs1* gene knockdown. A, Representative images of caudal fin at 0 hour-post-injection (hpi) of *in vivo* fluorescent morfolino in the dorsal half (D), and after 24 hours of regeneration (24hpi). (V) uninjected ventral half. **B,** Percentage of caudal fin regeneration inhibition in wild-type and telomerase-deficient adult zebrafish. Statistical significance was assessed using the Student's *t*-Test ($p<0.05$).

3.3.3. Inhibition of *ATR* gene affects larval fin primordia regeneration

It seems that inhibition of ATR, an activator of *Nbs1* causes ALT dysfunction [Flynn *et al.,* 2015], and we want to investigate the effect of ATR inhibition during the fin regeneration process. In this case, we performed a regeneration assay on zebrafish larvae inhibiting ATR expression using an ATR gene inhibitor by immersion immediately after amputation. We measured the regeneration area 24 hours later and observed that all the larvae treated with ATR inhibitor showed a lower percentage of regenerate area, although only statistically significant in the case of the telomerase-deficient genotypes.

.

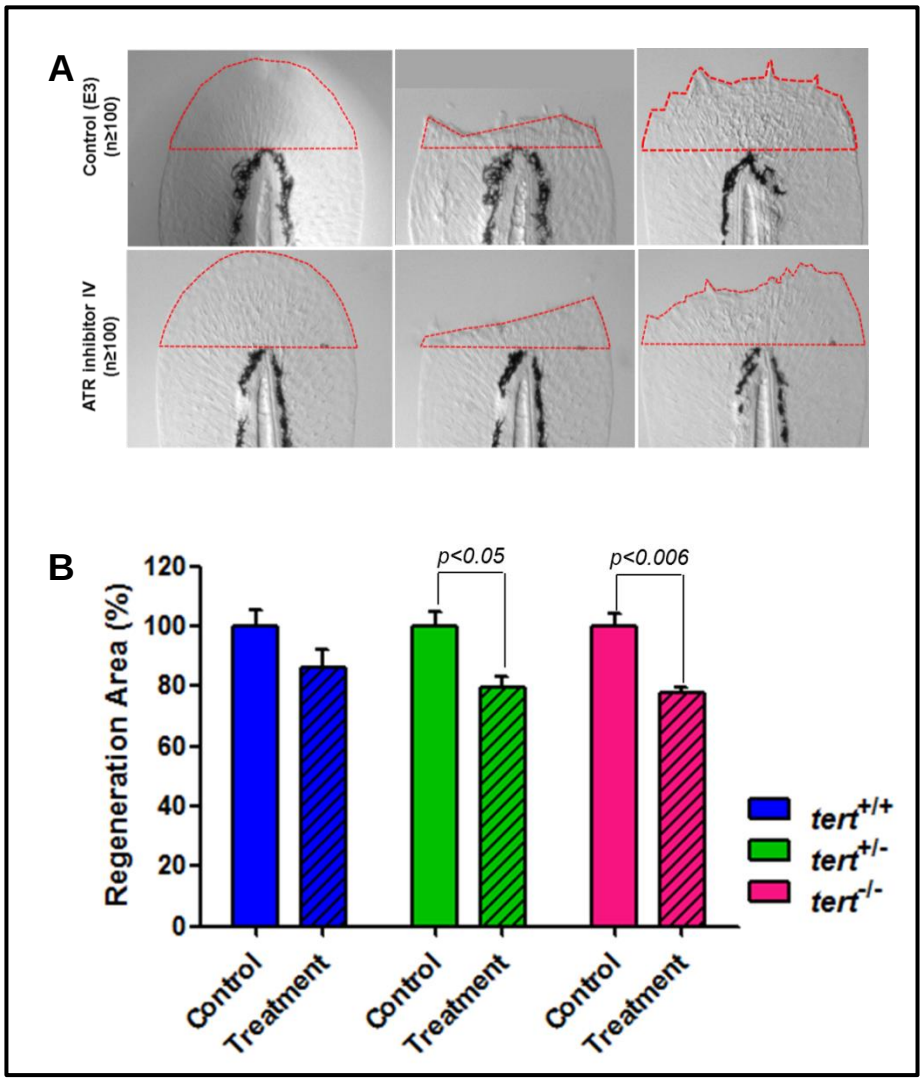


Figure 6. Measurement of the regeneration area after *ATR* gene inhibition in larvae from the three genotypes. A, Representative images of the regeneration assay. **B,** Percentage of regeneration area . Statistical significance was assessed using the Student's *t*-Test ($p<0.05$).

4. Discussion

The blastema is a proliferative mass of morphologically similar cells, formed through disorganization and distal migration of fibroblasts and osteoblasts proximal to the amputation plane [Gemberling *et al.,* 2013]. High proliferation rates accompanied by telomerase attrition required telomere maintenance to sustain replication and cell division.

We know the *tert* $^{-/-}$ specimens showed a telomeric length average much shorter than their wild-type siblings but, surprisingly, in contrast with those, they were able to maintain constant or even slightly increase their telomere length during aging [Anchelin *et al.,* 2013], then suggesting the possible involvement of the activation of an alternative telomere lengthening mechanism (ALT). This difference is noticeable when we compared cell telomere length from the whole fish, but much lower in the case of cells from caudal fin tissues, which makes difficult to compare telomere length from regenerative portion of caudal fin between genotypes.

Our results confirmed the notable regeneration capability of the zebrafish after a single or several successive fin amputations, even in the telomerase-deficient genotypes. In addition, the regeneration efficiency decrease with aging in all genotypes.

In the case of telomerase deficiency, homologous recombination constitute an alternative method (ALT 'alternative lengthening of telomeres') to maintain telomere DNA. Rolling-circle replication may use extrachromosomal t-circles, as has been shown in human ALT cells and in a wide variety of organisms including yeasts, higher plants and *Xenopus laevis*. From an evolutionary perspective, this widespread occurrence of t-circles may not only represent a back-up in the event of telomere dysfunction, but may be the primordial systems of telomere maintenance [Fajkus *et al.,* 2005]. This hypothesis and the fact that the activity binding to this external organ is crucial for the survival of the species, suggest the evolutionary explanation that short telomeres lead to recombination mechanism to ensure the telomere length maintenance of cells implicated in the regeneration after injury of such an organ essential for the fish survival.

A recent study about heart regeneration reported that absence of telomerase impairs proliferation leading to the accumulation of DNA damage, and *tert*$^{-/-}$ zebrafish hearts acquire a senescent phenotype [Bednarek *et al.,* 2015]. While, telomerase seems not to be essential for fin regeneration, conversely, telomerase is essential for zebrafish heart regeneration, suggesting telomere maintenance mechanism may be different

and/or specific of tissues and organs. Further studies are needed to elucidate a possible evolutionary explanation to this question.

The presence of extrachromosomal telomeric circles, similar in size to telomeric t-loops and proposed to result from homologous recombination events at the telomeres, has been used as a marker of ALT cells. At 24 hours post amputation, we detected telomeric DNA circles in the regenerative cells from the wild-type and the mutant zebrafish *tert*$^{-/-}$, although the amount of telomeric DNA circles is significantly higher in the mutant fish.

Moreover, we performed a regeneration assay using a *in vivo* morpholino against the *Nbs1* gene thereby inhibiting the formation of t-circles and also the maintenance of telomere by ALT, and we observed a lower regeneration efficiency after inhibition of *Nbs1* during caudal fin regeneration in adults from both and *tert*$^{+/+}$ and *tert*$^{-/-}$ genotypes.

Finally, by inhibiting ATR, a critical regulator of recombination recruited by RPA, we impede ALT during the regeneration process of the fin primordia from zebrafish larvae and then observed a lower regeneration efficiency in *tert*$^{+/+}$, *tert*$^{+/-}$ and *tert*$^{-/-}$ genotypes, although with values statistically significant in the case of telomerase-deficient genotypes.

Together, our results lead us to conclude that in the absence of telomerase, telomere length is maintained through a homologous recombination mechanism during the early regeneration. While in the wild-type zebrafish, the two mechanisms (telomerase-dependent and telomerase-independent) could coexist and participate to the maintenance of telomeres as ALT inhibition causes less severe effect than in *tert* mutant zebrafish. However, more studies are necessary to definitively conclude this issue.

In the present work, we confirmed the impressive capability of the zebrafish to heal and regenerate fin tissues after an injury, only limited with aging, even in the absence of telomerase. We showed that the ALT mechanism is involved in the telomere length maintenance during the regeneration process of the zebrafish caudal fin. As far as we know, this is the first work demonstrating ALT mechanism *in vivo* in a vertebrate model. Zebrafish has proven to be an undiscutible and useful vertebrate model system for further studies to deepen the understanding of the role of telomeres and telomerase in regeneration.

Conclusions

The results obtained in this work lead to the following conclusions:

1. Expression of telomerase and telomere length are closely related during the entire life cycle of the fish, and these two parameters can be used as biomarkers of aging in zebrafish.

2. Telomerase-deficient zebrafish show premature aging and reduced lifespan in the first generation, as occurs in humans and may be considered as a promising model to study telomere-driven aging

3. The second-generation embryos died in early developmental stages, and restoration of telomerase activity rescued telomere length and survival, indicating that telomerase dosage is crucial.

4. The *tert* mutant zebrafish line reproduces the genetic anticipation phenomenon, related to telomere shortening, observed in humans with DC

5. Genetic inhibition of p53 rescues the adverse effects of telomere loss, indicating that the molecular mechanisms induced by telomere shortening are conserved from fish to mammals.

6. Regeneration efficiency decreases with aging in wild-type and telomerase-deficient zebrafish

7. Wild-type and telomerase-deficient zebrafish regenerate caudal fin after single or successive amputations. *Tert* mutant line shows a lower regeneration rate than their wild-type counterpart, and maintains telomere length in the regenerative tissue

8. ALT mechanism is implicated in telomere maintenance during caudal fin regeneration in telomerase-deficient fish. Further studies are necessary to definitively elucidate the role of telomerase and the possible ALT implication in telomere maintenance during caudal fin regeneration in wild-type animals.

9. The zebrafish can be used to identify genes and drugs that affect the ability to restore aging phenotypes.

References

Abad JP, De Pablos B, Osoegawa K, De Jong PJ, Martín-Gallardo A & Villasante A (2004). **Genomic analysis of *Drosophila melanogaster* telomeres: full-length copies of HeT-A and TART elements at telomeres.** *Mol Biol Evol* **21** (9), 1613-1619.

Akimenko MA, Mari-beffa M, Becerra J & Geraudie J (2003). **Old questions, new tools, and some answers to the mystery of fin regeneration.** *Dev Dyn* **226,** 190-201.

Alter BP, Baerlocher GM, Savage SA, Chanock SJ, Weksler BB, Willner JP, Peters JA, Giri N & Lansdorp PM (2007). **Very short telomere length by flow-FISH identifies patients with dyskeratosis congenita.** *Blood* **110** (5), 1439-1447.

Anchelin, M., Murcia, L., Alcaraz-Pérez, F., García-Navarro, E. M. & Cayuela, M. L. (2011). **Behaviour of telomere and telomerase during aging and regeneration in zebrafish**. *PLoS ONE* **6**, e16955.

Anchelin M, Alcaraz-Pérez F, Martinez CM, Bernabé-García M, Mulero V & Cayuela ML (2013). **Premature aging in telomerase-deficient zebrafish**. *Dis Model Mech* **6**, 1101-1112.

Armanios M, Chen JL, Chang YP, Brodsky RA, Hawkins A, Griffin CA, Eshleman JR, Cohen AR, Chakravarti A, Hamosh A & Greider CW (2005). **Haploinsufficiency of telomerase reverse transcriptase leads to anticipation in autosomal dominant dyskeratosis congenita.** *Proc Natl Acad Sci USA* **102**, 15960–15964.

Au DW, Mok HO, Elmore LW & Holt SE, (2009). **Japanese medaka: a new vertebrate model for studying telomere and telomerase biology.** *Comp Biochem Physiol C Toxicol Pharmacol* **149** (2), 161-167.

Aubert G & Lansdorp PM (2008). **Telomeres and aging.** *Physiol Rev* **88**, 557-579.

Autexier (2008). **POT of gold: modeling dyskeratosis congenita in the mouse**. *Genes Dev.* **22**, 1731-1736.

Azevedo AS, Grotek B, Jacinto A, Weidinger G & Saúde L (2011) **The regenerative capacity of the zebrafish caudal fin is not affected by repeated amputations.** *PLoS ONE* **6** (7), e22820.

Azzalin CM, Reichenbach P, Khoriauli L, Giulotto E & Lingner J (2007). **Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends.** *Science* **318** (5851), 798-801.

Becker T, Wullimann MF, Becker CG, Bernhardt RR & Schachner M (1997). **Axonal regrowth after spinal cord transection in adult zebrafish**. *J Comp Neurol* **377**, 577-595.

Bednarek D, González-Rosa JM, Guzmán-Martínez G, Gutiérrez-Gutiérrez O, Aguado T, Sánchez-Ferrer C, Marques I, Galardi-Castilla M, de Diego I, Gómez MJ, Alfonso Cortés A, Zapata A, Jiménez-Borreguero LJ, Mercader N & Flores I (2015). **Telomerase is essential for zebrafish heart regeneration**. *Cell Reports* **12**, 1-13.

Beier F, Foronda M, Martinez P & Blasco MA (2012). **Conditional TRF1 knockout in the hematopoietic compartment leads to bone marrow failure and recapitulates clinical features of dyskeratosis congenita.** *Blood* **120** (15), 2990-3000.

Bernardes de Jesus B, Vera E, Schneeberge K, Tejera AM, Ayuso E, Bosch F & Blasco MA (2012). **Telomerase gene therapy in adult and old mice delays aging and increases longevity without increasing cancer.** *EMBO Mol Med* **4**, 691-704.

Bernhardt RR, Tongiorgi E, Anzini P & Schachner M (1996). **Increased expression of specific recognition molecules by retinal ganglion cells and by optic**

pathway glía accompanies the successfulregeneration of retinal axons in adult zebrafish. *J Comp Neurol* **376**, 253-264.

Blackburn EH (1991)**. Structure and function of telomeres**. *Nature* **350**, 569-573.

Blackburn EH (2005). **Telomeres and telomerase: their mechanism of action and the effects of altering their functions.** *FEBS Lett* **579**, 859-862. Review.

Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA & Greider CW (1997). **Telomere shortening and tumor formation by mouse cells lacking telomerase RNA**. *Cell* **91**, 25–34.

Blasco MA (2005). **Telomeres and human disease: ageing, cancer and beyond.** *Nat Rev Genet* **6**, 611-622.

Brittijn SA, Duive teijn SJ, Belmamoune M, Bertens LF, Bitter W, *et al*. (2009). **Zebrafish development and regeneration: new tools for biomedical research.** *Int J Dev Biol* **53**, 835-850.

Broccoli D, Young JW & de Lange T (1995). **Telomerase activity in normal and malignant hematopoietic cells.** *Proc Natl Acad Sci* U S A **92**, 9082-9086.

Brunet A & Rando TA (2007). **From stem to stern.** *Nature* **449**, 288-291.

Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA & Reddel RR (1997). **Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines.** *Nat Med* **3**, 1271-1274.

Bryan TM, Englezou A, Dunham MA & Reddel RR (1998). **Telomere length dynamics in telomerase-positive immortal human cell populations.** *Exp Cell Res* **239**, 370-378.

Calado RT and Young NS (2009). **Telomere diseases**. *N. Engl. J. Med.* **361**: 2353-2365.

Canela A, Vera E, Klatt P & Blasco MA (2007). **High-throughput telomere length quantification by FISH and its application to human population studies**. *Proc Natl Acad Sci* USA **104**, 5300-5305.

Canela A, Klatt P & Blasco MA (2007). **Telomere length analysis**. *Methods Mol Biol* **371**, 45-71.

Cayuela ML, Flores JM & Blasco MA (2005). **The telomerase RNA component Terc is required for the tumour-promoting effects of Tert overexpression**. *EMBO* **6**, 268-274.

Cerone MA, Londono-Vallejo JA & Bacchetti S (2001). **Telomere maintenance by telomeras and by recombination can coexist in human cells.** *Hum Mol Genet* **10** (18), 1945-1952.

Chablais F & Jazwinska A (2010). **IGF signaling between blastema and wound epidermis is required for fin regeneration.** *Development* **137**, 871-879.

Chan SW & Blackburn EH (2002). **New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin**. *Oncogene* **21**, 553-563. Review.

Cheung I, Schertzer M, Rose A & Lansdorp PM (2006). **High incidence of rapid telomere loss in telomerase-deficient Caenorhabditis elegans**. *Nucleic Acids Res.* **34**, 96-103.

Chiang YJ, Hemann MT, Hathcock KS, Tessarollo L, Feigenbaum L, Hahn WC & Hodes RJ (2004). **Expression of telomerase RNA template, but not telomerase reverse transcriptase, is limiting for telomere length maintenance in vivo**. *Mol. Cell. Biol.* **24**, 7024-7031.

Chin L, Artandi SE, Shen Q, Tam A, Lee SL, Gottlieb GJ, Greider CW & DePinho RA (1999). **p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis**. *Cell* **97**, 527-538.

Cohn M & Blackburn EH (1995). **Telomerase in yeast**. *Science* **269**, 396-400.

Collins K & Mitchell JR (2002). **Telomerase in the human organism**. *Oncogene* **21**, 564-579.

Compton S A, Choi JH, Cesare AJ, Ozgur S & Griffith JD (2007). **Xrcc3 and Nbs1 are required for the production of extrachromosomal telomeric circles in human alternative lengthening of telomere cells** *Cancer Res.* **67**, 1513–1519.

Conomos D, Pickett HA & Reddel RR (2013). **Alternative lengthening of telomeres: remodeling the telomere architecture.** *Frontiers in oncology* **3** (27), 1-7.

Counter CM, Gupta J, Harley CB, Leber B & Bacchetti S (1995). **Telomerase activity in normal leukocytes and in hematologic malignancies**. *Blood* **85**, 2315-2320.

Curado S, Anderson RM, Jungblut B, Mumm J, Schroeter E & Stainier DY (2007). **Conditional targeted cell ablation in zebrafish: a new tool for regeneration studies.** *Dev Dyn* **236** (4), 1025-1035.

De Lange T (2005). **Shelterin: The protein complex that shapes and safeguards human telomeres.** *Genes Dev* **19**, 2100-2110. Review.

Detrich HW 3rd (2008). **Fluorescent proteins in zebrafish cell and developmental biology.** *Methods Cell Biol* **85**, 219-241. Review.

Diotti R & Loayza D (2011). **Shelterin complex and associated factors at human telomeres.** *Nucleus* **2** (2), 119-135. Review.

Dokal I (2011). **Dyskeratosis congenita.** *Hematology Am Soc Hematol Educ Program* 480-486. Review.

Donate LE & Blasco MA (2011). **Telomeres in cancer and ageing.** *Philos Trans R Soc Lond B Biol Sci* **366** (1561), 76-84. Review.

Draskovic I & Londoño-Vallejo JA (2013). **Telomere recombination and alternative telomere lengthening mechanisms.** *Front Biosci* **18**, 1-20. Review.

Drummond MW, Balabanov S, Holyoake TL & Brummendorf TH (2007). **Concise review: Telomere biology in normal and leukemic hematopoietic stem cells.** *Stem Cells* **25**, 1853-1861.

Du HY, Idol R, Robledo S, Ivanovich J, An P, Londono-Vallejo A, Wilson DB, Mason PJ & Bessler M (2007). **Telomerase reversetranscriptase haploinsufficiency and telomere length in individuals with 5psyndrome.** *Aging Cell* **6**, 689-697.

Durant ST (2012). **Telomerase-independent paths to immortality in predictable cancer subtypes.** *J Cancer* **3**, 67-82. Review.

Ekker SC (2004). **Nonconventional antisense in zebrafish for functional genomics applications.** *Methods Cell Biol* **77**, 121-136.

Elmore LW, Norris MW, Sircar S, Bright AT & McChesney PA (2008)**. Upregulation of telomerase function during tissue regeneration**. *Exp Biol Med* (Maywood) **233**, 958–967.

Fajkus J, Sykorova E, & Leitch AR (2005). **Telomeres in evolution and evolution of telomeres** *Chromosome Research* **13**, 469-479.

Feitsma H & Cuppen E (2008). **Zebrafish as a cancer model**. *Mo.l Cancer Res* **6**, 685-694.

Finkel T, Serrano M & Blasco MA (2007). **The common biology of cancer and ageing.** *Nature* **448**,767-774. Review.

Fitzgerald MS, Riha K, Gao F, Ren S, McKnight TD & Shippen DE (1999). **Disruption of the telomerase catalytic subunit gene from Arabidopsis inactivates telomerase and leads to a slow loss of telomeric DNA**. *Proc. Natl. Acad. Sci. USA* **96**, 14813-14818.

Flores I, Cayuela ML & Blasco MA (2005). **Effects of telomerase and telomere length on epidermal stem cell behavior**. *Science* **309**, 1253-1256.

Flores I, Canela A, Vera E, Tejera A, Cotsarelis G, *et al*. (2008). **The longest telomeres: a general signature of adult stem cell compartments**. *Genes Dev* **22**, 654-667.

Flores I & Blasco MA (2009). **A p53-dependent response limits epidermal stem cell functionality and organismal size in mice with short telomeres**. *PLoS ONE* 4, e4934.

Flynn RL, Cox KE, Jeitany M, Wakimoto H, Bryll AR, Ganem NJ, Bersani F, Pineda JR, Suvà ML, Benes CH, Haber DA, Boussin FD & Zou L (2015) **Alternative lengthening of telomeres renders cancer cells hypersensitive to ATR inhibitors** *Science.* **347** (6219), 273-277.

Fu D & Collins K (2007). **Purification of human telomerase complexes identifies factors involved in telomerase biogénesis and telomere length regulation.** *Mol Cell* **28**, 773-785.

Galati A, Micheli E & Cacchione S (2013). **Chromatin structure in telomere dynamics.** *Front Oncol* **3**, 46. Review.

Gemberling M, **Bailey TJ**, **Hyde DR & Poss KD** (2013). **The zebrafish as a model for complex tissue regeneration** *Trends Genet* **29** (11).

Gerhard GS & Cheng KC (2002.) **A call to fins! Zebrafish as a gerontological model.** *Aging Cell* **1**, 104-111.

Gerhard GS, Kauffman EJ, Wang X, Stewart R, Moore JL, Kasales CJ, Demidenko E & Cheng, KC (2002). **Life spans and senescent phenotypes in two strains of Zebrafish (*Danio rerio*)**. *Exp. Gerontol.* **37**, 1055-1068.

Gerhard GS, (2003). **Comparative aspects of zebrafish *(Danio rerio)* as a model for aging research.** *Exp Gerontol.* **38** (11-12), 1333-1341.

Gerhard GS (2007). **Small laboratory fish as models for aging research.** *Ageing Res Rev*. **6** (1), 64–72.

Geurtzen K, Knopf F, Wehner D, Huitema LFA, Schulte-Merker S & Weidinger G (2014). **Mature osteoblasts dedifferentiate in response to traumatic bone injury in the zebrafish fin and skull.** *Development* **141**, 2225-2234.

Goessling W, North TE, Loewer S, Lord AM, Lee S, Stoick-Cooper CL, Weidinger G, Puder M, Daley GQ, Moon RT & Zon LI (2009). **Genetic interaction of PGE2 and wnt signaling regulates developmental specification of stem cells and regeneration.** *Cell* **136**, 1136-1147.

Goldman F, Bouarich R, Kulkarni S, Freeman S, Du HY, Harrington L, Mason PJ, Londono-Vallejo A & Bessler M (2005). **The effect of TERC haploinsufficiency on the inheritance of telomere length.** *Proc Natl Acad Sci USA* **102**, 17119-17124.

Goldsmith JR & Jobin C (2012). **Think small: zebrafish as a model system of human pathology.** *J Biomed Biotechnol* **2012.** Review.

Gómez DE, Armando RG, Farina HG, Menna PL, Cerrudo CS, Ghiringhelli PD & Alonso DF (2012). **Telomere structure and telomerase in health and disease.** *Int J Oncol* **41** (5), 1561-1569. Review.

González-Suárez E, Samper E, Flores JM & Blasco MA (2000). **Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis**. *Nature Genet* **26**, 114-117.

González-Suárez E, Samper E, Ramírez A, Flores JM, Martín-Caballero, Jorcano JL & Blasco MA (2001). **Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes**. *EMBO J* **20,** 2619-2630.

Gore AV, Monzo K, Cha YR, Pan W & Weinstein BM (2012). **Vascular development in the zebrafish.** *Cold Spring Harb Perspect Med* **2** (5), a006684. Review.

Goytisolo FA & Blasco MA (2002). **Many ways to telomere dysfunction: in vivo studies using mouse models.** *Oncogene* **21**, 584-591.

Greider CW & Blackburn EH (1985). **Identification of a specific telomere terminal transferase activity in Tetrahymena extracts**. *Cell* **43**, 405-413.

Greider CW & Blackburn EH (1987). **The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity**. *Cell* **51**, 887-898.

Greider CW & Blackburn EH (1989) **A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis**. *Nature* **337**, 331-337.

Greider CW (1996). **Telomere length regulation.** *Annu Rev Biochem* **65**, 337-365. Review.

Greider CW (1998). **Telomeres and senescence: the history, the experiment, the future.** *Curr Biol* **8** (5), 178-181. Review.

Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H & de Lange T (1999). **Mammalian telomeres end in a large duplex loop.** *Cell* **97** (4), 503-514.

Grobelny JV, Godwin AK & Broccoli D, (2000). **ALT-associated PML bodies are present in viable cells and are enriched in cells in the G2/M phase of the cell-cycle.** *J Cell Sci* **113**, 4577-4585.

Harley CB, Futcher AB & Greider CW (1990)**. Telomeres shorten during ageing of human fibroblasts**. *Nature* **345**, 458-460.

Harman D (1988). **Free radicals in aging.** *Mol Cell Biochem* **84**, 155-161.

Hatakeyama H, Nakamura KI, Izumiyama-Shimomura N, Ishii A, Tsuchida S, Takubo K &Ishikawa N (2008). **The teleost *Oryzias latipes* shows telomere shortening with age despite considerable telomerase activity throughout life.** *Mech Ageing Dev* **129**, 550-557.

Heiss NS, Knight SW, Vulliamy TJ, Klauck SM, Wiemann S, Mason PJ, Poustka A & Dokal I (1998). **X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions.** *Nat Genet* **19.**

Hemann MT, Rudolph KL, Strong MA, DePinho RA, Chin L & Greider CW (2001). **Telomere dysfunction triggers developmentally regulated germ cell apoptosis**. *Mol. Biol. Cell* **12**, 2023-2030.

Herbert BS, Hochreiter AE, Wright WE & Shay JW (2006). **Nonradioactive detection of telomerase activity using the telomeric repeat amplification protocol**. *Nat Protoc* **1** (3), 1583–1590.

Herrera E, Samper E, Martín-Caballero J, Flores JM, Lee HW & Blasco MA (1999). **Disease states associated with telomerase deficiency appear earlier in mice with short telomeres**. *EMBO J.* **18**, 2950-2960.

Hirose K, Shiomi T, Hozumi S & Kikuchi Y (2014). **Mechanistic target of rapamycin complex 1 signaling regulates cell proliferation, cell survival, and differentiation in regenerating zebrafish fins.** *BMC Developmental Biology* **14** (42).

Hiyama K, Ishioka S, Shirotani Y, Inai K, Hiyama E *et al.*, (1995). **Alterations in telomeric repeat length in lung cancer are associated with loss of heterozygosity in p53 and Rb**. *Oncogene* **10**, 937–944.

Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Piatyszek MA *et al.*, (1995). **Correlating telomerase activity levels with human neuroblastoma outcomes.** *Nat Med* **1**, 249-255.

Hockemeyer D, Palm W, Wang RC, Couto SS & de Lange T (2008). **Engineered telomere degradation models dyskeratosis congenita.** *Genes & Dev* **22**, 1773-1785.

Imamura S, Uchiyama J, Koshimizu E, Hanai J, Raftopoulou C, Murphey RD, Bayliss PE, Imai Y, Burns CE, Masutomi K *et al.*, (2008). **A non-canonical function of zebrafish telomerase reverse transcriptase is required for developmental hematopoiesis**. *PLoS ONE* **3**, e3364.

Isogai S, Hitomi J, Yaniv K & Weinstein BM (2009). **Zebrafish as a new animal model to study lymphangiogenesis.** *Anat sci Int* **84** (3), 102-111. Review.

Jaskelioff M, Muller FL, Paik JH, Thomas E, Jiang S, Adams AC, Sahin E, Kost-Alimova M, Protopopov A, Cadiñanos J *et al.* (2011). **Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice.** *Nature* **469**, 102-106.

Johnson SL & Weston JA (1995). **Temperature-sensitive mutations that cause stage-specific defects in zebrafish fin regeneration.** *Genetics* **141, 1583-1595.**

Kawakami K (2004). **Transgenesis and gene trap methods in zebrafish by using the Tol2 transposable element.** *Methods Cell Biol* **77**, 201-222.

.

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, *et al.* (1994). **Specific association of human telomerase activity with immortal cells and cancer**. *Science* **266**, 2011-2015.

Kirkwood TB (2005). **Understanding the odd science of aging.** *Cell* 120 **(4)**, 437-447.

Kirwan M & Dokal I (2009). **Dyskeratosis congenita, stem cells and telomeres.** *Biochim. Biophys. Acta* **1792**, 371-379.

Kishi S, Uchiyama J, Baughman AM, Goto T, Lin MC, *et al.* (2003). **The zebrafish as a vertebrate model of functional aging and very gradual senescence**. *Exp Gerontol* **38**, 777-786.

Kishi S (2004). **Functional aging and gradual senescence in zebrafish**. *Ann N Y Acad Sci* **1019**, 521-526.

Kishi S, Bayliss PE, Uchiyama J, Koshimizu E, Qi J, Nanjappa, P, Imamura S, Islam, A, Neuberg D, Amsterdam, A *et al.* (2008). **The identification of zebrafish mutants showing alterations in senescence-associated biomarkers**. *PLoS Genet* **4**, e1000152.

Kishi S, Slack BE, Uchiyama J & Zhdanova IV (2009). **Zebrafish as a genetic model in biological and behavioral gerontology: where development meets aging in vertebrates–a mini-review.** *Gerontology* **55**, 430-441.

Knudson M, Kulkarni S, Ballas ZK, Bessler M & Goldman F (2005). **Association of immune abnormalities with telomere shortening in autosomaldominant dyskeratosis congenita**. *Blood* **105**, 682-688.

Kuilman T, Michaloglou C, Mooi WJ & Peeper DS (2010). **The essence of senescence.** *Genes & Development* **24**, 2463-2479. Review.

Lansdorp PM, Verwoerd NP, van de Rijke FM, Dragowska V, Little MT, Dirks RW, Raap AK & Tanke HJ (1996). **Heterogeneity in telomere length of human chromosomes**. *Hum Mol Genet* **5**, 685-691.

Lau BWM, Wong AOL, Tsao GSW, So KF & Yip HKF (2008). **Molecular cloning and characterization of the zebrafish (*Danio rerio*) telomerase catalytic subunit (telomerase reverse transcriptase, TERT)**. *J Mol Neurosci* **34**, 63-75.

Lau LMS, Dagg RA, Henson JD, Au AYM, Royds JA & Reddel RR (2013**). Detection of alternative lengthening of telomeres by telomere quantitative PCR.** *Nucleic Acids Research* **41** (2), e34.

Lee HW, Blasco MA, Gottlieb GJ, Horner JW, 2nd, Greider CW & DePinho RA (1998). **Essential role of mouse telomerase in highly proliferative organs**. *Nature* **392**, 569-574.

Lee C & Smith A (2004). **Molecular cytogenetic methodologies and a bacterial artificial chromosome (BAC) probe panel resource for genomic analyses in zebrafish**. *Methods Cell Biol.* **77**, 241-254.

Lee Y, Grill S, Sanchez A, Murphy-Ryan M & Poss KD (2005). **Fgf signaling instructs position-dependent growth rate during zebrafish fin regeneration** *Development* **132**, 5173-5183.

Leong IU, Lai D, Lan CC, Johnson R & Love DR (2011). **Targeted mutagenesis of zebrafish: use zinc-finger nucleases.** *Birth Defects Res C Embryo Today* **93** (3), 249-255. Review.

.

Leri A, Franco S, Zacheo A, Barlucchi L, Chimenti S, Limana F, Nadal-Ginard B, Kajstura J, Anversa P & Blasco MA (2003). **Ablation of telomerase and telomere loss leads to cardiac dilatation and heart failure associated with p53 upregulation**. *EMBO J.* **22**, 131-139.

Lieschke GJ & Currie PD (2007). **Animal models of human disease: zebrafish swim into view.** *Nature Genet* **8**, 353-367.

Liu Y, Snow BE, Hande, MP, Yeung D, Erdmann NJ, Wakeham A, Itie A, Siderovski DP, Lansdorp M & Robinson MO (2000). **The telomerase reverse transcriptase is limitin, P and necessary for telomerase function in vivo**. *Curr. Biol.* **10**, 1459-1462.

Londoño-Vallejo JA, Der-Sarkissian H, Cazes L, Bacchetti S & Reddel RR (2004). **Alternative lengthening of telomeres is characterized by high rates of telomeric exchange.** *Cancer Res* **64** (7), 2324-2327.

López de Silanes I, Stagno d'Alcontres M & Blasco MA (2010). **TERRA transcripts are bound by a complex array of RNA-binding proteins.** *Nat Commun* **1**, 33.

Lund TC, Glass TJ, Tolar J & Blazar BR (2009). **Expression of telomerase and telomere length are unaffected by either age or limb regeneration in Danio rerio**. *PLoS ONE* **4**, (11).

Lundblad V & Blackburn EH (1993). **An alternative pathway for yeast telomere maintenance rescues est1- senescence.** *Cell* **73**, 347-360.

Martin CS, Moriyama A & Zon LI (2011). Hematopoietic stem cells, hematopoiesis and disease: lessons from the zebrafish model. ***Genome Med*** 3 **(12), 83. Review.**

Martin-Rivera L, Herrera E, Albar JP & Blasco MA (1998). **Expression of mouse telomerase catalytic subunit in embryos and adult tissues.** *Proc Natl Acad Sci USA* **95**, 10471-10476.

Martínez P, Thanasoula M, Muñoz P, Liao C, Tejera A, McNees C, Flores JM, Fernández-Capetillo O, Tarsounas M & Blasco MA **(**2009). **Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice.** *Genes Dev.* **23**, 2060-2075.

Maser RS & DePinho RA (2004). **Telomeres and the DNA damage response: why the fox is guarding the henhouse.** *DNA Repair (Amst.)* **3**, 979-988.

Mason PJ & Bessler M (2004). **Heterozygous telomerase deficiency in mouse and man: when less is definitely not more**. *Cell Cycle* **3**, 1127-1129.

Mason PJ & Bessler M (2011). **The genetics of dyskeratosis congenita.** *Cancer Genet* **204.**

McChesney PA, Elmore LW & Holt SE (2005). **Vertebrate marine species as model systems for studying telomeres and telomerase**. *Zebrafish* **1**, 349-355.

McEachern MJ & Iyer S (2001). **Short telomeres in yeast are highly recombinogenic.** *Mol Cell* **7** (4), 695-704.

Meng X, Noyes MB, Zhu LJ, Lawson ND & Wolfe SA (2008). **Targeted gene inactivation in zebrafish using zinc-finger nucleases.** *Nat Biotechnol* **26** (6), 695-701.

Mione MC & Trede NS (2010). **The zebrafish as a model for cancer.** *Dis Model Mech* **3** (9-10), 517-523. Review.

Mitchell JR, Wood E & Collins K (1999). **A telomerase component is defective in the human disease dyskeratosis congenita**. *Nature* **402**, 551-555.

Miyake Y, Nakamura M, Nabetani A, Shimamura S, Tamura M,Yonehara S, Saito M & Ishikawa F (2009). **RPA-like mammalian Ctc1-Stn1-Ten1 complex binds to single-stranded DNA and protects telomeres independently of the Pot1 pathway.** *Mol Cell* **36** (2), 193-206.

Morrish TA & Greider CW (2009). **Short telomeres initiate telomere Recombination in primary and tumor cells.** *PLoS Genet* **5** (1), e1000357.

Morrison JI, Sara Lööf S, Pingping He P & András Simon A (2006).**Salamander limb regeneration involves the activation of a multipotent skeletal muscle satellite cell population.** *JCB* **172** (3), 433-440.

Murphey Rd & Zon LI (2006). **Small molecule screening in the zebrafish.** *Methods* **39**, 255-261.

Nabetani A & Ishikawa F (2011). **Alternative lengthening of telomeres pathway: recombination-mediated telomere maintenance mechanism in human cells.** *J Biochem* **149** (1), 5-14. Review.

Ogami M, Ikura Y, Ohsawa M, Matsuo T, Kayo S, Yoshimi N, Hai E, Shirai N, Ehara S, Komatsu R, Naruko T & Ueda M (2004). **Telomere shortening in human coronary artery diseases.** *Arterioscler Thromb Vasc Biol* **24** (3), 546-550.

Olovnikov AM (1973). **A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon.** *J Theor Biol* **41** (1), 181-190. Review.

Patton EE & Zon LI (2001). **The art and design of genetic screens: zebrafish.** *Nat Rev Genet* **2**, 956-966. Review.

Pereboom TC, van Weele LJ, Bondt A & MacInnes AW (2011). **A zebrafishmodel of dyskeratosis congenita reveals hematopoietic stem cell formation failure resulting from ribosomal protein-mediated p53 stabilization**. *Blood* **118**, 5458-5465.

.

Poleo G, Brown CW, Laforest L & Akimenko MA (2001). **Cell proliferation and movement during early fin regeneration in zebrafish.** *Dev Dyn* **221**, 380-390.

Poon SS, Martens UM, Ward RK & Lansdorp PM (1999). **Telomere length measurements using digital fluorescence microscopy**. *Cytometry* **36**, 267-278.

Porro A, Feuerhahn S, Reichenbach P & Lingner J (2010). **Molecular dissection of telomeric repeat-containing RNA biogenesis unveils the presence of distinct and multiple regulatory pathways.** *Mol Cell Biol* **30** (20), 4808-4817.

Porta EA (2002). **Pigments in aging: an overview.** *Ann N Y Acad Sci* **959**, 57-65.

Poss KD, Shen J, Nechiporuk A, McMahon G, Thisse B, Thisse C & Keating MT (2000). **Roles for Fgf signaling during zebrafish fin regeneration** *Developmental Biology* **222,** 347-358

Poss KD, Wilson LG & Keating MT (2002). **Heart regeneration in zebrafish.** *Science* **298**, 2188-2190.

Poss KD, Nechiporuk A, Hillam AM, Johnson SL & Keating MT (2002). **Mps1 defines a proximal blastemal proliferative compartment essential for zebrafish fin regeneration** *Development* **129**, 5141-5149.

Poss KD, Keating MT & Nechiporuk A (2003). **Tales of regeneration in zebrafish.** *Dev. Dyn.* **226**, 202-210. Review.

Prowse KR & Greider CW (1995). **Developmental and tissue specific regulation of mouse telomerase and telomere length.** *Proc Natl Acad Sci USA* **92**, 4818-4822.

Quaife NM, Watson O & Chico TJ (2012). **Zebrafish: an emerging model of vascular development and remodeling.** *Curr Opin Pharmacol* **12** (5), 608-614. Review.

Redon S, Zemp I & Lingner J (2013). **A three-state model for the regulation of telomerase by TERRA and hnRNPA1.** *Nucleic Acids Res* **41** (19), 9117-9128.

Reimschuessel R (2001). **A fish model of renal regeneration and development**. *ILAR J* **42**, 285-291.

Renshaw SA & Trede NS (2012). **A model 450 million years in the making: zebrafish and vertebrate immunity.** *Dis Model Mech* **5** (1), 38-47. Review.

Rohner N, Perathoner S, Frohnhöfer HG & Harris MP (2011). **Enhancing the efficiency of N-ethyl-N-nitrosourea-induced mutagenesis in the zebrafish.** *Zebrafish* **8** (3), 119-123.

Rowlerson A, Radaelli G, Mascarello F & Veggetti A (1997). **Regeneration of skeletal muscle in two teleost fish: Sparus aurata and Brachydanio rerio**. *Cell Tissue Res* **289**, 311-322.

Samper E, Flores JM & Blasco MA (2001). **Restoration of telomerase activity rescues chromosomal instability and premature aging in Terc-/- mice with short telomeres.** *EMBO Rep.* **2**, 800-807.

Savage SA, Giri N, Baerlocher GM, Orr N, Lansdorp PM & Alter BP (2008). **TINF2, a component of the shelterin telomere protection complex, is mutated in dyskeratosis congenita.** *Am J Hum Genet* **82**, 501-509.

Schmidt R, Strahle U & Scholpp S (2013). **Neurogenesis in zebrafish – from embryo to adult.** *Neural Dev* **8**, 3. Review.

Schoeftner S & Blasco MA (2008). **Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II.** *Nat Cell Biol* **10** (2), 228-236.

Sfeir A, Kosiyatrakul ST, Hockemeyer D, MacRae SL, Karlseder J, Schildkraut CL & de Lange T (2009). **Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication**. *Cell* **138**, 90-103.

Sfeir A (2012). **Telomeres at a glance.** *J Cell Sci* **125**, 4173-4178. Review.

Shao J, Chen D, Ye Q, Cui J, Li Y & Li L (2011). **Tissue regeneration after injury in adult zebrafish. The regenerative potential of the caudal fin.** *Dev Dyn* **240**, 1271-1277.

Shay JW & Roninson IB (2004). **Hallmarks of senescence in carcinogenesis and cancer therapy.** *Oncogene* **23** (16), 2919-2933.

Starr JM, McGurn B, Harris SE, Whalley LJ, Deary IJ & Shiels PG (2007). **Association between telomere length and heart disease in a narrow age cohort of older people.** *Exp Gerontol* **42** (6), 571-573.

Stewart JA, Chaiken MF, Wang F & Price CM (2012). **Maintaining the end: roles of telomere proteins in end-protection, telomere replication and length regulation.** *Mutat Res* **730** (1-2), 12-19. Review.

Strong MA, Vidal-Cardenas SL, Karim B, Yu H, Guo N & Greider CW (2011). **Phenotypes in mTERT$^{+/-}$ and mTERT$^{-/-}$ mice are due to short telomeres, not telomere-independent functions of telomerase reverse transcriptase.** *Cell Biol* **31** (12), 2369-2379.

Swanberg SE, O'Hare TH, Robb EA, Robinson CM, Chang H & Delany ME (2010). **Telomere biology of the chicken: a model for aging research**. *Exp. Gerontol.* **45**, 647-654.

Terman A & Brunk UT (1998). **Lipofuscin: mechanisms of formation and increase with age.** APMIS 106 (**2**), 265-276.

Tsai SB, Tucci V, Uchiyama J, Fabian NJ, Lin MC, *et al.* (2007). **Differential effects of genotoxic stress on both concurrent body growth and gradual senescence in the in the adult zebrafish.** *Aging Cell* 6, 209–224.

Tümpel S & Rudolph KL (2012). **The role of telomere shortening in somatic stem cells and tisssue aging: lessons from telomerase model systems.** *Ann N Y Acad Sci* **1266**, 28-39. Review.

Ulaner GA & Giudice LC (1997). **Developmental regulation of telomerase activity in human fetal tissues during gestation.** *Mol Hum Reprod* **3** (9), 769-773.

Veinteicher AS, Meng Z, Mason PJ, Veenstra TD & Artandi SE (2008). Identification of ATPases pontin and reptin as telomerase components essential holoenzyme. Cell 132, 945-957.

Vera E, Bernardes de Jesus B, Foronda M, Flores JM & Blasco MA (2012). **The rate of increase of short telomeres predicts longevity in mammals.** *Cell Rep* **2**, 732-737.

Vulliamy T, Knight SW, Mason PJ & Dokal I (2001a). **Very short telomeres in the peripheral blood of patients with X-linked and autosomal dyskeratosis congenita.** *Blood Cells Mol Dis* **27**, 353-357.

Vulliamy T, Marrone A, Goldman F, Dearlove A, Bessler M, Mason P & Dokal I (2001b).**The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita.** *Nature* **413,** 432-435.

.**V**ulliamy T, Marrone A, Szydlo R, Walne A, Mason PJ & Dokal I (2004). **Disease anticipation is associated with progressive telomere shortening in families with dyskeratosis congenita due to mutations in TERC**. *Nat. Genet.* **36**, 447-449.

Vulliamy T, Marrone A, Knight SW, Walne AJ, Mason PJ & Dokal I (2006). **Mutations in dyskeratosis congenita: their impact on telomere length and the diversity of clinical presentation.** *Blood* **107**, 2680-2685.

Vulliamy T, Beswick R, Kirwan M, Marrone A, Digweed M, Walne AJ & Dokal I (2008). **Mutations in the telomerase component NHP2 cause the premature ageing síndrome dyskeratosis congenita.** *Proc Natl Acad Sci USA* **105**, 8073-8078.

Vulliamy T, Kirwan M, Beswick R, Hossain U, Baqai C, Ratcliffe A, Marsh J, Walne AJ & Dokal I (2011). **Differences in disease severity but similar telomere lengths in genetic subgroups of patients with telomerase and shelterin mutations.** *PLoS One* **6** (9), e24383.

Wallace DC. (2005). **A mitochondrial paradigm of metabolic and degenerative diseases, aging, cancer: a dawn for evolutionary medicine.** *Annu Rev Genet* **39**, 359–407.

Walne AJ, Vulliamy T, Marrone A, Beswick R, Kirwan M, Masunari Y, Al Qurashi FH, Aljurf M & Dokal I (2007). **Genetic heterogeneity in autosomal recessive dyskeratosis congenita with one subtype due to mutations in the telomerase associated protein NOP10.** *Hum Mol Genet* **16**, 1619-1629.

Watson JD (1972). **Origin of concatemeric T7 DNA.** *Nat New Biol* **239** (94), 197-201. Review.

Wege H, Chui MS, Le HT, Tran JM & Zern MA (2003). **SYBR Green real-time telomeric repeat amplification protocol for the rapid quantification of telomerase activity**. *Nucleic Acids Res* **31** (2).

Wellinger RJ, Ethier K, Labrecque P & Zakian VA (1996). **Evidence for a new step in telomere maintenance.** *Cell* **85** (3), 423-433.

Westerfield M (2000). **The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish *Danio (Brachydanio) rerio***. Eugene, OR: University of Oregon Press.

Wicky C, Villeneuve AM, Lauper N, Codourey L, Tobler H & Müller F (1996). **Telomeric repeats (TTAGGC)n are sufficient for chromosome capping function in Caenorhabditis elegans**. *Proc. Natl. Acad. Sci. USA* **93**, 8983-8988.

Wright WE, Piatyszek MA, Rainey WE, Byrd W & Shay JW (1996). **Telomerase activity in human germline and embryonic tissues and cells**. *Dev Genet* **18**, 173–179.

Wu G, lee WH, Chen PL (2000). **NBS1 and TRF1 colocalize at promyelocytic leukemia bodies during late S/G2 phases in immortalized telomerase-**

negative cells. Implications of NBS1 in alternative lengthening of telomeres. *J Biol Chem* **275**:30618-22.

Wyatt HDM, West SC & Beattie TL (2010). **InTERTpreting telomerase structure and function.** *Nucleic Acids Res* **38**, 5609-5612.

Xie M, Mosig A, Qi X, Li Y, Stadler PF & Chen JJ (2008). **Structure and function of the smallest vertebrate telomerase RNA from teleost fish.** *J. Biol. Chem.* **283**, 2049-2059.

Yeager TR, Neumann AA, Englezou A, Huschtscha LI, Noble JR & Reddel RR (1999). **Telomerase-negative immortalized cells contain a novel type of promyelocytic (PML) body.** *Cancer Res* **59**, 4175-4179.

Yu L, Tucci V, Kishi S & Zhdanova IV (2006). **Cognitive aging in zebrafish.** *PLoS One* **1**, e14.

Yuan X, Ishibashi S, Hatakeyama S, Saito M, Nakayama J, Nikaido R, Haruyama T, Watanabe Y, Iwata H, Iida M *et al.* (1999). **Presence of telomeric G-strand tails in the telomerase catalytic subunit TERT knockout mice.** *Genes Cells* **4**, 563-572.

Zhang Y, Morimoto K, Danilova N, Zhang B & Lin S (2012). **Zebrafish models for dyskeratosis congenita reveal critical roles of p53 activation contributing to hematopoietic defects through RNA processing**. *PLoS ONE* **7**, e30188.

Zhong F, Savage SA, Shkreli M, Giri N, Jessop L, Myers T, Chen R, Alter BP & Artandi SE (2011). **Disruption of telomerase trafficking by TCAB1 mutation causes dyskeratosis congenita.** *Genes Dev* **25**, 11-16.

Zhong ZH, Jiang WQ, Cesare AJ, Neumann AA, Wadhwa R & Reddel RR (2007) **Disruption of telomere maintenance by depletion of theMRE11/RAD50/NBS1 complex in cells that use alternative lengthening of telomeres.** *JBC* **282** (40), 29314-29322.

Zijlmans JM, Martens UM, Poon SS, Raap AK, Tanke HJ, Ward RK & Lansdorp PM (1997). **Telomeres in the mouse have large inter-chromosomal variations in the umber of T2AG3 repeats.** *Proc Natl Acad Sci* USA **94**, 7423-7428.

Zon LI & Peterson RT (2005). **In vivo drug discovery in the zebrafish**. *Nat. Rev.Drug Discov.* **4**, 35-44.

Publications

Anchelin M, Murcia L, Alcaraz-Pérez F, García Navarro EM, Cayuela ML (2011). Behaviour of telomere and telomerase during aging and regeneration in zebrafish *PLoS One* 6 (2):e16955.

Anchelin M, Alcaraz-Pérez F, Martínez CM, Bernabé-García M, Mulero V, Cayuela ML (2013). Premature aging in telomerase-deficient zebrafish *Dis Model Mech* 6(5):1101-12.

Jelassi B, **Anchelin M,** Chamouton J, Cayuela ML, Clarysse1 L, Li J, Goré1 J, Jiang1 LH, and Roger S (2013) Anthraquinone emodin inhibits MDA-MB-435s human cancer cell invasiveness by antagonizing P2X7 receptors. *Carcinogenesis* 34(7):1487-96.

Alcaraz-Pérez F, García-Castillo J, García-Moreno D, López-Muñoz A, **Anchelin M**, Angosto D, Zon LI, Mulero V, Cayuela ML (2014). A non-canonical function of telomerase RNA in the regulation of developmental myelopoiesis in zebrafish. *Nat Commun.* 5:3228.

Resumen en español

1. Introducción

En eucariotas, cada cromosoma consiste normalmente en una molécula de ADN lineal enormemente larga y asociada a proteínas cromosómicas. En todos vertebrados, los extremos de los cromosomas denominados "telómeros" consisten en cientos de repeticiones en tándem de una secuencia hexamérica de nucleótidos, rica en Guanina: "TTAGGG". Esta secuencia telomérica, de longitud variable según la especie, aparece conservada desde eucariotas inferiores hasta mamíferos [Greider, 1998]. El ADN telomérico de doble cadena presenta en el extremo 3' una hebra de cadena simple de unos 50 a 300 nucleótidos de largo. Con el fin de no dejar expuesta esta hebra, el extremo terminal del telómero se dobla, formando un bucle de ADN telomérico (t-loop, telomeric-loop), mientras que el extremo 3' invade la secuencia doble formando una estructura de triple cadena y un bucle de desplazamiento (D-loop Displacement-loop). Estas estructuras junto con un grupo de proteínas teloméricas forman complejos ribonucleoproteínicos que protegen los extremos libres de los cromosomas evitando ser reconocidos como roturas en el ADN de doble cadena y asegurando así la integridad del cromosoma. En humanos, se han descrito un grupo de seis proteínas asociadas al telómero que unidas forman un complejo denominado "Shelterin".

Debido al problema fundamental de la replicación semi-conservativa del ADN [Watson, 1972; Olovnikov, 1973], las ADN polimerasas convencionales no pueden replicar los extremos de las cadenas y los cromosomas lineales se acortan en cada división celular por la perdida de 50 a 100 pares de bases (pb). La secuencia telomérica no contiene genes y el acortamiento telomérico no significaría perdida de información genética, lo que es una ventaja, sin embargo cuando los telómeros alcanzan una longitud mínima critica, pierden su función protectora, lo que desencadena una respuesta de daño en el ADN (DNA Damage Response DDR). Esos cambios pueden inducir un parada transitoria de la proliferación celular, permitiendo a las células reparar el daño, o por el contrario, llevar la célula hacía una muerte celular programada o senescencia replicativa si el daño excede cierto límite.

Los organismos eucariotas pueden compensar el acortamiento telomérico gracias a la "telomerasa", una enzima celular capaz de añadir repeticiones "TTAGGG" en los extremos de los telómeros [Blackburn, 2005]. La telomerasa es un complejo ribonucleoproteico compuesto por una subunidad catalítica con actividad

retrotranscriptasa (Telomerase Reverse Transcriptase, TERT), una subunidad de RNA que actúa como molde para la síntesis de la secuencia telomérica (Telomerase RNA Component, TERC o TR), además de proteínas accesorias específicas de las especies. En humanos, se han descrito siete proteínas: Disquerina, NHP2, NOP10, GAR1, TCAB1 [Fu & Collins, 2007], Pontin y Reptin, [Veinteicher *et al*., 2008].

La telomerasa es activa en las células madre pluripotentes, durante la embriogénesis y el desarrollo, etapas de alta tasa de proliferación celular. Sin embargo en el organismo adulto, las células somáticas normales carecen de actividad telomérica, limitándose está a células muy proliferativas, células madre adultas y/o progenitoras, y células de la línea germinal. Las células somáticas normales y las células madre sufren un acortamiento progresivo de los telómeros debido a la replicación celular asociada al envejecimiento del organismo, hasta alcanzar una longitud mínima crítica y sufrir apoptosis o senescencia. Sin embargo, debido a alteraciones en los mecanismos de control de daño en el ADN y a la inducción de la expresión de la telomerasa, algunas células no entran en senescencia sino proliferan sin control llegando a desarrollar un tumor.

El mantenimiento de los telómeros puede ser constitutivamente defectivo debido a mutaciones que reducen severamente la capacidad de la telomerasa para alargar los telómeros desembocando a un acortamiento acelerado de los telómeros, varias enfermedades, llamadas "síndromes teloméricos", han sido descritas en relación con telómeros cortos.

La disqueratosis congénita (DC) es una enfermedad rara hereditaria causada por defectos en el mantenimiento de los telómeros. La mutación en alguno de los componentes del complejo Telomerasa o del complejo Shelterin produce la enfermedad. Se trata de un síndrome de envejecimiento prematuro donde todos los pacientes muestran acortamiento telomérico. Los síntomas que aparecen en la infancia: pigmentación anormal de la piel, distrofía de las uñas y leucoplaquia oral suelen ir acompañados de retraso en el desarrollo, perdida de pelo y fallo de la medula ósea. La muerte prematura de estos enfermos se debe en el 85% de los casos a fallos en la hematopoyesis/ inmunodeficiencia, en el 10% al cáncer, y en el 5% a complicaciones pulmonares [Mason & Bessler, 2011; Dokal, 2011]. Existen tres modos de herencia de la DC: ligada al sexo con mutaciones en el gen de la disquerina, [Heiss *et al*., 1998], autosómica dominante con mutaciones en *TERT*, *TR* y TIN2 [Armanios *et al*., 2005; Vulliamy *et al*., 2001b; Savage *et al*., 2008] y autosómica recesiva con mutaciones en

NOP1, NHP2 y TCAB1 [Walne *et al*., 2007; Vulliamy *et al*., 2008; Zhong *et al.,* 2011]. En los casos de DC por mutaciones en *TERT* y *TR*, se observa un fenómeno de anticipación genética, y la enfermedad se presenta y se agrava cada vez más temprano en las sucesivas generaciones lo que parece ser una consecuencia directa del acortamiento telomérico [Vulliamy *et al*., 2004; Armanios *et al*., 2005].

La telomerasa es activa durante la embriogénesis y el desarrollo [Ulaner *et al.,* 1997], pero restringida a los tipos celulares con alta tasa proliferativa, células de la línea germinal y células madre en el contexto de un organismo adulto [Blasco, 2005]. Las células somáticas donde no se expresa la telomerasa están abocadas a una perdida irremediable de su potencial de división. El acortamiento telomérico es considerado como uno de los mecanismos responsables del envejecimiento de las células. Cuando los telómeros alcanzan una longitud mínima crítica, pierden su estructura protectora, lo que desencadena unas respuestas de daño en el ADN (DDR), con la consiguiente activación de proteínas de DDR (53BP1, NBS1 y MDC1) y de las quinasas ATM y ATR en células senescentes. Además, al amplificarse los señales de DDR, se activan las quinasas CHK1 y CHK2, causando reacciones de fosforilación y activación de varias proteínas del ciclo celular como CDC25 y p53. En conjunto, una cascada de señalización que puede inducir una parada transitoria de la proliferación para permitir a las células reparar el daño, o si el daño es persistente, desembocar en la apoptosis o en la parada permanente del ciclo celular o senescencia replicativa.

Las células madre adultas que si expresan telomerasa, residen en compartimentos específicos o “niches” enriquecidos en células con telómeros suficientemente largos en organismos jóvenes y adultos para reparar lesiones de manera eficiente. Sin embargo, en organismos viejos, las células madre con telómeros muy cortos pueden impedir la movilización de las células madre y en consecuencia impedir la renovación celular, lo que puede conducir finalmente a fallos orgánicos.

En resumen, el acortamiento telomérico asociado al envejecimiento es suficiente para impedir la movilización de las células madre y la regeneración de los tejidos y se considera como un determinante clave de la longevidad del organismo.

En la mayoría de los casos, las células tumorales y células inmortalizadas utilizan la sobre-expresión de la telomerasa para mantener la longitud telomérica y asegurar su proliferación, aunque se han publicado varios estudios sobre tumores humanos y líneas celulares derivadas de tumores [Bryan *et al.,* 1997; 1998] que describen un mecanismo

independiente de la telomerasa, basado en recombinación homóloga, el mecanismo ALT (alternative lengthening of telomere).

Aunque las primeras evidencias de alargamiento telomérico por recombinación homóloga entre cromosomas fueron descritas en levaduras *Saccharomyces cerevisae* deficientes en telomerase [Lundblad & Blackburn, 1993], sorprendentemente, levaduras *Kluyveromyces lactis* positivas en telomerase pero con telómeros cortos [McEachern & Iyer, 2001], y células primarias de ratón, con y sin telomerasa, pero con telómeros cortos [Morrish & Greider, 2009], utilizan ALT para el mantenimiento de sus telómeros. Estos estudios sugieren que el mecanismo de recombinación homologa se inicia por la disrupción de la estructura protectora de telómeros cortos y no por la pérdida de la función telomérica. Otro dato interesante, es que los dos mecanismos de mantenimiento de la longitud telomérica, por telomerase y por recombinación homóloga pueden coexistir en células humanas [Cerone *et al*., 2001].

Se han descritos varios marcadores del mecanismo ALT, como heterogeneidad en el tamaño de los telómeros, presencia de círculos teloméricos extracromosómicos (t-circles), presencia de unas estructuras nucleares llamadas APBs (ALT-associated PML (ProMyelocytic Leukemia) bodies) que pueden contener: PML, ADN telomérico (TTAGGG)n, proteínas unidas a telómero (TRF1, TRF2, hRAP1, TIN2) el complejo proteínico de recombinación y reparación del ADN (MRE11/RAD50/NBS1 (MRN)) [Yeager *et al*., 1999; Grobelny, 2000; Wu, 2000]. Además, se han detectado intercambios entre cromatidas hermanas al nivel de los telómeros (Telomere -Sister Chromatid Exchanges, T-SCEs) en tumores humanos ALT [Londoño-Vallejo *et al.*, 2004].

Estudios posteriores orientados hacia la búsqueda de nuevas terapias anti-tumorales muestran que la depleción del gen Nbs1 inhibe la formación de los circulos teloméricos [Compton *et al.*, 2007] impidiendo el mecanismo ALT [Zhong *et al.*, 2007], y que la inhibición de ATR, un regulador de la recombinación inhibe ALT causando la fragmentación del cromosoma y la apoptosis de las células ALT [Flynn *et al.*, 2015].

Comparado con otros modelos vertebrados, el pez cebra (*Danio rerio*), ofrece muchas ventajas para su mantenimiento y manejo en el laboratorio, como su pequeño tamaño, su alta fecundidad, su rápido desarrollo extrauterino. Además, presenta una gran similitud fisiológica con los mamíferos, y comparte una gran similitud genética con los humanos, y por ello, se ha convertido en un modelo excepcional para estudios

de desarrollo, regeneración, envejecimiento, cáncer, hematopoyesis, sistema immune, entre otros.

La importancia del acortamiento telomérico de las células es incuestionable y directamente relacionado con el proceso de envejecimiento del organismo. Hasta ahora, el ratón ha sido el modelo más utilizado para estudios de telomerasa, pero la longitud de sus telómeros, de 8 a 10 veces más larga que los telómeros humanos, es un inconveniente notable a la hora de realizar estudios de envejecimiento, y el fenotipo de envejecimiento "prematuro" en modelo de ratón deficiente en telomerasa no aparece antes de la 4ª generación de ratones [Blasco *et al.*, 1997; Lee *et al.*, 1998]. Además, por la misma razón, no reproduce el fallo de la medula ósea, principal causa de muerte en pacientes de disqueratosis congénita, ni el fenómeno de anticipación de la enfermedad en generaciones sucesivas [Zijlmans *et al.*, 1997].

La longitud telomérica del pez cebra (15-20 kb) es relativamente similar a la de los telómeros humanos (10-15 kb), por todas estas razones, el pez cebra se presenta como un modelo alternativo ideal para estudiar la biología de los telómeros.

La vida media del pez cebra es de 42 meses y de 66 meses para los individuos más longevos [Gerhard *et al.*, 2002]. A pesar de tener una actividad de telomerasa alta durante todo su ciclo de vida, el pez teleósteo *Oryzias latipes* muestra un acortamiento de sus telómeros con el envejecimiento [Hatakeyama *et al.*, 2008]. Sin embargo, otro estudio longitudinal en órganos de pez cebra no aprecia cambios de longitud telomérica a lo largo del ciclo de vida del pez [Lund *et al.*, 2009].

El pez cebra, como muchos otros teleósteos, sufre una senescencia progresiva similar a la de los mamíferos, con una lenta disminución de las funciones y de los sistemas fisiológicos. Biomarcadores del envejecimiento tales como la actividad β-galactosidasa (β-SA-gal, del inglés Senescence-associated β-galactosidase), y la acumulación de proteínas oxidadas se detectaron en la piel y en el músculo respectivamente durante el envejecimiento en pez cebra [Kishi *et al.*, 2003]. Además, el pez cebra muestran una clara disminución en el rendimiento cognitivo en edades avanzadas [Yu et al., 2006].

Los estudios anteriores, incompletos y a veces con resultados no concluyentes, hacen necesarios estudios adicionales para arrojar luz sobre el comportamiento de los telómeros y la telomerasa en el proceso de envejecimiento. El pez cebra se ha establecido como un modelo complementario al modelo de ratón para estudiar el envejecimiento y las enfermedades relacionadas con la disfunción de la telomerasa.

El pez cebra es capaz de regenerar diferentes tipos de tejidos como aletas, retina, higado, corazón, hasta edades avanzadas [Becker *et al*., 1997; Rowlerson *et al.,* 1997; Poss *et al*., 2002, 2003; Reimschuessel *et al*., 2001]. Además, presenta una actividad telomérica constitutiva en tejidos somáticos desde la etapa embrionaria hasta la etapa de adulto viejo [Kishi *et al*., 2003; McChesney *et al*., 2005]. Un estudio sobre varios tejidos de especies acuáticas incluyendo el pez cebra sugiere que la telomerasa podría tener un papel más relevante para la renovación de tejidos y la regeneración después de un daño que para la longevidad del organismo en estas especies con alta capacidad de regeneración [Elmore *et al*., 2008].

El proceso de regeneración implica la participación de células progenitoras/células madre [Brittijn *et al.,* 2009; Flores *et al.,* 2005], y la expresión constante de la actividad de la telomerasa podría ser crucial para el mantenimiento de los telómeros de estas células durante el proceso regenerativo. En un estudio de regeneración, Lund *et al.,* (2009) observan que la longitud telomérica no cambia después de escisiones sucesivas [Lund *et al.,* 2009].

De hecho, los fenotipos asociados a la pérdida prematura de la capacidad de regeneración de tejidos, incluyendo la piel (pérdida de cabello, encanecimiento del cabello, disminución en la eficiencia de cicatrización de heridas) se encuentran en ratones deficientes en telomerasa [González-Suárez *et al.,* 2000; 2001; Cayuela *et al.,* 2005].

Por su fácil manejo y su rápida y total regeneración después de una escisión (3 días en larvas y 14-15 días en adultos), la aleta caudal del pez cebra es un órgano muy conveniente para estudios de regeneración. Hasta ahora se conoce poco sobre el origen y el destino de las células implicadas en la formación del blastema después de la escisión de la aleta caudal del pez. Unos estudios apuntan la activación de células madre y/o progenitoras que migran para formar distalmente el blastema, mientras otros apuntan la dediferenciación de células adultas diferenciadas. En ambos casos, el proceso de regeneración de los tejidos requiere la participación de células con una longitud telomérica adecuada para soportar altas tasas de proliferación celular, y ensayos de regeneración serían muy convenientes para dilucidar el papel de la telomerasa en el mantenimiento de los telómeros durante un proceso de regeneración.

Comparado con otros modelos, el pez cebra ofrece muchas ventajas para la manipulación experimental y se presenta como un modelo vertebrado ideal para estudiar los telómeros y la telomerasa a lo largo de la vida de un organismo, así como

durante un proceso de regeneración de un órgano, como la aleta caudal, de vital importancia para la supervivencia del animal.

2. Objetivos

En el presente trabajo se proponen los siguientes objetivos:

1. Caracterización de la longitud telomérica y de la expresión y actividad de la telomerasa en el pez cebra (*Danio rerio*) a lo largo de su ciclo de vida.

2. Caracterización de la línea de pez cebra deficiente en telomerasa.

3. Estudio de la longitud telomérica y de la expresión y actividad de la telomerasa en el pez cebra (*Danio rerio*) durante el proceso de regeneración de la aleta caudal del pez cebra en presencia o deficiencia de telomerasa.

3. Materiales y Métodos

Animales

Peces cebra (*Danio rerio*) fueron cedidos por el centro internacional de recursos del pez cebra (ZIRC, Oregón, EEUU) y mantenidos como se describe en el manual del pez cebra (Westerfield, 2000). La línea mutante para *tert* (alelo hu3430) fue obtenida del Instituto Sanger y la línea mutante para *p53* (*zdf1*) (P53M214K) (Berghmans *et al*., 2005) fue amablemente proporcionada por el Dr. Leonard I. Zon (HSCRB, Harvard University, Cambridge, MA).

Los experimentos desarrollados cumplen con la directiva de la Unión Europea (86/609/EU) y han sido aprobados por el Comité de Bioética del Hospital Clínico Universitario "Virgen de la Arrixaca" (España).

Análisis de la expresión génica

El ARN total fue extraído de embriones/larvas/adultos con TRIzol (Invitrogen) siguiendo las instrucciones del fabricante, seguido de un tratamiento con DNasa I libre de RNasa (Invitrogen). La retrotranscriptasa Superscript III RNasa H2 (Invitrogen) se usó para sintetizar el cDNA con un cebador oligo-dT_{18} a partir de 1µg de RNA total a 50ºC durante 60 min. La PCR a tiempo real fue realizada en un aparato ABI PRISM 7700 (Applied Biosystems) usando SYBR Green PCR Core Reagents (Applied Biosystems). Las mezclas de reacción fueron incubadas durante 10 min a 95ºC, seguido de 40 ciclos de 15s a 95ºC, 1 min a 60ºC, y finalmente 15s a 95ºC, 1 min a 60ºC,y 15s a 95ºC. Para cada ARN, la expresión génica fue corregida por la proteína ribosómica S11 (*rps11*) contenida en cada muestra usando el método *Ct* comparativo ($2^{-\Delta\Delta}$ *Ct*). En todos los casos, cada PCR se llevó a cabo con cada muestra por triplicado y fue repetida al menos con dos muestras independientes.

Obtención de suspensiones celulares

Peces cebra anestesiados en benzocaína al 0.05%, y seguidamente sacrificados en hielo, fueron desmenuzados e incubados en agitación durante 30 min en tampón fosfato salino (PBS), centrifugados (600***g***, 5min), incubados durante 1 min en tripsina (0.5

mg/ml) / EDTA (0.1 mg/ml) en PBS, de nuevo centrifugados e incubados con agitación durante 30 min en colagenasa (0.5 mg/ml) en un medio RPMI suplementado con $CaCl_2$ $2H_20$ (0.7 mg/ml). Los tejidos así digeridos fueron filtrados (100µm) y las suspensiones celulares obtenidas, lavadas y resuspendidas en PBS. Ese mismo protocolo de extracción enzimática se utilizó para la obtención de suspensiones celulares de tejidos de aleta caudal.

Ensayo de longitud telomérica (TRF)

El ensayo de longitud telomérica se modificó a partir de Blasco *et al*., 1997. Las células aisladas y embebidas en bloques de agarosa siguiendo las instrucciones del fabricante (CHEF agarose plung kit de BioRad). Las células o el ADN embebidos en bloques de agarosa fueron digeridos con MboI y la electroforesis se realizó a través de un gel de agarosa al 0.5% en TBE (0.5X), a 120V durante 6h. El gel se transfirió y se sondeó con un fragmento de 1.6 kb con la secuencia (TTAGGG) n. El Southern blot se hibridó con la sonda marcada radiactivamente a 65ºC en albumina de suero bovino (BSA) (0.01 gm $^{-1}$), fosfato de sodio (200 mM), formamida al 15%, EDTA (1 mM), dodecil sulfato de sodio al 7% (SDS). El filtro (Hybond N + Amersham Pharmacia Biotech) se lavó tres veces en SSC (0.2 X), SDS al 0.1% a 65ºC durante 30 min.

La sonda telomérica específica se marcó con [a-32P] dCTP usando bolas de marcaje de ADN-Ready to-Go de acuerdo a las instrucciones del proveedor (GE Healthcare).

Ensayo de actividad telomérica (TRAP)

Se utilizó el kit de detección de telomerasa TRAPezeH (Millipore, Cat.#S7707) para medir cualitativamente la actividad telomérica de extractos de embriones de pez cebra. Se obtuvieron los extractos de proteínas siguiendo las instrucciones del fabricante. Células de carcinoma humano (incluidas en el kit de detección de telomerasa) se utilizaron como control positivo. El extracto con mayor concentración de proteína se incubó con 1 µg de RNAse A (QIAGEN) a 37ºC durante 20 min como control negativo específico.

Ensayo de actividad telomérica (Q-TRAP)

Para determinar cuantitativamente la actividad de la telomerasa de los extractos de órganos y de larvas de pez cebra, se realizó un TRAP cuantitativo a tiempo real (Q-TRAP) según lo descrito por Herbert *et al.,* (Herbert *et al.,* 2006). Los extractos proteicos se obtuvieron como describieron los autores. Se utilizaron diluciones en serie (1:10) de muestra de células positivas para la telomerasa (células de carcinoma humano) para obtener una curva estándar. Para cuantificar la actividad telomérica, se realizó la amplificación por PCR como indicado por los autores. Después de la PCR, se recogieron los datos a tiempo real y se convirtieron en unidades de Actividad de Telomerasa Relativa (RTA) realizando el cálculo: RTA de la muestra =10 (Ct muestra-Yint)/pendiente. La curva estándar obtenida fue: y=23.2295x+23.802.

Q-FISH

El ensayo Q-FISH sobre células interfásicas y metafásicas fue realizado como ha sido descrito por Canela *et al*. (Canela *et al*., 2007). Las imágenes de Cy3 y DAPI fueron capturadas con objetivos de 100X y 60X, respectivamente, utilizando una Cámara Digital Nikon DXM 1200C de un microscopio de fluorescencia Nikon Direct Eclipse. Los señales de fluorescencia de los telómeros fueron cuantificados utilizando el programa TFL-TELO (Peter Lansdorp, Vancouver, Canada).

Los valores de fluorescencia de los telómeros fueron convertidas en kb por calibración externa con la línea de células HeLa L con longitud telomérica conocida de 23.82 kb y con las líneas de linfocitos L5178Y-S, L5178Y-R con longitudes teloméricas conocidas de 10.2 y 79.7 kb, respectivamente.

Flow-FISH

Una suspensión celular (10^6 células) obtenida a partir de diferentes tejidos con el método descrito anteriormente se resuspendió en BSA al 0.1% en PBS, la mitad se resuspendió en 500 µl de solución de hibridación con una sonda telomérica marcada con FITC (PNA) y la otra mitad en una solución de hibridación sin sonda como control negativo. Las muestras fueron denaturalizadas durante 10 min a 80ºC en agitación, seguido de 2h de hibridación en oscuridad y temperatura ambiente. A continuación, las

células fueron lavadas 2 veces en una solución de lavado (formamida deionizada al 70%, Tris (10mM), BSA al 0.1% y Tween-20 al 0.1% en dH_2O, pH 7.2). Por último, las células fueron resuspendidas en una solución de ioduro de propidio, incubadas 2h a temperatura ambiente, mantenidas a 4ºC y analizadas por citometría de flujo.

Ensayo de regeneración de la aleta caudal en peces cebra adultos

Peces cebra de la estirpe AB fueron anestesiados en benzocaína al 0.05%. El tejido de la aleta caudal fue amputado a 2 mm aproximadamente de la base del pedúnculo caudal utilizando una hoja de bisturí. Los peces se mantuvieron a 32ºC durante el experimento. Se capturaron imágenes de la aleta caudal de cada pez antes de la escisión y de nuevo a diferentes días de regeneración. El porcentaje de regeneración de la aleta fue determinado en base al área de aleta regenerada dividida por el área original de la aleta ± el error estándar.

Para el estudio de la longitud telomérica de las células de la aleta caudal por flow-FISH, se muestreo por amputación el tejido de aleta caudal (clip 1) de peces cebra de la estirpe AB de diferentes edades. Una segunda y tercera amputación se realizó con 5 días de intervalo para obtener la porción de tejido regenerado (clip2 y clip3 respectivamente).

Análisis histológico

Varias muestras de tejido de pez cebra adulto (ojo, hígado, testículo) fijadas en formalina al 4% (Panreac Quimica) durante 24h, fueron procesadas y embebidas en parafina.

Las tinciones histológicas de hematoxilina-eosina (H&E) y de ácido periódico de Schiff (PAS) se realizaron sobre secciones de 4 μm utilizando protocolos estándar. Las secciones histológicas teñidas se examinaron utilizando un microscopio convencional a 200X, 400X y 630X.

El ensayo de TUNEL se realizó sobre tejido de testículo de pez cebra de genotipo *tert*$^{+/+}$ y *tert*$^{-/-}$, utilizando un sistema colorimétrico de detección de apoptosis DeadEndTM (Promega) siguiendo las instrucciones del fabricante. Las secciones histológicas teñidas fueron examinadas utilizando un microscopio convencional a 400X.

Metafases

El método para la obtención de metafases fue adaptado de Lee and Smith (Lee & Smith, 2004). Embriones de pez cebra de 23 hpf previamente decorionados, fueron incubados en colchicina (4 mg/ml) durante 8 h. Se añadió una solución fría de PBS (0.9X) suplementada con suero bovino fetal al 10% y se pasaron los embriones por filtros de 100 y 40 µm sucesivamente. Después de centrifugar (250 ***g***, 10 min, 4°C), las células fueron incubadas en una solución hipotónica (citrato de sodio al 1.1%, colchicina (4 mg/ml)) durante 25 min a 28.5ºC. De nuevo centrifugadas (450 ***g***, 10 min, 4°C), las células se fijaron con una solución de Carnoy fría (metanol: ácido acético glacial (3:1)). El último paso se repitió y a continuación, los cromosomas se tiraron sobre unos porta-objetos que se dejaron secar a 37ºC durante toda la noche.

Microinyección

Morfolinos específicos (MOs) (Gene Tools) se prepararon en la mezcla de microinyección (0.5x de tampon Tango y rojo fenol al 0.05%) y se microinyectaron en el saco vitelino de embriones de pez cebra en el estadio de desarrollo de 1-8 células utilizando un microinyector Narishige IM300 (0.5-1 nL por embryo). Una vez microinyectados, los embriones se incubaron en *egg water* a 28.5ºC.

Ensayo de apoptosis en el pez cebra

Realizamos un ensayo de TUNEL sobre larvas G2 de 48 hpf de genotipos *tert*$^{+/+}$y *tert*$^{-/-}$, teniendo el genotipo *tert*$^{-/-}$ un fenotipo normal (grupo I) y un fenotipo defectivo (leve: grupo II, grave y muy grave: grupo III). Utilizamos el kit de detección de apoptosis Apop Tag Red *In Situ* (S7165-Millipore) siguiendo las instrucciones del fabricante. Las células apoptóticas se contaron utilizando el software Image J.

Ensayo de detección de círculos de ADN telomérico (CCassay)

El Ensayo de detección de círculos de ADN telomérico se realizó siguiendo el protocolo descrito por Lau *et al*., 2013. Tejidos de aleta caudal de peces cebra de genotipos *tert*$^{+/+}$ y *tert*$^{-/-}$ a 0 hora post amputación (hpa) y las correspondientes partes

regeneradas a 24 y/o 72 hpa se muestrearon y mantuvieron a -80ºC. Para la extracción del ADN genómico (gDNA) de las muestras, se utilizó el kit "WizardGenomic DNA Purification kit (Promega). A continuación, se realizó una amplificación de los círculos C de ADN con BSA (0.2µg/µl), Tween-20 al 0.1%, dithiotreitol (4µM) (DTT), dNTPs (1 µM), 3.75U de ϕ29 polymerase, ϕ29 polymerase buffer (1X) y 16 ng de gDNA, con incubación de 8h a 30ºC, seguido de 20 min a 65ºC. El ensayo se hizó con y sin ϕ29 polymerase para cada muestra. Posteriormente se utilizó la reacción de amplificación de círculos C como molde en una q-PCR para la detección de secuencias teloméricas. Los primers usados fueron: telomere directo: 5'-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3' y telomere reverso: 5'-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3'. Finalmente las mismas reacciones de amplificación se utilizaron como molde en una segunda qPCR para detectar rps11 como valor estándar, usando los primers rps11 directo: 5'-ACAGAAATGCCCCTTCACTG-3' y rps11 reverso: 5'-GCCTCTTCTCAAAACGGTTG-3'. Ambas qPCRs se realizaron para las muestras con y sin ϕ 29 polimerasa.

Ensayo de regeneración

Peces cebra adultos *tert*$^{+/+}$, *tert* $^{-/-}$ fueron anestesiados en benzocaína al 0.05% antes de la amputación de la parte distal de su aleta caudal y mantenidos a 32ºC para su regeneración. Se utilizaron morfolinos *in vivo* (MOs) (Gene Tools) unidos a un fluoroforo de fluoresceina (FITC), a una concentración de 1.5mM, con las siguientes secuencias:

Nbs1: 5'-GATTTACACAGAGAAGATTACCTCC-3'

Control negativo: 5'-CCTCTTACCTCAGTTACAATTTATA-3'

Los MOs se inyectaron en el blastema de regeneración de la mitad dorsal de la aleta caudal de cada pez 48 h después de la amputación (48 hpa), considerando la mitad ventral no inyectada como control interno. A continuación, se electroporó el tejido microinyectado utilizando un electroporador (NEPAGENE), con 5 pulsos consecutivos de 50ms, a 15V y con una pausa de 30 sec entre cada pulso.

Se capturaron imágenes de las aletas inmediatamente después de la electroporación, y a las 24 horas de regeneración y se midieron las áreas de cola regenerada. Con el fin de calcular el porcentaje de inhibición de regeneración entre la

parte inyectada y la parte no-inyectada, se utilizó la formula siguiente: (Exp 3 días-Exp 2 días)/(Cont 3 días-Cont 2 días)*100, Exp representando el área regenerada de la parte de la aleta transfectada y Cont, la área regenerada de la parte no inyectada.

Ensayo de regeneración en larvas de pez cebra

Larvas de 48 horas post-fertilización (hpf) obtenidas de cruces de pez cebra (*tert* $^{+/-}$ x *tert* $^{+/-}$) y decorionadas, se anestesiaron en tricaina al 0.008%. A continuación, y con ayuda de una hoja de bisturí se les recortó la aleta caudal en un punto distal a la notocorda. Cada larva se individualizo en pocillo de placas de 96 pocillos conteniendo un inhibidor de ATR (10µm) (ATR Inhibitor IV, Calbiochem) o E3 como control negativo. Se capturaron imágenes de cada aleta caudal antes y después de la escisión y 24 horas después de la amputación para medir y calcular el área de regeneración.

Genotipage de larvas de pez cebra obtenidas de cruces de *tert* $^{+/-}$ x *tert* $^{+/-}$

Para la extracción del ADN genómico, las larvas se incubaron individualmente en una solución de lisis alcalina a 95ºC durante 1 h, a continuación, se enfriaron en hielo, se les añadió una solución de neutralización y después de centrifugar (1.000 rpm**,** 1 min), el sobrenadante se utilizó como molde de ADN para realizar una amplificación por PCR. Los cebadores utilizados para distinguir las larvas *tert* $^{-/-}$ de las larvas *tert*$^{+/+}$ y *tert* $^{+/-}$ fueron: Zf TERT directo: 5'-TGCCGGAGGTCTTGGCG-3', y Zf TERT reverso: 5'-CGCACACCTGCAGAACA-3' para el genotipo *tert*$^{+/+}$ y Zf TERT F: 5'-TGCCGGAGGTCTTGGCG-3' y Zf TERT reverso: 5'-CGCACACCTGCAGAACT-3' para el genotipo *tert* $^{-/-}$. Los productos de la PCR se examinaron por electroforesis en gel teñido con red safe (Intron Biotechnology).

Análisis estadístico

Los datos fueron analizados mediante el análisis de la varianza (ANOVA) y el test de Tukey para determinar las diferencias entre los grupos. Las diferencias entre dos muestras se analizaron mediante el Test-*t* de Student. El test Log-Rank fue usado para determinar las diferencias estadísticas en las curvas de supervivencia de distintos grupos experimentales.

4. Resultados y discusión

4.1. Caracterización de la longitud telomérica y de la expresión y actividad de la telomerasa del pez cebra (*Danio rerio*) en el envejecimiento y durante el proceso de regeneración de la aleta caudal.

Aunque, se ha demostrado ampliamente que los telómeros y la telomerasa están involucrados en el envejecimiento y la promoción del cáncer en mamíferos y que el pez cebra se ha convertido en un modelo muy utilizado para el estudio de estos procesos complejos, hasta el momento sólo hay unos pocos sobre los telómeros y la biología de la telomerasa en esta especie [Gerhard & Cheng, 2002; Gerhard, 2007; Kishi *et al*., 2009]. El pez cebra con una vida media de 3 años, es un modelo vertebrado ideal para estudiar la función de la telomerasa a gran escala. Hemos utilizado esta especie para llevar a cabo un estudio exhaustivo de la expresión de la telomerasa y la longitud de los telómeros en diferentes tejidos y a lo largo de su ciclo de vida.

En contraste con los mamíferos, los tejidos del pez cebra muestran un alto nivel de expresión génica de *TERT* [Lau *et al.,* 2008]. Hemos detectado expresión de *TERT* en diferentes tejidos y diferentes etapas (larva, juvenil, adulto jóven y adulto viejo) y una caida drástica de está en todos los tejidos del pez adulto viejo con excepción del músculo, estos resultados estarían en línea con un estudio de envejecimiento [Kishi, 2004] que muestra la proliferación continua de miocitos pero sin acumulación de granulos de lipofucsina en el músculo de pez cebra viejo. Los resultados de expresión de *TERT* se correlacionaban bien con los resultados de Q-TRAP detectando la presencia de actividad telomérica en tejidos de peces adultos, así como una disminución significativa de esta actividad en adultos viejos con excepción del músculo y del ovario.

En línea con estos resultados, observamos un aumento de la longitud de los telómeros durante la etapa de desarrollo del pez y un acortamiento significativo en peces adultos viejos. Nuestros resultados discrepan con unos datos publicados recientemente [Lund *et al.,* 2009] en los que los autores concluyen que los telómeros no se acortan con la edad. Estas discrepancias podrían explicarse por la metodología utilizada. El ensayo de TRF no es lo suficientemente sensible para detectar cambios en la longitud del telómero y menos para detectar telómeros cortos, algo que es crucial en estos estudios de envejecimiento [Canela et al., 2007]. Por lo tanto, hemos combinado esta técnica con otra más precisa el Q-FISH .

Por otra parte, todos nuestros estudios se han realizado utilizando organismos enteros de varias cepas (AB, TL y Wik) obtenidas de diferentes centros con el fin de evitar una longitud telomérica asociada a una cepa y / o a un centro dado. Estos resultados también se confirmaron utilizando un tejido específico, la aleta caudal, donde observamos por Flow-FISH que la longitud del telómero se incrementó hasta los 18 meses de edad y disminuyó gradualmente después de esta edad. En general, las tres cepas de tipo salvaje analizadas mostraron longitudes teloméricas ligeramente diferentes. TL es el fondo genético que mostró los telómeros más cortos y tales diferencias podrían tener un impacto en la susceptibilidad al cáncer y en el envejecimiento. De hecho, hemos observado que la vida media de los individuos de la cepa TL es de menos de 3,5 años, mientras que las cepas AB y WIK tienen vidas más largas en nuestras instalaciones. Se deben realizar más estudios para determinar si estas diferencias son importantes en los procesos de envejecimiento y de cáncer.

En este estudio, hemos establecido que existe una relación directa entre los niveles de expresión de la telomerasa, la actividad de la telomerasa y la longitud del telómero en el pez cebra. La haploinsuficiencia de *TERT* conduce al acortamiento de los telómeros en humanos y causa la enfermedad de envejecimiento prematuro conocida como la disqueratosis congénita [Armanios *et al*., 2005; Du *et al*., 2007]. Esto significa que los niveles de telomerasa controlan la longitud del telómero en humanos y en el pez cebra, y por lo tanto, la sobre-expresión de *TERT* podría prevenir la erosión de los telómeros y retrasar la senescencia en los animales adultos.

A diferencia de los mamíferos, los vertebrados inferiores son capaces de regenerar estructuras complejas después de un daño, incluyendo corazón, médula espinal, retina y aletas. Este proceso implica células progenitoras / células madre residentes [Brittijn *et al.*, 2009; Flores *et al.*, 2005]. En los mamíferos, la telomerasa se expresa en las células germinales y en el compartimento o “niche” de células madre de varios tejidos adultos. Se ha propuesto que la telomerasa puede ser importante para la regeneración de tejidos después de una lesión. De hecho, los fenotipos asociados con la pérdida prematura de la capacidad regenerativa de tejidos, incluyendo la piel (pérdida de cabello, encanecimiento del cabello, peor cicatrización de heridas) se observan en ratones deficientes en telomerasa [González-Suárez *et al.*, 2000; 2001; Cayuela *et al*., 2005]. Nuestros resultados muestran que el pez cebra es capaz de regenerar la aleta caudal amputada en todas las edades, aunque los peces más viejos, que a su vez tienen un nivel más bajo de expresión de *TERT*, muestran una regeneración deficiente. De hecho, la

eficiencia de la regeneración mostró una correspondencia directa con la expresión de la *TERT* (3días / peces jóvenes frente a 12 días / peces viejos). Además, hemos observado un aumento en la expresión de *TERT* en los tejidos regenerados tanto en jóvenes (3 meses de edad) como en viejos (24 meses), pero sólo significativo en el caso de los peces jóvenes (58% de la regulación positiva). Por lo tanto, los peces jóvenes respondieron mejor que los peces viejos después del daño. Es importante destacar que la regulación positiva de la expresión de *TERT* se correlaciona con el comportamiento de la longitud telomérica después de una segunda escisión, ya que los diferentes grupos de edad respondieron alargando sus telómeros. Nuestros datos fueron consistentes con la idea de que los telómeros necesitan mantenerse frente al aumento de la proliferación celular asociada a la renovación de tejidos. Aunque la fuerte regulación positiva de la expresión de la telomerasa observado en peces de 3 meses de edad no se correlacionó con un fuerte aumento de la longitud del telómero, este grupo fue el único capaz de alargar sus telómeros después de tres amputaciones consecutivas . Sin embargo, los peces mayores de 3 meses, a pesar de mostrar un aumento de la longitud de los telómeros después de una segunda escisión (clip2), no son capaces de mantener este alargamiento después de una tercera escisión (clip 3). Este comportamiento podría estar relacionado con una ineficiente activación de expresión de *TERT* en los peces mayores de 3 meses. Sin embargo, la regeneración es un proceso complejo en el que hay muchos genes factores involucrados. Por lo tanto, se necesitan más estudios para aclarar el papel de la telomerasa y de la longitud telomérica en un proceso de regeneración. Además, unos experimentos de ganancia y pérdida de función de los dos componentes de la telomerasa arrojarían luz sobre el papel de la telomerasa en la regeneración.

Hasta ahora, la actividad SA B-Gal, la deficiencia de melatonina y la función cognitiva fueron utilizados como biomarcadores del envejecimiento [Kishi *et al.*, 2008; Yu *et al*., 2006; Tsai *et al*., 2007]. Nuestro estudio demuestra que el acortamiento de los telómeros, la caida de la expresión de la telomerasa, y la menor eficiencia regenerativa son también biomarcadores fiables del proceso del envejecimiento. Estos resultados indican que la longitud del telómero, la expresión de la telomerasa y la capacidad de regeneración son parametros altamente dependientes de la edad del pez, y por lo tanto, son útiles para evaluar el proceso de envejecimiento del pez cebra.

Son necesarios más estudios para establecer si todas las células expresan telomerasa o sólo una población específica. Un pez cebra transgénico expresando un reportero inducido por el promotor de *TERT* podría ser útil para la identificación *in vivo*

de células con alta/baja actividad telomerasa, es decir células progenitoras/madre/células envejecidas. Aunque hay diferencias obvias entre humanos y pez cebra, tales como la alta expresión de *TERT* a lo largo de su ciclo de vida útil, ambas especies muestran una disminución en la expresión de la telomerasa y en la longitud de los telómeros con el envejecimiento. Por tanto, proponemos que el pez cebra puede ser utilizado para identificar los genes y drogas que afectan a la capacidad de restaurar los fenotipos de envejecimiento utilizando la longitud del telómero o expresión de la telomerasa, que han sido identificados como buenos biomarcadores de envejecimiento.

4.2. Caracterización de la línea mutante del pez cebra deficiente en telomerase

El estudio de los telómeros y de la biología de la telomerasa es crucial para la comprensión de los procesos de envejecimiento y de cáncer. Aunque el ratón ha sido el modelo más utilizado para estos propósitos, existen diferencias fundamentales entre ratones y seres humanos. Por lo tanto, hemos caracterizado un modelo vertebrado para estudiar el papel de la telomerasa y los telómeros en el proceso de envejecimiento [Anchelin *et al.*, 2011].

Siguiendo la línea de estudios de nuestro grupo de investigación, en este trabajo, hemos caracterizado la línea mutante de pez cebra deficiente en telomerasa obtenida a través del Instituto Sanger (línea hu3430). Esta línea mutante $tert^{-/-}$ es el resultado de una mutación puntual (T>A) que da lugar a un codón de stop prematuro y como consecuencia a una proteína truncada. Como era de esperar, este mutante carece de actividad telomerasa y presenta unos telómeros más cortos que el pez cebra de genotipo $tert^{+/+}$. Sin embargo, hemos observado un incremento en la expresión de ARN mensajero de *TERT*, lo que sugiere que la ausencia de actividad de la telomerasa se compensa por la inducción de la activación de su propio promotor. Este resultado indica la existencia de mecanismos de regulación de la expresión génica de *TERT* que sería interesante explorar en futuros estudios.

En estudios anteriores, observamos un acortamiento de los telómeros en los peces wild-type ($tert^{+/+}$) viejos [Anchelin *et al.*, 2011]. Inesperadamente, la longitud telomérica del pez cebra mutante $tert^{-/-}$, aunque más corto que el del genotipo $tert^{+/+}$, se mantuvo constante o incluso aumentó ligeramente durante el envejecimiento. Sin

embargo, la dinámica de la longitud de los telómeros de los tejidos parece ser específica de cada tejido. En el músculo, se observa una tendencia a la disminución de la longitud del telómero con el envejecimiento, mientras en el testículo esa disminución fue mucho más pronunciada, y las células de riñón cefálico mantuvieron la longitud de sus telómeros durante todo el ciclo de vida del pez. Aunque las diferencias en tasas de proliferación celular entre tejidos podrían explicar estos resultados [Lee *et al*., 1998], es tentador especular en la posible participación de activación de los mecanismos de mantenimiento de los telómeros independientes de la telomerasa (ALT por Alternative Lengthening of Telomere), por lo menos para las células hematopoyéticas altamente proliferativas del riñón cefálico. Por lo tanto, el modelo de pez cebra deficiente en telomerasa podría ser un excelente modelo para investigar el papel de ALT en el mantenimiento de los telómeros en ausencia de actividad de la telomerasa.

Los ratones tienen unos telómeros muy largos, y el fenotipo de envejecimiento "prematuro" en el modelo de ratón deficiente en telomerasa no aparece antes de las generaciones G5-G6 [Blasco *et al.*, 1997; Liu *et al.*, 2000]. Sin embargo, los seres humanos y los peces cebra tienen una longitud telomérica similar, y hemos podido observar síntomas de envejecimiento prematuro en la G1 de pez cebra deficiente en telomerasa que como consecuencia, tiene una vida media mucho más corta que el genotipo $tert^{+/+}$.

Varios signos de envejecimiento, tales como extrema delgadez, acumulación de lipofucsina en el hígado, y degeneración de las células de la retina, se observaron en adultos $tert^{-/-}$ jóvenes (10 meses de edad). El acortamiento telomérico y la inestabilidad genómica en las generaciones tardías de ratones deficientes en telomerasa es asociado al agotamiento de células germinales, igual que en los humanos [Hemann *et al.*, 2001; Lee *et al.*, 1998]. El pez cebra deficiente en telomerasa muestra degeneración prematura de los testículos e infertilidad en la 1ª generación.

Las larvas obtenidas por cruces de peces $tert^{-/-}$ x $tert^{-/-}$ (G2), con una supervivencia muy corta, presentaban un porcentaje de telómeros cortos muy alto (58,73% frente al 15,13% en el tipo silvestre) y además un tamaño inferior al de las larvas $tert^{+/+}$, como sucede en regeneraciones tardías de ratones deficientes en telomerasa debido al efecto de los telómeros cortos sobre la funcionalidad de las células madre [Flores *et al*., 2005; Herrera *et al.*, 1999]. Curiosamente, las larvas de cruces $tert^{+/+}$ x $tert^{-/-}$ mostraron un aumento de la supervivencia indicando que la reintroducción de *TERT* en peces cebra deficientes de telomerasa y con telómeros cortos

por herencia, rescato la viabilidad de la descendencia impidiendo más acortamiento de los telómeros, como ocurre en ratones [Bernardes de Jesús et al., 2012; Jaskelioff *et al*., 2011; Samper *et al*., 2001]. Este resultado indica que una longitud telomérica mínima es necesaria para mantener la homeostasis de los tejidos y que nuevos fármacos capaces de inducir la expresión de *TERT* o de un alelo de TR tendrían beneficios terapéuticos en pacientes heterocigotos con DC. Las poderosas ventajas del pez cebra para realizar escrutinio de drogas a gran escala [Zon & Peterson, 2005] podrían contribuir a la identificación de esas drogas. Otro dato muy interesante, es que estas larvas G2 obtenidas por cruces $tert^{+/+}$ x $tert^{-/-}$ resultaron tener una longitud telomérica media más larga y una mejor supervivencia, cuando el progenitor macho $tert^{-/-}$ era joven (5meses) que cuando era viejo (11 meses), lo que es indicativo del mismo fenómeno de anticipación relacionado con la longitud de los telómeros descrito en individuos con DC [Armanios *et al*., 2005; Vulliamy *et al.*, 2004]. Además, los histogramas de frecuencia de longitud telomérica nos indican que los heterocigotos aún tenían una alta proporción de los telómeros cortos en comparación con el tipo silvestre, lo que indica que la dosis de la telomerasa es crucial. Por lo tanto, la línea de pez cebra deficientes en *TERT* reproduce los mecanismos de la edad en ratones y en personas con DC deficientes en telomerasa [Armanios *et al.*, 2005; Knudson *et al.*, 2005;. Vulliamy *et al.*, 2004].

Un estudio muy reciente demostró que el ratio de incremento del porcentaje de telómeros cortos era un predictor de la vida media de los ratones, tanto silvestres como deficientes en telomerasa, más significativo que el ratio de acortamiento del telómero por mes, y que los individuos que mostraron mayor tasa de incremento en el porcentaje de telómeros cortos eran también los de vida media más corta [Vera *et al*., 2012]. Nuestros resultados están en línea con esta idea, los peces $tert^{-/-}$ de la G1 mostraron una vida media más corta que la de tipo salvaje y las larvas $tert^{-/-}$ de la G2 murieron prematuramente. Además, observamos varios fenotipos en la G2 de $tert^{-/-}$ y el grado de severidad de los defectos de cada fenotipo se correlacionó directamente con la longitud telomérica y más específicamente con la proporción de telómeros cortos. Los resultados de Q-FISH en células metafásicas mostraron que el acortamiento de los telómeros iba acompañado del aumento en el número de extremos cromosómicos libres de señal telomérica, de fusiones cromosómicas y de señales multiteloméricas (MTS). Por lo tanto, se especula que el pez cebra heterocigotos $tert^{+/-}$ que presenta mayor proporción de telómeros cortos podría mostrar una anticipación en las señales de envejecimiento y una longevidad más corta. Sin embargo, se necesitan más estudios para caracterizar ese

genotipo y estudiar si sería capaz de recapitular el proceso de anticipación observado en individuos con DC.

Las patologías que se producen en el modelo de ratón deficiente en telomerasa están acompañadas por una reducción en el potencial proliferativo debido a una activación de p53, lo que conduce a la detención del crecimiento y / o la apoptosis en los tejidos afectados [Leri *et al*., 2003]. Además, la deficiencia de p53 rescata los efectos adversos de la pérdida de telómeros [Chin *et al.*, 1999; Flores & Blasco, 2009]. Estos resultados son consistentes con la inducción de la expresión del gen *p53* en los tejidos de pez cebra con una alta tasa de proliferación, tales como los de los testículos. En este tejido, la expresión del gen *p53* fue mayor en los peces *tert*$^{-/-}$ en comparación con sus hermanos *tert*$^{+/+}$, coincidiendo con el acortamiento de sus telómeros. Del mismo modo, las larvas G2 también mostraron una activación de la expresión del gen *p53* y un mayor número de células apoptóticas en los fenotipos anormales (grupos II y III) en comparación con el fenotipo normal (grupo I), lo que indica una vez más la importancia de los telómeros críticamente cortos y la inestabilidad genómica. La relevancia de p53 en el senescencia prematura y en la reducción de la fertilidad de la G1, y los defectos en el desarrollo y la mortalidad temprana de las larvas G2 *tert*$^{-/-}$ fue confirmada por el rescate completo de la supervivencia de las larvas G2 a través de la inactivación transitoria de p53 y la generación de la G1 de doble mutantes: *tert*$^{-/-}$; *p53*$^{-/-}$, que mostraron ausencia de alteraciones morfológicas y ausencia de células germinales TUNEL $^{+}$ en los testículos. Es importante destacar que las larvas G2 *tert*$^{-/-}$; *p53*$^{-/-}$ aumentaron su supervivencia durante la primera semana, pese a tener la misma longitud telomérica que las larvas G2 *tert*$^{-/-}$ lo que podría explicar el alto porcentaje de mortalidad entre 10 y 20 días post-fertilización (dpf). Aunque la deficiencia de p53 fue capaz de rescatar inicialmente los efectos adversos de la pérdida de telómeros, una proliferación celular sostenida en ausencia de p53 podría dar lugar a un acortamiento de los telómeros y a la disfunción progresiva que, a su vez, reduciría la vida media. Se requieren más estudios de envejecimiento y carcinogénesis con la G2 de doble mutantes.

En resumen, el pez cebra deficiente en telomerasa, caracterizado en este estudio, es un modelo prometedor para investigar el envejecimiento asociado al telómero, ya que recapitula el telómero humano y la biología de la telomerasa. Además, es un excepcional modelo vertebrado para el descubrimiento de nuevos tratamientos capaces de reactivar temporalmente la expresión de la telomerasa en las personas con DC.

4.3. Estudio de la longitud telomérica durante el proceso de regeneración de la aleta caudal en la linea mutante del pez cebra deficiente en telomerasa.

La formación del blastema de regeneración requiere una proliferación celular activa y el mantenimiento de los telómeros para sostener la alta tasa de replicación y división. Nuestros anteriores resultados revelaron una relación directa entre la expresión de la telomerasa, la longitud telomérica y la eficiencia de la regeneración de los tejidos de la aleta caudal del pez cebra después de una escisión (Anchelin *et al.,* 2011).

La longitud telomérica del pez cebra mutante *tert*$^{-/-}$ es más corta que la del genotipo silvestre *tert*$^{+/+}$ y no disminuye con el envejecimiento [Anchelin *et al.,* 2013], lo que sugiere la posible activación de los mecanismos de mantenimiento de los telómeros independientes de la telomerasa (ALT 'alternative lengthening of telomeres'). La diferencia de longitud telomérica entre los dos genotipos es notable cuando analizamos las células del pez entero, pero es muy estrecha si analizamos las células de la aleta caudal, lo que dificulta la comparación de longitud telomérica entre genotipos cuando analizamos las células obtenidas de los blastemas de regeneración.

Nuestros resultados confirman la alta capacidad de regeneración de la aleta caudal del pez cebra después de una única amputación, así como después de amputaciones sucesivas, aun en ausencia de la telomerasa. Además, observamos que la eficiencia de la regeneración disminuye con el envejecimiento de los peces en los tres genotipos.

En caso de deficiencia en telomerasa, la recombinación homóloga constituye un método alternativo (ALT) para el mantenimiento de los telómeros. Un trabajo de Fajkus *et al.,* (2005) examina los telómeros desde una perspectiva evolutiva, e hipotetiza que la presencia de círculos de ADN extracromósomicos en células humanas ALT y en varios organismos (levaduras, plantas superiores, *Xenopus laevis*) podría indicar que son sistemas primordiales de mantenimiento de los telomeres. Esta hipotesis sugiere una possible explicación evolutiva, los telómeros muy cortos mantendrian una longitud adecuada por un mecanismo de recombinación asegurando así su integridad y la correcta proliferación cellular para la regeneración en el caso de la amputación de un órgano esencial para la supervivencia del pez en su medio natural de vida.

Un estudio reciente determina que la telomerasa es imprescindible para la regeneración del corazón del pez cebra después de un daño [Bednarek *et al.,* 2015], lo que sugiere que el mecanismo de mantenimiento de la longitud telomérica puede ser

diferente y / o específico de tejidos y órganos, y que son necesarios más estudios para elucidar una posible explicación evolutiva.

La detección de círculos teloméricos extracromosómicos ha sido utilizada como marcador de células ALT. Veinticuatro horas después de la amputación (24 hpa) de la aleta caudal, hemos detectado círculos de ADN telomérico tanto en las células regenerativas del pez cebra silvestre como en las del pez cebra mutante *tert*$^{-/-}$, aunque la cantidad de círculos de ADN fue más significativa en el pez deficiente en telomerasa.

En otro ensayo de regeneración, hemos utilizado un morfolino contra el gen *Nbs1* para inhibir la formación de los círculos teloméricos y así impedir el mantenimiento de los telómeros y hemos observado una menor eficiencia de regeneración tanto en el genotipo *tert*$^{+/+}$ como en el mutante *tert*$^{-/-}$.

Finalmente, hemos realizado un ensayo de regeneración en larvas de pez cebra, inhibiendo por inmersión un regulador de la recombinación homóloga (ATR) impidiendo ALT durante el primer día de la regeneración. Hemos observado una menor eficiencia de regeneración en los tres genotipos, aunque con diferencias estadísticamente significativas en el caso de los genotipos deficientes para la telomerasa.

Podemos concluir que, en ausencia de telomerasa, la longitud telomérica se mantiene por un mecanismo de recombinación homóloga durante la regeneración, mientras que en el pez silvestre podrían coexistir y participar los dos mecanismos de alargamiento de telómeros, se requieren más estudios para aclarar esta cuestión.

Nuestros resultados confirman la gran capacidad del pez cebra para reparar y renovar los tejidos de la aleta después de una o de varias amputaciones. Los peces deficientes en telomerasa regeneran los tejidos con un ratio de regeneración más bajo. La eficiencia de regeneración disminuye con el envejecimiento de los peces en los tres genotipos. Además, nuestros resultados demuestran que el mecanismo ALT está implicado en el mantenimiento de los telómeros durante la regeneración de la aleta en el zebrafish deficiente para la telomerasa. Hasta la fecha, los estudios sobre ALT, se han realizado *in vitro* en líneas celulares inmortalizadas o células tumorales. El pez cebra demuestra de nuevo ser un excelente modelo para futuros estudios *in vivo* del mantenimiento de los telómeros y del papel de la telomerasa en el proceso de regeneración.

5. Conclusiones

Los resultados de este trabajo conducen a las siguientes conclusiones:

1. La expresión de la telomerasa y la longitud de los telómeros están estrechamente relacionados a lo largo del ciclo de vida del pez cebra, y esos dos parametros pueden ser utilizados como biomarcadores de envejecimiento en el pez cebra.

2. El pez cebra deficiente en telomerasa muestra un envejecimiento prematuro y una vida media más corta desde la primera generación, como ocurre en los seres humanos, y puede ser considerado como un modelo prometedor para estudiar el envejecimiento inducido por el telómero.

3. Los embriones deficientes en telomerasa de segunda generación mueren en las primeras etapas de desarrollo y la restauración de la actividad telomerasa rescata la longitud de los telómeros y la supervivencia, lo que indica que la dosis de telomerasa es crucial.

4. La línea de pez cebra deficiente en telomerasa reproduce el fenómeno de anticipación genética, ligado al acortamiento telomérico observado en humanos con DC.

5. La inhibición genética de p53 rescata los efectos adversos de la perdidad de telómeros, lo que indica que los mecanismos moleculares inducidos por el acortamiento telomérico están conservados desde los peces hasta los mamíferos.

6. La eficiencia de regeneración disminuye con el envejecimiento en el pez cebra salvaje y en el pez cebra deficiente en telomerasa.

7. El pez cebra wild-type y el pez cebra deficiente en telomerasa regeneran los tejidos de la aleta caudal después de una o varias escisiones. La línea *tert* mutante tiene un ratio de regeneración menor que el de los peces de tipo salvaje, y mantiene la longitud telomérica en los tejidos regenerados.

8. El mecanismo ALT está implicado en el mantenimiento de la longitud telomérica durante la regeneración de la aleta caudal en el pez cebra deficiente para la telomerasa. Son necesarios más estudios para elucidar definitivamente el papel de la telomerasa y la posible implicación de ALT en el mantenimiento de

la longitud telomérica durante la regeneración de la aleta caudal en el pez cebra de tipo salvaje.

9. El pez cebra puede ser utilizado para la identificación de genes y fármacos que permitan restaurar los fenotipos de envejecimiento.