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ESCUELA INTERNACIONAL DE DOCTORADO

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Desarrollo de nuevas terapias basadas en las funciones no canónicas del componente de RNA de la telomerasa

Dña. Elena Martínez Balsalobre

2020

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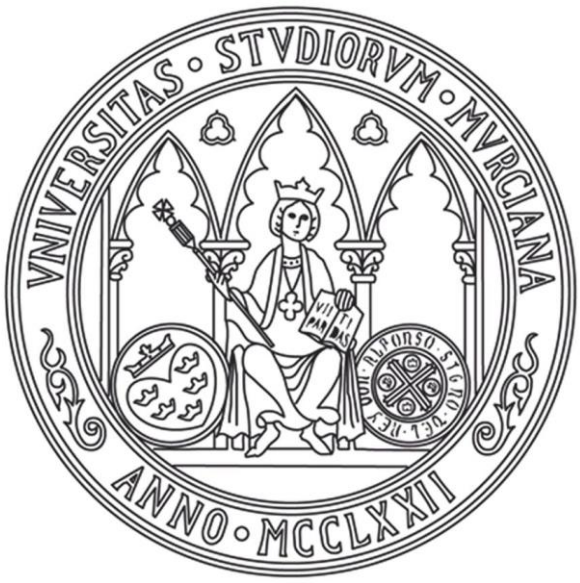

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TABLE OF CONTENTS

ABBREVIATIONS

AA	Aplastic anemia
AGM	Aorta-gonadmesonephros
ALM	Anterior Lateral Mesoderm
ALT	Alternative Lengthening of Telomeres
as	Antisense
BIBR1532	(E)-2-(3-(naphthalen-2-yl)but-2-enamido)benzoic acid
bp	Base pairs
cDNA	complementary DNA
CFU	Colony Forming Units
CHT	Caudal Hematopoietic Tissue
ChIRP	Chromatin Isolation by RNA Purification
CMV	Cytomegalovirus
CSF	Colony Stimulating Factor
D-loop	Displacement loop
DC	Dyskeratosis congenita
DDR	DNA damage response
DKC1	Dyskerin 1
DMEN	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfphoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide Triphosphate
dpf	days post-fertilization
dsDNA	double-stranded DNA
EBs	Embryoid Bodies
EMPs	Erythromyeloid Progenitors
EMT	Epithelial–Mesenchymal Transition
F	Direct primer
FACS	Fluorescence-Activated Cell Sorting
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase encoding gene

Abbreviations

GATA1	GATA binding protein 1 (globin transcription factor 1) encoding gene
gDNA	Genomic DNA
GFP	Green fluorescent protein
GCSF	Granulocyte- colony stimulating factor
h	Hours
HEPs	Hemogenic progenitors
hpf	Hours post-fertilization
HPs	Hematopoietic Progenitors
HSCs	Hematopoietic Stem Cells
IMC	Intermediate Cell Mass
iPSCs	induced Pluripotent Stem Cells
kb	Kilobases
kDa	Kilodaltons
LC	Liquid Chromatography
LNA	Locked Nucleic Acid
Luc	Luciferase
MCSF	Macrophage- Colony-Stimulating Factor
min	Minutes
MO	Morpholino
Mpx	Myeloperoxidase
mRNA	Messenger RNA
MS	Mass Spectrometry
MYC	V-myc avian myelocytomatosis viral oncogene homolog
n.s.	not significant
nt	nucleotides
PBI	Posterior Blood Island
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Q-TRAP	Quantitative-TRAP Telomeric Repeat Amplification Protocol
R	Reverse primer
RNA	Ribonucleic Acid

RNA Pol II	RNA Polymerase II
RNase	Ribonuclease
RNP	Ribonucleoprotein
ROS	Reactive Oxygen Species
rps11	Ribosomal protein subunit 11 encoding gene
RT	Room Temperature
RTA	Relative Telomerase Activity
qPCR	Quantitative Polymerase Chain Reaction
SEM	Standard Error of Mean
SPI1	Spi-1 proto-oncogene encoding gene
ssDNA	single-stranded DNA
Std	Standard
TBS	Tris-Buffered Saline
TCA	Tricarboxylic Acid Cycle
TTBS	Tween-Tris-Buffered Saline
TERC	Telomerase RNA Component
TERT	Telomerase catalytic subunit
T-loop	Telomeric DNA loop
TMM	Telomere Maintenance Mechanism
TSA	Tyramide Signal Amplification
TSS	Transcriptional Start Sites
VEGF	Vascular Endothelial Growth Factor
wt	Wildtype
ZIRC	Zebrafish International Resource Center
zf	Zebrafish

SUMMARY

During the development of this Doctoral Thesis, we have exploited the unquestionable advantages of the zebrafish to study the role of *terc* in myelopoiesis, characterizing the functional involvement of each *terc* domain in this process. To investigate the function of each domain, different mutations that are frequently found in patients with dyskeratosis congenita (DC) were reproduced and we observed that only the mutations affecting the CR4/CR5 domain impaired myelopoiesis.

Taking advantage of the functional knowledge of the roles played by the CR4/CR5 domain of *TERC* in myelopoiesis, several aptamers were developed. Two of the aptamers, *CR4CR5* and *AA*, were able to stimulate myelopoiesis by increasing the number of neutrophils and macrophages, without affecting the number of erythrocytes, and independently of *terc* expression or telomerase activity. In addition, they functioned as *terc*; that is, they interacted with RNA polymerase II and with the *terc* binding sequences present in the promoter of master myelopoiesis genes, enhancing their expression in a *terc* binding site-dependent manner. Importantly, aptamers harbouring CR4/CR5 mutations found in DC patients failed to perform all these functions. In addition, preclinical zebrafish models of DC (*terc* deficiency) and poikiloderma with neutropenia (*usb1* deficiency) diseases demonstrated the therapeutic potential of the developed aptamers to treat neutropenia. Finally, the corresponding human aptamers also increased myeloid gene expression promoting myelopoiesis in human induced pluripotent stem cells. Therefore, two potential therapeutic agents for DC and other neutropenic diseases have been developed in this study.

Although we delved into *TERC* structural and functional characteristics during this thesis, we decided that it was still possible to explore its potential beyond its role in telomeric biology and its non-canonical role in myelopoiesis. For this reason, a proteomic approach was developed to fully exploit the potential of *TERC*. By this way, it has been discovered that *TERC* potentially interacts with a multitude of proteins, involved in several cellular processes, such as protein folding, degradation (ubiquitination) and translation, carbon and lipid metabolism, nonsense-mediated mRNA decay, mitochondrial biogenesis and cell cycle. Notably, *terc* harbouring the mutation of the CR4/CR5 domain found in DC patients showed a similar interactome as wild type *terc*, despite these mutations impair both telomerase activity and *TERC*-dependent regulation of myelopoiesis. This study paves the way for future functional studies aimed at the characterization of the non-canonical roles of *terc* that could help in improving diagnosis and treatment of patients with *TERC* mutations.

INTRODUCTION

1. Telomeres

Genomic stability is the main requisite of species survival to ensure that all required information will be passed on to the next generations. Therefore, cells have to develop new abilities to recognize and repair DNA damage and progress through the cell cycle, adequately.

One of the evolutionary adaptations is the emergence of telomeres, 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 conserved long tandem repeats at chromosomal ends composed of a guanine rich sequence that differ in length in diverse species (Greider, 1998). They were found in the extrachromosomal ribosomal DNA of *Tetrahymena thermophile*, a protozoan. Usually the protozoan telomeres contain 20–70 hexameric TTGGGG tandem repeats (Blackburn et al., 2006). The telomeres in yeast comprise GGTTACA repeat sequences extending up to 300 bp. In plants, TTTAGGG repeats typically range between 2 to 100 kb. In vertebrates, chromosomal ends consist of TTAGGG repeats, which in humans are between 10 and 15 kb, but in rodents extend up to 150 kb (Srinivas et al., 2020).

1.1. Structure and function

Telomeric DNA is double stranded with a single-stranded terminal that averages 50-300 nucleotides (nt) in length in human cells. At each cell division the telomeres are shortened in most somatic cells of an adult organism about 50-150 base pair (bp) in humans (Figure 1) (Zhu et al., 2011).

In order to protect the single stranded overhang, this G-rich strand fold back and invade the double-stranded telomere helix, forming a large T-loop (telomere 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 (Figure 2). As a result, the T-loop protects the G-tail from dsDNA break by sequestering the 3'-overhang into a higher order DNA structure (Griffith et al., 1999). The formation of this structure preventing its recognition by the DNA damage response (DDR) machinery.

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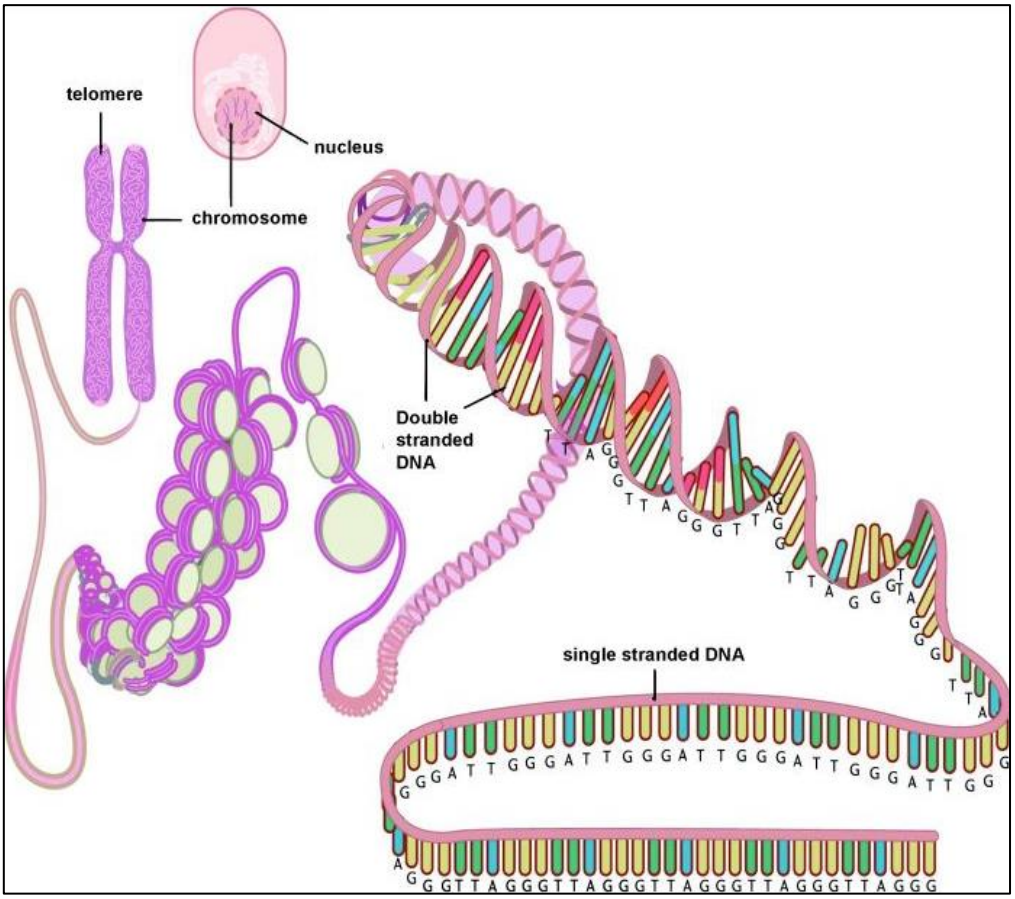

Figure 1. Telomere structure and its location on the chromosome and in the cell. *Adapted from (Zhu et al., 2011).*

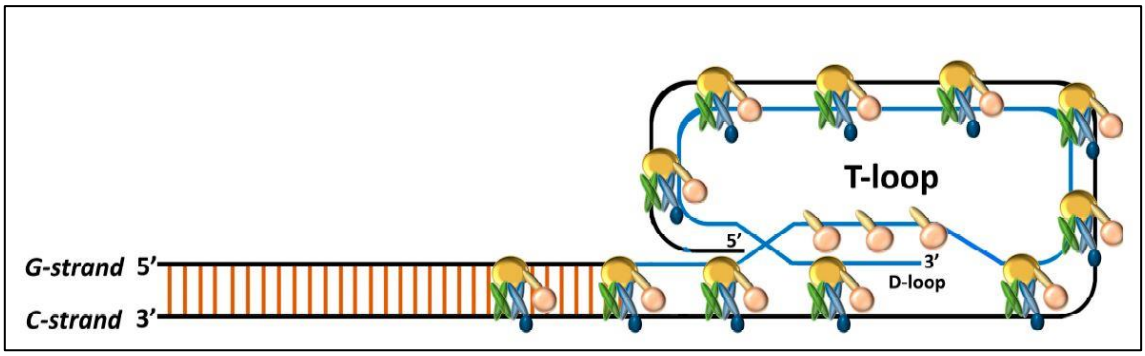

Figure 2. Telomere secondary structure scheme. Location of T-loop and D-loop in the telomeres to protect the 3'-overhang. *Adapted from (Srinivas et al., 2020).*

However, in vertebrates, the ends of the chromosomes need to be protected from being confused with chromosome breaks. To carry out this function there is a specific group of proteins collectively called shelterin. Shelterin complex is basically composed by six proteins: TRF1 (telomeric repeat-binding factor 1), its homodimer TRF2 (telomeric repeat-binding factor 2), RAP1 (repressor activator protein 1), POT1 (protection of telomeres 1), TIN2 (TRF1-interacting nuclear factor 2) and TPP1 (TIN2 and POT1-interacting protein 1). Three shelterin subunits, TRF1, TRF2, and POT1, directly recognize TTAGGG repeats. They are interconnected by three additional shelterin proteins, TIN2, TPP1, and RAP1, forming a complex that allows cells to distinguish telomeres from sites of DNA damage (Figure 3). TRF1 and TRF2 bind directly to double stranded telomeric sequence. TRF2 interacts with and recruits RAP1. On the other hand, TIN2 connects TPP1 and POT1 to the TRF1 and TRF2 components, but also stabilizes them. POT1 binds to the 3′ ssDNA overhang of telomeres, while TIN2 binds the ssDNA and dsDNA binding complexes, especially in the area of telomeric D-loop formation (reviewed by (de Lange, 2005).

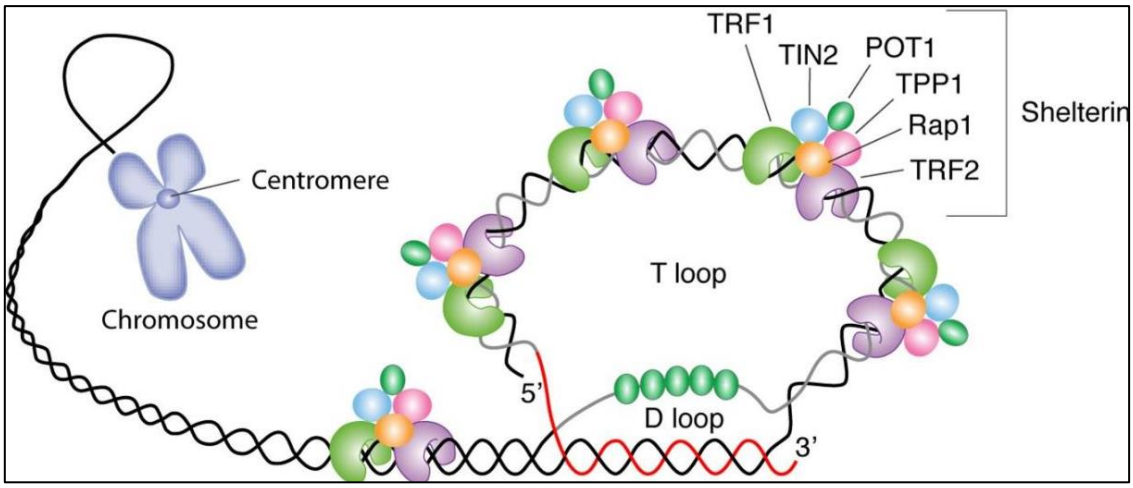

Figure 3. DNA protection model through shelterin complex in a T-loop. *Adapted from (Calado and Young, 2008).*

In addition to its role in DNA protection, shelterin also regulates access to telomeric DNA restoration processes after each genome replication. In general, shelterin complex functions as a dynamic unit in regulating telomere length, protects the chromosomal ends from being recognized as DNA damage and represses DNA damage response (DDR) signals (Diotti and Loayza, 2011, Srinivas et al., 2020).

Shelterin proteins mutations contribute to human hematopoietic failure syndromes, developmental defects and cancer. Every single sheltering protein plays a critical role *in vivo*, especially in tissues that require active renewal and robust stem cell compartments, as hematopoietic tissues. For example, TRF1 inactivation in bone marrow results in hematopoietic failure within weeks in mice, and TRF2 overexpression in the skin resulted in hyperpigmentation and predisposed mice to squamous cell carcinomas (reviewed by (Jones et al., 2016).

Apart from shelterin and interacting partners, there are other more recent discovery complexes that also play an important role in telomere biology, as the CST complex. CST is a heterotrimeric protein complex, consisting of CTC1 (conserved telomere maintenance component 1), STN1 (suppressor of CDC13A) and TEN1 (CST complex subunit TEN1). It localizes at single strand and functions in telomere capping and length regulation (Chen et al., 2012).

In front of the telomeres (subtelomeres) we have the main third component in telomere biology: TERRA RNAs (Figure 4). Subtelomeres are chromatin regions between main chromosomal sequences and telomeres. TERRA RNAs are packed into constitutive heterochromatin that contains H3K9me3, H4K20me3 and HP1 heterochromatin marks and also harbors transcriptional start sites (TSS) for TERRA RNAs. These RNAs are transcribed by RNA polymerase II (Schoeftner and Blasco, 2008). TERRA facilitates the replication of telomeric repeats and promotes chromosome stability. At telomeres, TERRA forms RNA-DNA hybrids, or R-loops, which play a key role in promoting homology-directed repair at telomeres. The depletion or overexpression of TERRA activates DNA damage response and results in a telomere dysfunction, confirming its role in maintaining telomeric homeostasis (reviewed by (Lalonde and Chartrand, 2020, Srinivas et al., 2020)).

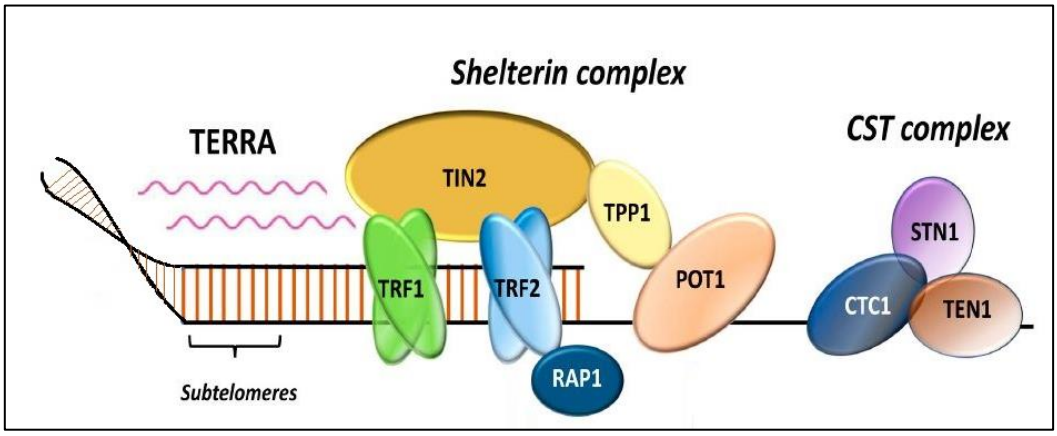

Figure 4. Representation of shelterin complex, CST complex and telomeric repeat containing RNA (TERRA). *Adapted from (Srinivas et al., 2020)*.

The structure of the telomeres is dynamic, has to switch between a protected and a deprotected state throughout the cell cycle and cell differentiation. 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.

1.2. The 'end-replication' problem

Incomplete replication at chromosomal ends by DNA polymerase results in progressive shortening of telomeres with each successive cell division and is termed as the "end replication problem" (Ohki et al., 2001). Each DNA strand requires a template and an RNA primer to generate a new complementary strand. This is not a problem for the strand in 5' to 3' direction, because with a single RNA primer can synthesize a new strand (leading strand), but the other strand (lagging strand) requires annealing of multiple primers that elongate into short Okazaki fragments opposite to the replication fork. When the primers degrade, the gaps are filled by Polδ followed by ligation of the discontinuous fragments. The problem is the gap at 3´end, because it remains unfilled (Figure 5). This region would be susceptible to enzymes that degrade single strand DNA (ssDNA) (Turner et al., 2019).

In the absence of any compensatory mechanism, the result would be that the length of the chromosome would be shortened about 250 nucleotides after every cell division, resulting in accumulative telomere attrition during ageing (reviewed by (Srinivas et al., 2020)).

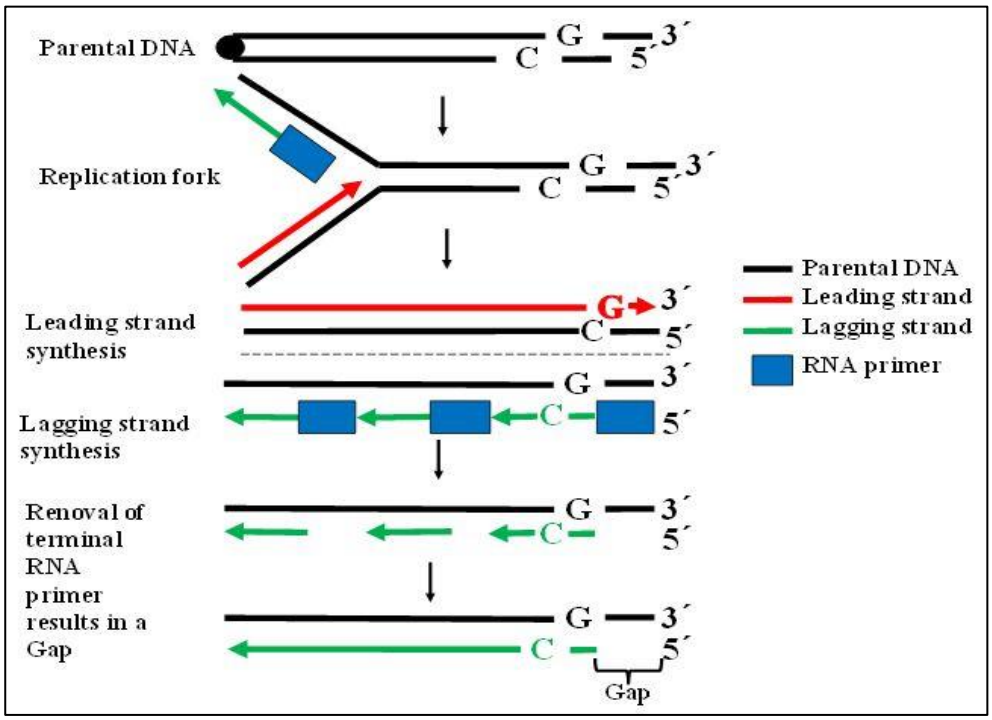

Figure 5. Scheme of the 'end-replication problem'. The strand 3´-5´ cannot be synthesized directly because it needs an RNA primer to initiate synthesis. Therefore, it is done in a discontinuous manner (Okazaki fragments). The problem is that the mechanism leaves a gap in 3´end and there is a telomere shortening at each round of DNA replication. *Adapted from the website http://jcs.biologists.org.*

2. Telomerase complex

Eukaryotic organisms have developed a mechanism that solves the end-replication problem. This mechanism is telomerase complex, a specialized RNA-dependent DNA polymerase that synthesizes telomeric repeats at the end of eukaryotic chromosomes (reviewed by (Blackburn, 2005)), preventing telomeric shortening after each cell division (Figure 6).

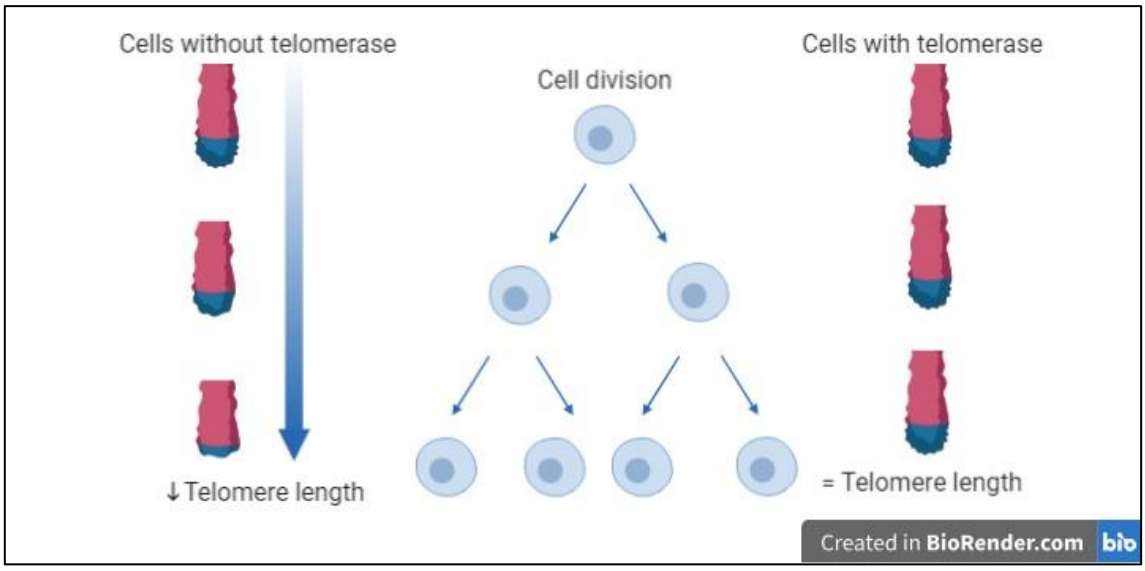

Figure 6. Telomeric maintenance through telomerase. Without telomerase, chromosomes shorten and prevent cells from continuing to divide. With telomerase, the telomeric length is maintained in the different divisions, allowing proliferation. *Created in BioRender.com.*

Telomerase is routinely active in tissues during embryogenesis and development, while in adults is expressed only in proliferating cells or tissues that are continuously renewed, such as epithelial cells, activated lymphocytes, specific bone marrow stem cells and dividing male germ cell lineages (Ulaner and Giudice, 1997). In most adult cells, telomerase is not expressed. Consequently, after several cell divisions, telomeres reach a critical length and chromosomes become uncapped. This limits cell proliferation by activating checkpoints that induce replicative senescence (permanent cell cycle arrest) or apoptosis (programmed cell death) (Figure 7). The telomere shortening also induces a DNA damage response (DDR) that results in chromosomal end-to-end fusions. Telomeres and telomere shortening act as a strong tumor suppressor mechanism in human somatic cells (Tümpel and Rudolph, 2012, Tomita, 2018, Gonzalez-Suarez et al., 2000).

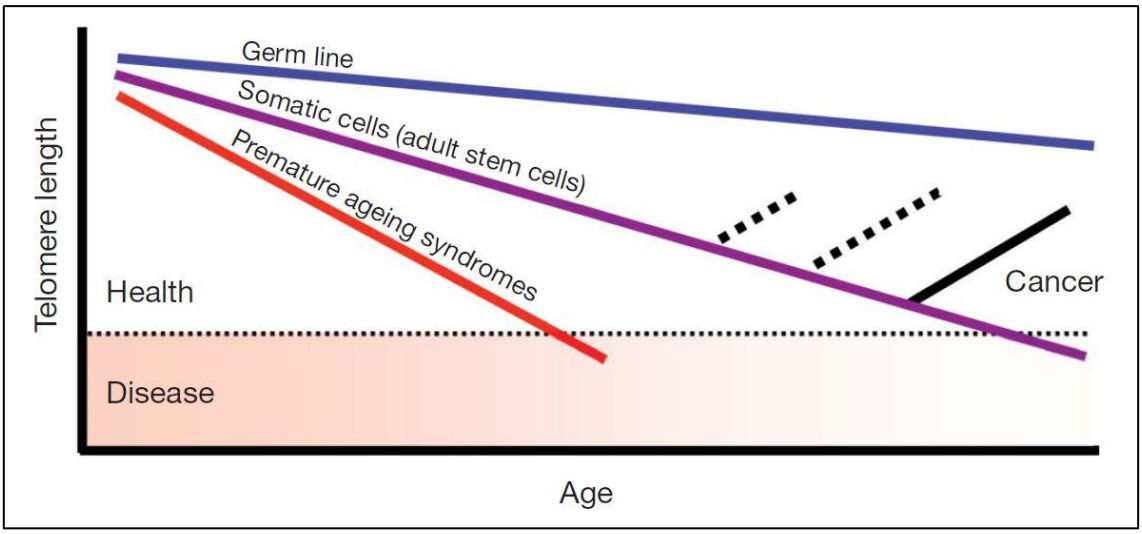

Figure 7. Telomere length in health and disease. Normal somatic cells, including adult stem cells, undergo progressive telomere attrition coupled to cell division. This attrition is attenuated in germline cells with high levels of telomerase activity. Telomere shortening in somatic cells contributes to ageing-related pathologies. Mutations in telomerase complex accelerate the telomere shortening, developing premature ageing syndromes. On other hand, telomerase reactivation in somatic cells can develop cancer. Therefore, the key is to maintain a telomeric homeostasis. *Adapted from (Finkel et al., 2007).*

In most of tumor cells genomic stability must be re-established and telomere length must be restored by a telomere maintenance mechanism (TMM). In approximately 90% of cancerous cells telomerase is up-regulated or reactivated (Wright et al., 1996). There are a small percentage of tumors that do not depend on telomerase for telomere elongation, but on another mechanism called Alternative Lengthening of Telomeres (ALT) (reviewed by (Draskovic and Londono Vallejo, 2013)). ALT cells are characterized by heterogeneous telomere length with extremely long (>50 kb) and short (<5 kb) telomeres. This mechanism is based on homologous recombination (HR) dependent exchange and/or HR-dependent synthesis of telomeric DNA (Londono-Vallejo et al., 2004).

There are two proteins that are indispensable for ALT function: α-thalassemia/mental retardation syndrome X-linked protein (ATRX) and death domain-associated protein (DAXX). Mutations in these proteins have been associated with ALT-positive tumors, because they function as a chromatin remodeling complex that leads to a repressed heterochromatin state that activates recombination and initiation of ALT (reviewed by (Srinivas et al., 2020)).

Telomerase and ALT are not two exclusive mechanisms, there are also some cell types that utilize both mechanisms (Figure 8) (Bojovic et al., 2015, Xu et al., 2014, Artandi and Cooper, 2009), although the capability of cells to switch from telomerase to ALT is poorly understood (reviewed by (De Vitis et al., 2018).

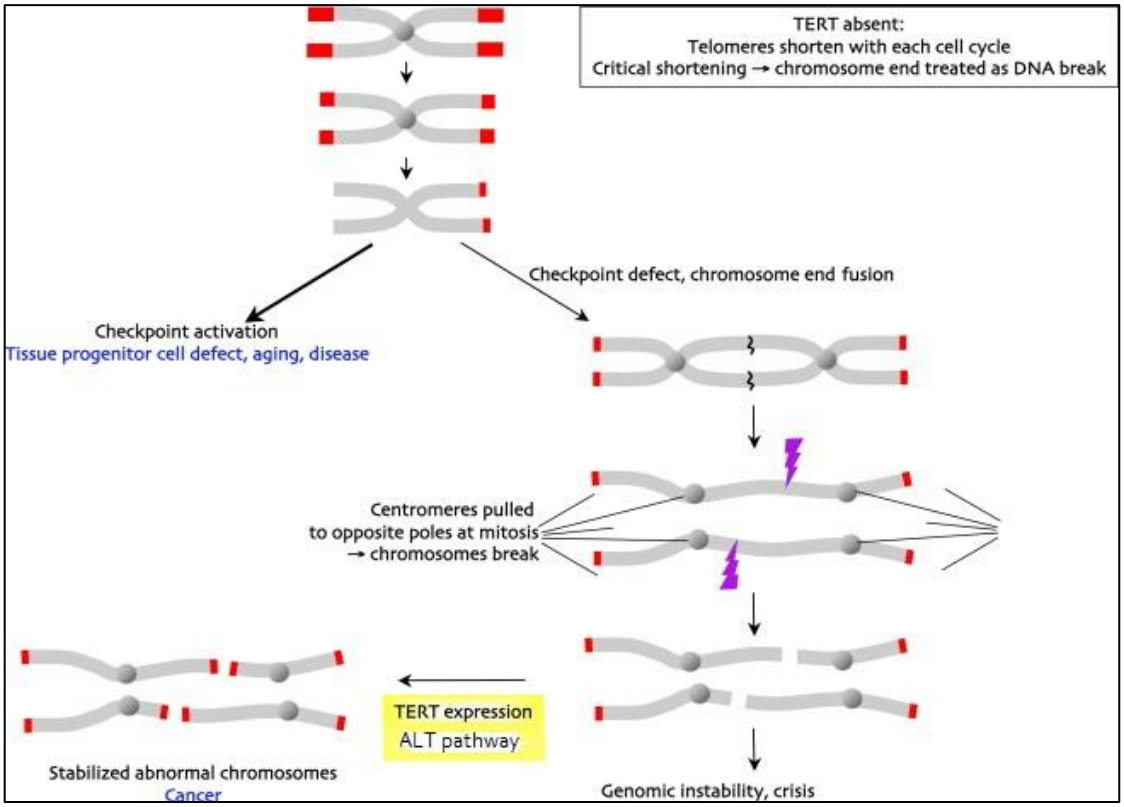

Figure 8. Telomere Shortening Causes Senescence and Chromosome Instability. In cells with critically short telomeres the DNA-damage response is activated. However, in cells lacking a robust DNA-damage checkpoint response, inappropriate "repair" reactions act on telomeres, which can drive tumor development. Telomerase upregulation (or, rarely, the ALT pathway) stabilizes the telomeres, allowing the propagation of aberrant genomes associated with many cancers. *Adapted from (Artandi and Cooper, 2009)*.

2.1. Structure and function

The telomerase complex is a ribonucleoprotein composed mainly of a catalytic subunit (telomerase reverse transcriptase, TERT) and an RNA component (*TERC*) which acts as a template for the addition of the telomere sequence in the 3' end of the telomere. Furthermore, there are several accessory molecules that regulate telomerase biogenesis, subcellular localization, and function *in vivo* (Wyatt et al., 2010, Podlevsky and Chen, 2012). 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 (Gomez et al., 2012)) (Figure 9).

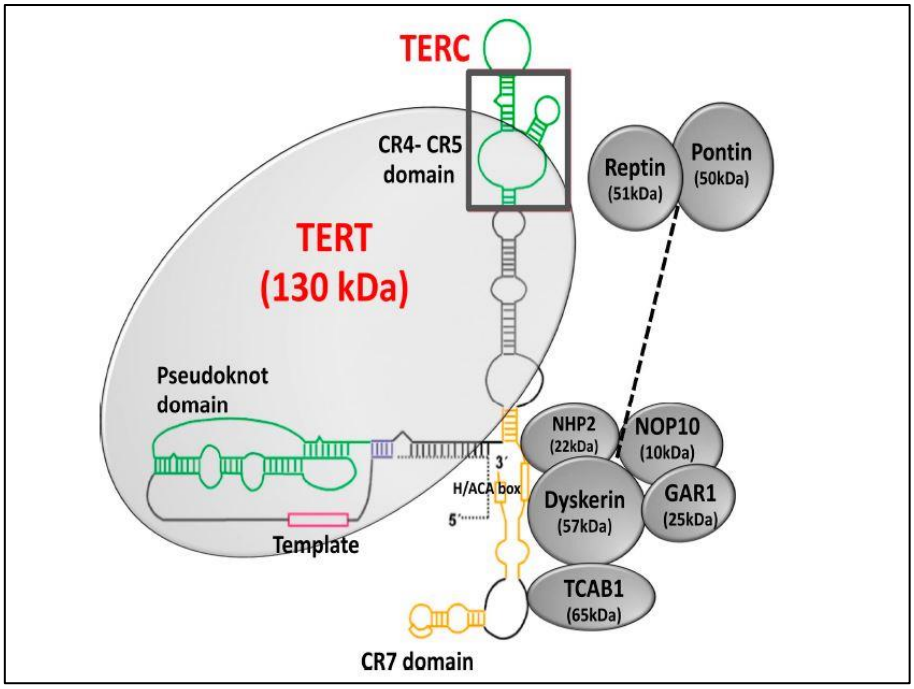

Figure 9. Schematic representation of human telomerase and its associated proteins. *Adapted from (Srinivas et al., 2020).*

Dyskerin, NHP2, NOP10 and GAR1 bind to the 3' end of *TERC* that contains a conserved H/ACA domain, because there are required for the stability and accumulation of human *TERC in vivo*. NOP10 and GAR1 bind to Dyskerin, and NHP2 binds to the RNA directly. (Fu and Collins, 2007, Egan and Collins, 2012).

TCAB1 regulates the subcellular location of telomerase complex assembly in the Cajal body (spherical nuclear bodies of 0.3–1.0 μm in diameter found in the nucleus of proliferative cells with a high protein and RNA content). TCAB1 stably associates with active telomerase enzyme (TERT, *TERC* and DKC1) and directs it through Cajal bodies to telomeres. In this manner, TCAB1 may act as a Cajal body-targeting or retention factor, may facilitate additional assembly steps of the enzyme in Cajal bodies, and/or may facilitate translocation of telomerase to telomeres (Venteicher et al., 2009). Pontin and Reptin are two closed ATPases necessary for the stability of Dyskerin and *TERC in vivo*. Dyskerin, Pontin and Reptin form a scaffold that recruits and stabilizes *TERC* and assembles the telomerase ribonucleoprotein particle. After this complex is formed, Pontin and Reptin are dissociate from the complex and release the catalytically active enzyme (Venteicher et al., 2008).

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. The first stage is the annealing, where the RNA template of the telomerase hybridizes with the 3' extreme of the telomeric DNA. The template sequence of 11 nucleotides is complementary to almost two telomeric repeats. The second phase is the extension, where the template is completed by synthesis, using triphosphate nucleotides in the catalytic site of the TERT enzyme. The third phase is translocation, which causes the enzyme telomerase to move in the direction of the 3' end, allowing for greater elongation. This cycle is repeated several times and the 5'-3' chain can be elongated. Finally, the DNA polymerase can synthesize the lagging strand by Okazaki fragments and the end of the chromosome is faithfully replicated (Figure 10) (Sfeir, 2012).

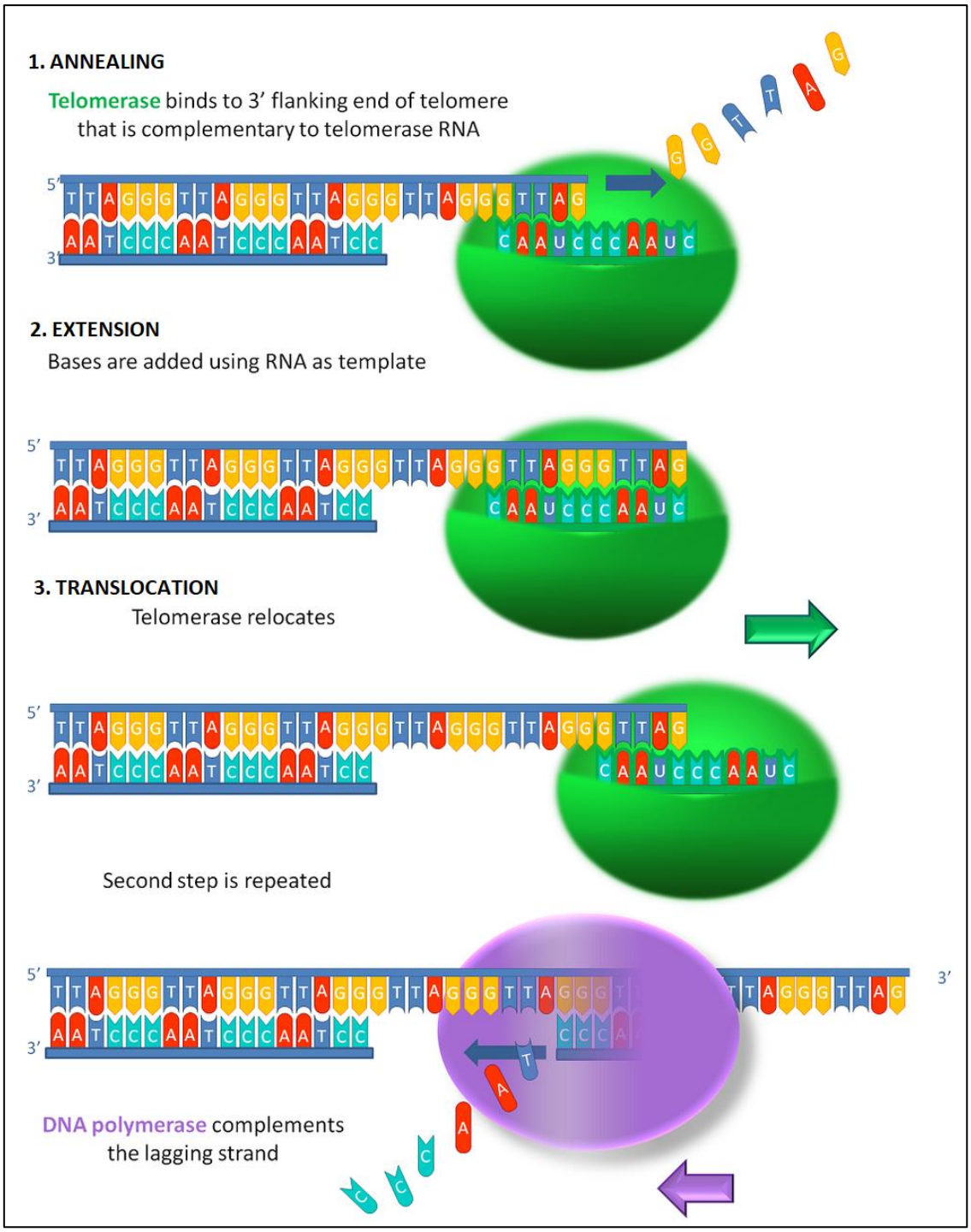

Figure 10. Telomerase-dependent telomere maintenance mechanism. Telomerase binds to 3'-flanking end of telomere that is complementary to *TERC*, and bases are added using *TERC* as template. Then, telomerase relocates, and telomere is extended in the 3'-direction. Finally, the DNA polymerase can synthesize the lagging strand. *Adapted from the website* [*http://wikipedia.org/*](http://wikipedia.org/).

2.2. The catalytic subunit

The catalytic subunit, TERT, is a major component of the telomerase, together with the RNA component. In humans, the *hTERT* gene is located in chromosomal 5p15.33 region and consists of 16 exons and 15 introns, spanning 35 Kb. Bioinformatics and mutational studies have collectively established that *TERT* contains three main structural elements: (i) a long N-terminal extension that contains conserved DNA and RNA-binding domains; (ii) a central catalytic reverse transcriptase (RT) domain; and (iii) a short C-terminal extension (Figure 11) (Hahn et al., 1999, Wyatt et al., 2010).

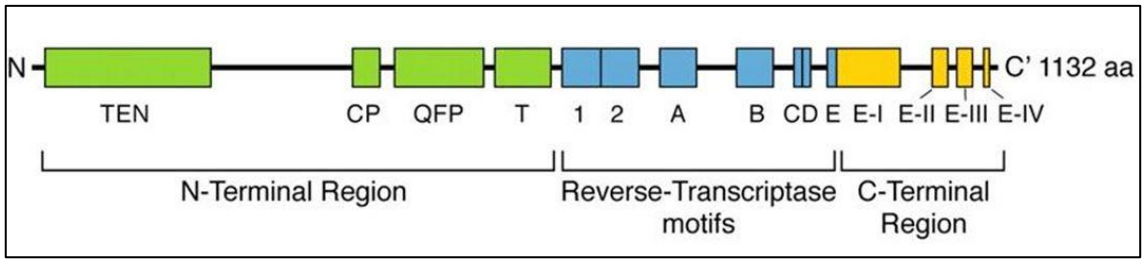

Figure 11. Schematic representation of the linear structure of *hTERT* gene. The N-terminal region comprises a telomerase-essential N-terminal domain (TEN), the CP, and the QFP domains, required for RNA interaction, and a telomerase-specific T motif. The central domain is the catalytic RT domain. The C-terminal region contains 4 conserved domains (E-I to E-IV). *Adapted from (Hahn et al., 1999)*.

Telomerase reactivation in tumorigenesis can be for multiple genetic and epigenetic mechanisms, depending on the tumor, such as increase of copy number of the *TERT* and *TERC* genes, somatic mutations in *TERT* promoter and transcripcional regulation of *TERT*. The increase of copy number is the most frequently alteration in cancer, especially in neuroblastomas, lung, cervical, breast and colorectal cancer (Cao et al., 2008). *TERT* promoter mutations are also relatively frequent in specific types of human cancers, such as central nervous system (43%), bladder (59%), thyroid (follicular cell-derived, 10%) and skin (melanoma, 29%) (Vinagre et al., 2013). These mutations are mainly at 124-146 bp from the ATG start site generate *de novo* binding sites for ETS/TCF (E-twenty-six/ternary complex) transcription factors (Horn et al., 2013). On the other hand, the transcripcional regulation of *TERT* is mainly through *TERT* hypermethylated oncological region (THOR). THOR is hypermethylated in malignant tumors and hypomethylated in normal tissues and stem cells. Hypermethylation prevents binding of transcriptional repressors and involves telomerase activation (Lee et al., 2019).

Telomerase activation is a crucial prerequisite for immortalization and plays an important role during the malignant progression of cancer cells by maintaining telomere length. However, telomerase complex is involved in several cell signaling pathways without apparent involvement of its well established function in telomere maintenance (reviewed by (Cong and Shay, 2008, Martínez and Blasco, 2011)). There are several studies to elucidate the extracurricular role of TERT in cancer, for example, a study on the transcriptional response of the entire genome to acute changes in TERT levels in the skin of mice, discover that TERT controls tissue progenitor cells via transcriptional regulation of a developmental program converging on the Myc and Wnt pathways (Choi et al., 2008). In addition, TERT contains a mitochondrial localization signal peptide at its N-terminal that targets TERT to mitochondria where it is active and has an important role in apoptosis (Santos et al., 2004) and Reactive Oxygen Species (ROS) production (Santos, Meyer et al. 2004). Actually, we know that telomerase is involved in cancer not only for telomere maintenance, but also regulation of gene expression, mitochondrial function, cell survival, cell transformation, and epithelial–mesenchymal transition (EMT), which may provide transformed cells with cancer-specific capacities at multiple stages of tumor development (Ding et al., 2013) (Figure 12).

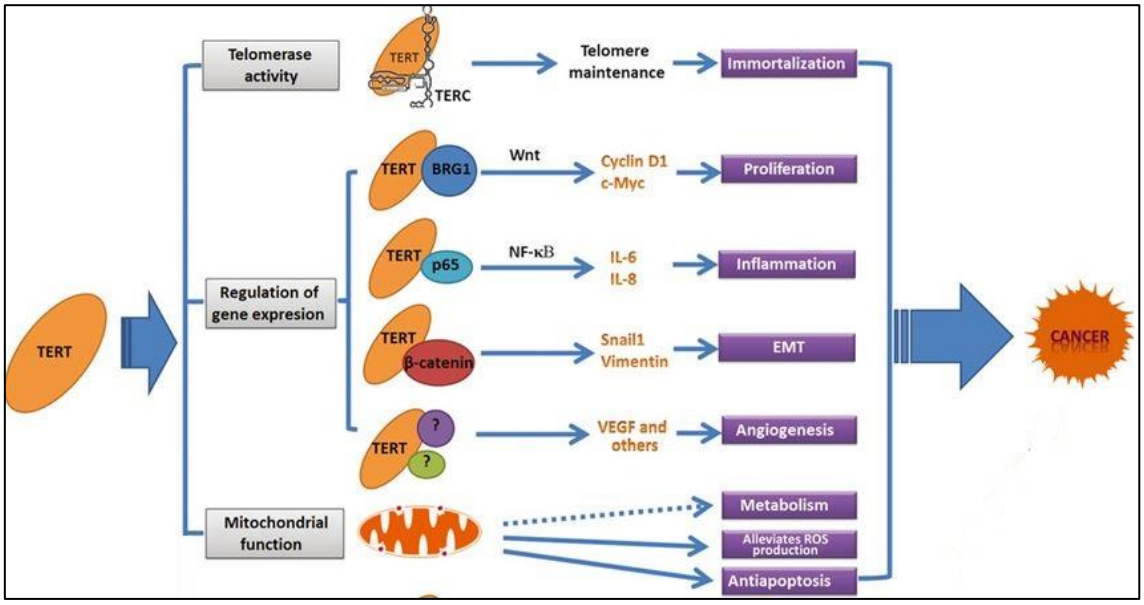

Figure 12. Canonical and non-canonical functions of hTERT in cancer progression. hTERT functions as the catalytic component of telomerase in maintaining telomere homeostasis and forms complexes with different factors involved in several cellular functions in a telomere-independent way, which provide transformed cells with cancer-specific capacities. *Adapted from (Ding et al., 2013).*

2.3. RNA component

The main components of telomerase are the catalytic subunit (TERT) and the RNA component (*TERC*). *TERC* has been isolated from ciliates and yeasts to mammals and is essential for telomerase activity (Blasco et al., 1996). The TERT subunit is highly conserved across species, but *TERC* has far more divergent sequence conservation. It is variable in size across phyla, ranging from over 2000 bases in Neurospora to just under 150 bases in killifish. However, there are some very well-preserved domains: a template region, a pseudoknot (PK) fold, a template boundary element (TBE) and a stem terminus element (STE) (Figure 13). The template is a single-stranded region that is used as a template during addition of telomere repeats to chromosome ends. The PK domain has an essential role in stabilize RNA folding, so mutations in this part giving rise to several pre-mature ageing syndromes. The TBE acts to stop run-through reverse transcription past the template. Finally, the STE is required for telomerase activity, but it role is not well understood, like the PK-fold (reviewed by (Musgrove et al., 2018)).

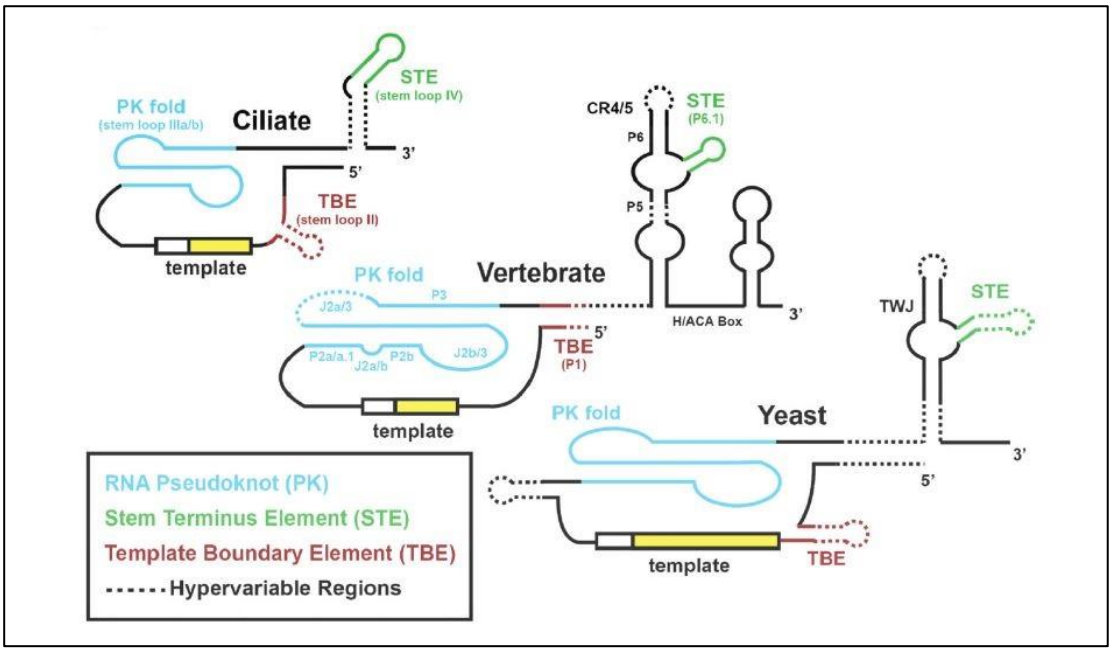

Figure 13. Telomerase RNA structure in ciliates, vertebrates and yeasts. Template boundary element (TBE) (red), template (yellow), RNA pseudoknot (PK) fold (blue), and stem terminus element (green) are indicated for each organism. A dashed line indicates regions that are hyper-variable between species within each category. *Adapted from (Musgrove et al., 2018).*

In humans, *TERC* has an extension of 451 nucleotides of length and contains a sequence of 11 bp (5-CUAACCCUAAC-3') that encodes for telomeric repeats. The secondary structure for human, and vertebrates in general, have four conserved structural domains: the pseudoknot/core domain, the conserved regions 4 and 5 (CR4/CR5), the box H/ACA and the CR7 domain (these last two domains can be grouped in ScaRNA domain) (Figure 14) (reviewed by (Zhang et al., 2011)).

The pseudoknot domain (nucleotide 33–191) consists of a large loop containing the template sequence, a P1 loop-closing helix and a P2/P3 regions (Figure 14), which interacts with human telomerase holoenzyme (Figure 15). The template is in the TERT active site. This domain is the only one essential for the *in vitro* reconstitution of catalytically active telomerase (Zhang et al., 2011, Nguyen et al., 2018).

The CR4/CR5 domain (nucleotides ∼243 to ∼326) consists of P5, P6 and P6.1 regions connected as a three-way junction (Figure 14). The P6.1 hairpin is the most conserved region, because is the only part interacting with TERT (Figure 15) and is critical for TERT association and telomerase catalytic activity (Zhang et al., 2011, Nguyen et al., 2018).

The ScaRNA domain (nucleotides ∼211 to ∼237 and ∼334 to 451) has a P7 and P8 regions (Figure 14). The H/ACA motif has a hairpin-hinge-hairpin-tail secondary structure, where the conserved H box is located at the hinge region and the ACA box is located at the 3’-end tail. H/ACA motif is not required for telomerase activity *in vitro*, but *in vivo* plays essential roles in biogenesis and regulation of telomerase holoenzyme, including localization, 3’-end processing and accumulation of *TERC*. These unique features of the domain are due to the fact that it is able to interact with telomerase proteins, such as Dyskerin, GAR1, NOP10, TCAB1 and NHP2 (Figure 15) (Nguyen et al., 2018, Zhang et al., 2011).

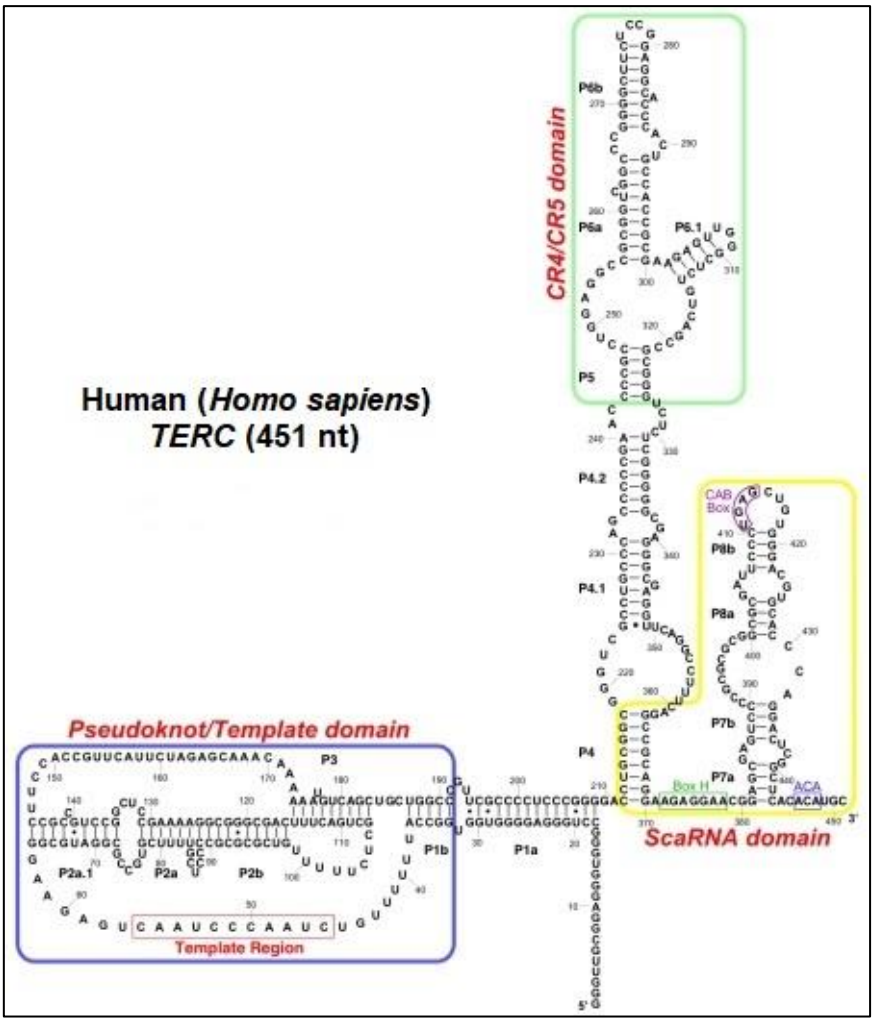

Figure 14. Secondary structure of human telomerase RNA. *TERC* has three conserved structural domains: the pseudoknot/core domain (blue), the conserved regions 4 and 5 (CR4/CR5) (green) and the ScaRNA domain (yellow). *Adapted from (Chen et al., 2000).*

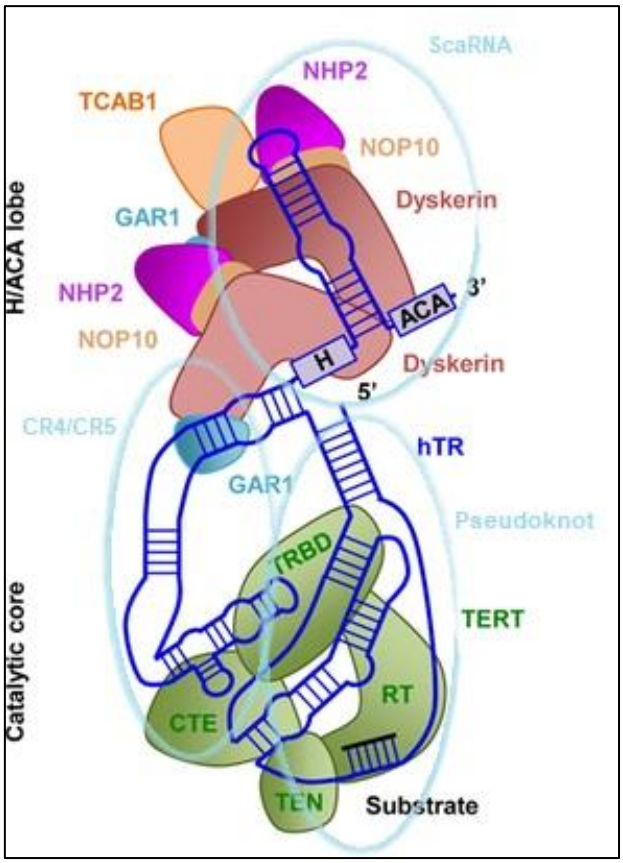

Figure 15. Cryo-EM structure of the substrate-bound human telomerase holoenzyme. Human *TERC* contains three major domains: the Pseudoknot/core domain, the CR4/CR5 domain and the ScaRNA domain. The Pseudoknot and CR4/CR5 domains bind TERT. The ScaRNA domain binds telomerase associated proteins, such as Dyskerin, GAR1, NOP10, NHP2 and TCAB1. TCAB1 binds both Dyskerin and the CAB box located at the CR7 region within the H/ACA ScaRNA domain. *Adapted from (Nguyen et al., 2018).*

The gene encoding human *TERC* is a single copy gene, which was located on chromosome 3, in region 3q26.3. The genetic regulation of telomerase RNA is not clear *in vivo*, because *TERC* is constitutively expressed in most somatic cells, even when TERT is repressed at the transcriptional level (Cairney and Keith, 2008), suggesting a non-canonical role also for *TERC*.

There is evidence to suggest that *TERC* may have a role in the development of cancer, beyond its role in telomeres (Fragnet et al., 2003, Cayuela et al., 2005, Li et al., 2005). Recent studies reveal that *TERC* also has a function in stimulating inflammatory response by interacting with the promoter of four NK-κB pathway genes through the formation of RNA-DNA triplexes, thereby enhancing their transcription (Liu et al., 2019). In addition, it has been demonstrated in our

laboratory that *terc* plays an extracurricular role in zebrafish myelopoiesis. *terc* deficiency induces neutropenia and monocytopenia, regardless of telomere length and telomerase activity. *terc* is required for myelopoiesis, but dispensable for the emergence of hematopoietic stem cells and does not compromise neutrophil functionality. This new role in myelopoiesis is due to the regulation of the expression of the myelopoietic cytokine colony stimulating factor 3 (granulocyte) b (*csf3b*, also known as *gcsf*) and by maintaining an appropriate balance between the major myeloid transcription factor Spi-1 proto-oncogene b (*spi1b*, also known as *pu.1*) and the erythroid transcription factor Gata binding protein 1a (*gata1a*) (Figure 16) (Alcaraz-Perez et al., 2014).

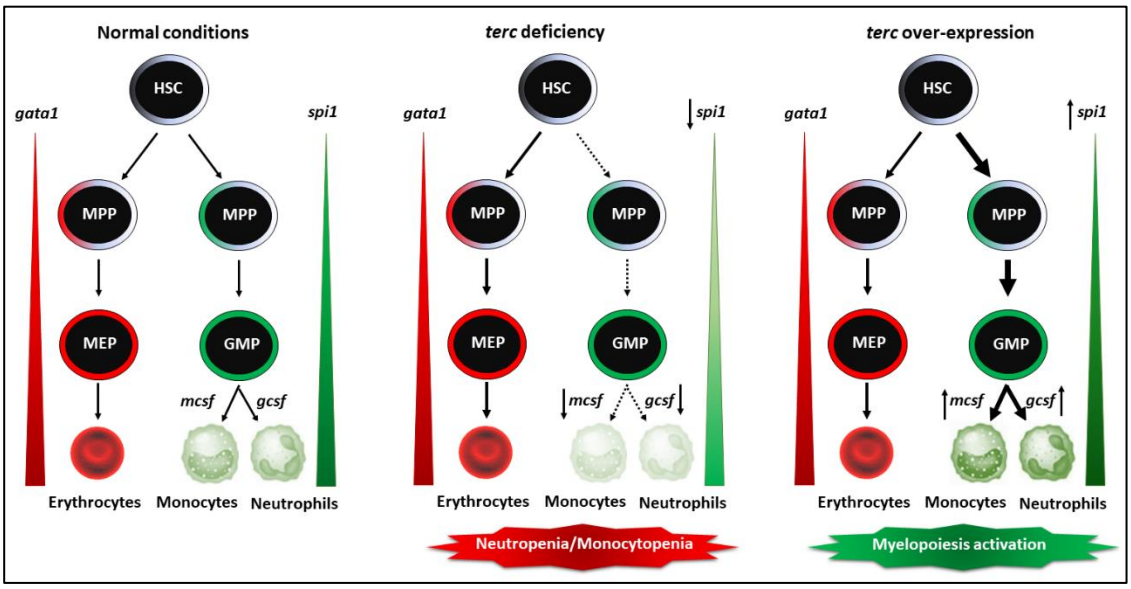

Figure 16. Schematic representation of the role of *terc* in zebrafish myelopoiesis. Under normal conditions there is a balance between *spi1* and *gata1*, the main myeloid and erytroid transcription factor, respectively. Therefore, a balanced number of myeloid and erythroid cells are produced. However, in *terc* deficiency, *spi1* expression decreased and neutrophil and macrophage numbers decrease, originating a neutropenia and/or monocytopenia. Consequently, in *terc* over-expression conditions, there is an increase in the expression of *spi1b*, *gcsf* and *mcsf*, resulting in myelopoiesis activation.

Although the mechanism involved in this activity of *TERC* is largely unknown, a study identified that *TERC* was able to bind 2198 sites across the human genome using chromatin isolation by RNA purification (ChIRP), which represents a large resource to study potential non-canonical functions of telomerase RNA. *TERC* could bind multiple genes and binding sites through a cytosine-rich motif (Figure 17) (Chu et al., 2011).

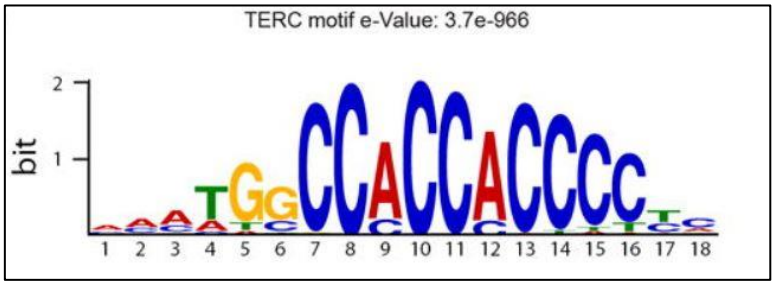

Figure 17. A cytosine-rich motif enriched among *TERC* binding sites (e-Value = 3.7e-966). *Adapted from (Chu et al., 2011).*

The fact that *TERC* is able to bind to a consensus binding sites present in whole genome can lead to hypothetical that *TERC* binds the genome near to myelopoietic genes and recruit proteins to regulate myelopoietic gene expression. Because one of the main lines of work in our laboratory is the extracurricular activity of *TERC*, we wanted to delve deeper into the regulatory mechanism of *TERC* in zebrafish and human myelopoiesis. For that, we found that *TERC* regulates the expression of *csf3b*, *spi1a* and *spi1b* genes in zebrafish, and their human counterparts *CSF2*, *CSF3* and *SPI1*. Telomerase RNA binds to DNA through its consensus binding sites and to RNA Polymerase II (RNA Pol II), recruiting it to the promoters of myeloid genes, assuring an efficient RNA Pol II occupancy around the TSS of these genes, their appropriate expression and, therefore, robust myelopoiesis (Figure 18) (García-Castillo, et al., unpublished).

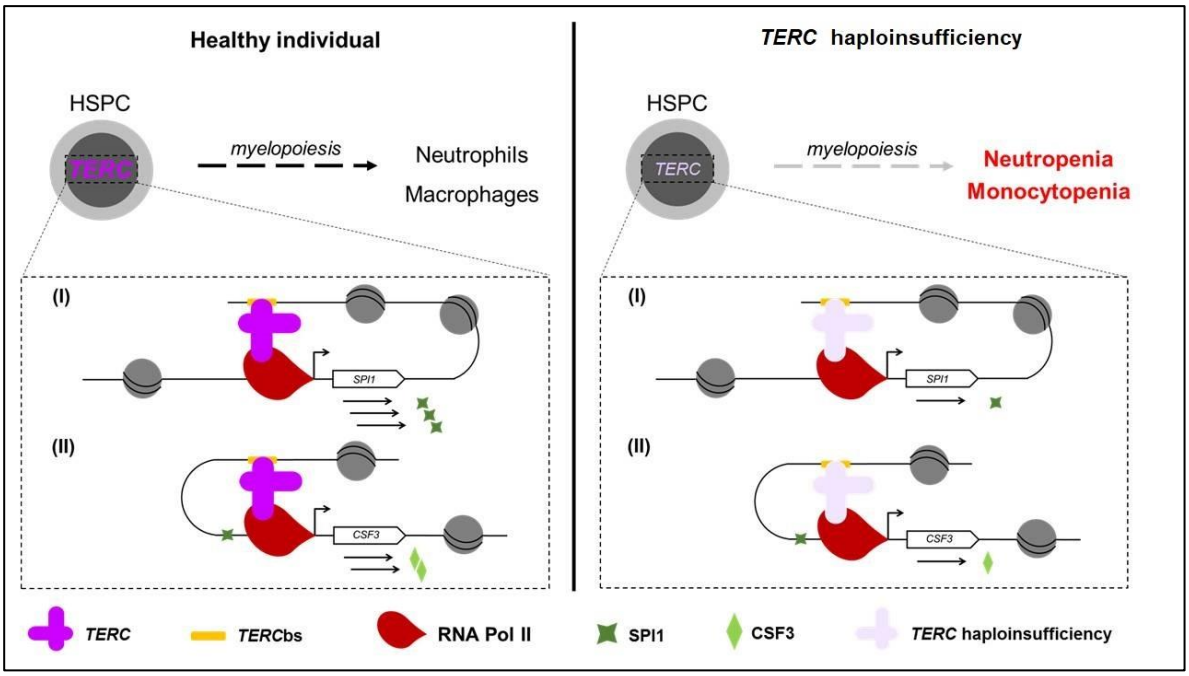

Figure 18. Model of telomerase RNA regulation of myeloid gene expression. In a healthy individual *TERC* binds to specific regulatory sequences, recruiting RNA Pol II and stimulating *SPI1* and *CSF3* gene expression to produce an appropriate number of neutrophils and macrophages. However, in *TERC* haploinsufficiency, *SPI1* and *CSF3* gene expression decreases, resulting in neutropenia and/or monocytopenia.

3. Telomere diseases

Telomeres shorten progressively throughout an individual's lifetime and this is directly implicated in cellular senescence and, therefore, is expected to play a fundamental role in ageing processes (reviewed by (Steffens et al., 2013)). The first demonstration that telomere shortening is at the origin of ageing pathologies and determines longevity came from the study of telomerase-deficient mice, which show premature ageing phenotypes in low proliferating tissues like heart or brain and high-proliferating tissues like bone marrow (BM), gut, skin, and testis (Blasco et al., 1997).

The diseases characterized by presenting with critically short telomeres are called as telomeropathies. The first telomeropathy detected was dyskeratosis congenita (DC). Patients have a rare, inherited multisystem disorder. 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 reticular skin pigmentation abnormalities, oral leukoplakia and dystrophic nails. The symptoms are accompanied by a spectrum of other diseases, like immune deficiency, pulmonary complications, hematologic disease and cancer. The 90% of patients with DC developed at least a single lineage cytopenia by the fourth decade of life. Bone marrow failure is the principal cause of premature mortality, followed by pulmonary disease and cancer (reviewed by (Dokal, 2011, Mason and Bessler, 2011).

Studies on families with *TERC* mutations showed that in these families there is a higher probability to present hematological abnormalities than pulmonary disease and a higher incidence of cancer than in patients with TERT mutations (Vulliamy et al., 2011). All the patients showed telomere shortening so the cause of this difference could be explain by the extracurricular role of *TERC* in myelopoiesis (Alcaraz-Perez et al., 2014) (García-Castillo et al., unpublished).

Others human telomeropathies are mainly aplastic anemia (AA, a bone marrow failure state characterized by low blood counts and an insufficiency of hematopoietic cells in the BM), myelodysplastic syndrome (MDS, another BM failure which bone marrow cells do not develop into mature blood cells), idiopathic pulmonary fibrosis (IPF, characterized by cough, dyspnea, impaired gas exchange and reduced lung volume), liver disease (LD, fibrosis with inflammation and nodular regenerative hyperplasia, a leading cause of noncirrhotic portal hypertension), Hoyeraal-Hreidarsson syndrome (HH, a severe variant of DC, with very short telomeres, which occurs in early childhood with cerebellar hypoplasia, microcephaly, immunodeficiency, BM failure and intrauterine growth retardation) and Revesz syndrome (RS, another severe variant of DC, similar to HH) (reviewed by ((Martinez and Blasco, 2017, Dokal, 2011).

Telomerase and shelterin protein mutations have been detected in all these diseases, usually in heterozygosis (TERT, *TERC*, TIN2 and Dyskerin), although there are also mutations in homozygosis (NOP10) or biallelic mutations (TCAB1 and NHP2) (Figure 19). Further work in this area will continue to provide new insights into the pathophysiology of degenerative diseases and human ageing (reviewed by (Dokal, 2011)).

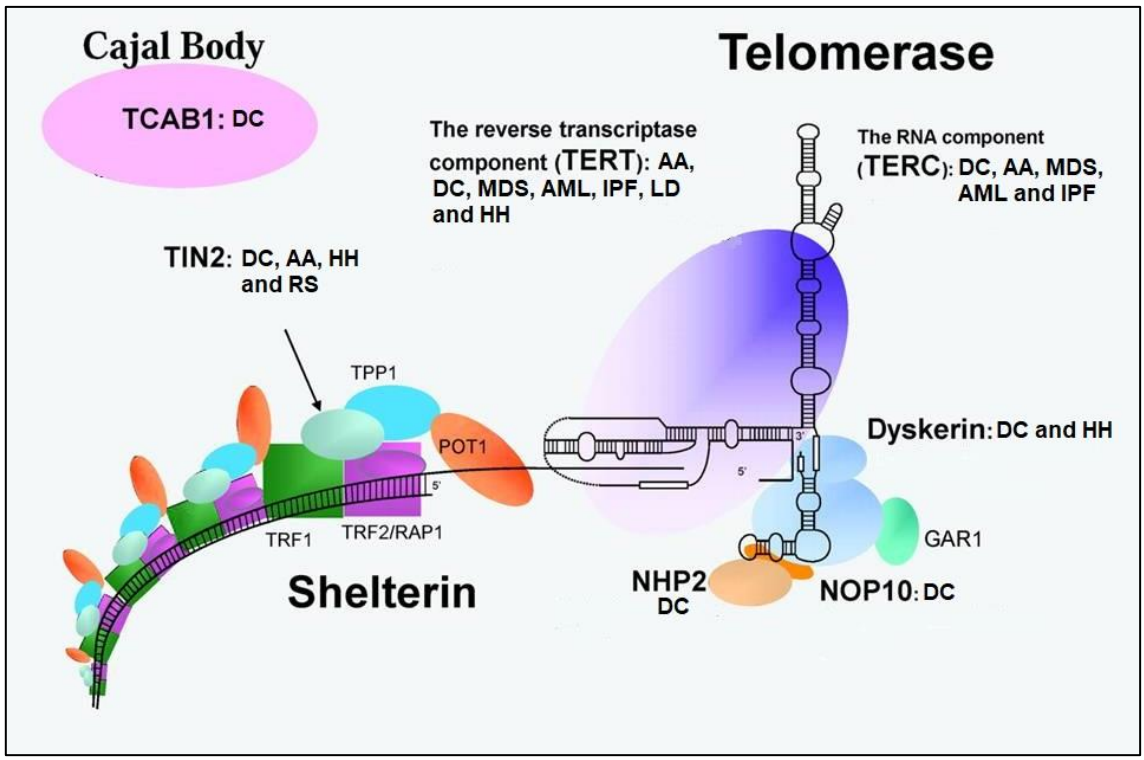

Figure 19. Schematic representation of the telomerase and shelterin complexes involved in diseases associated with telomere shortening. *Adapted from (Dokal, 2011).*

The current treatment for these telomeropathies is organ transplantation, such as BM, liver, and lung. However, this improves the physical conditions of the patient, but does not treat the cause of the symptoms: telomere shortening. Therefore, the best therapy is the increase of telomerase activity (reviewed by (Martinez and Blasco, 2017)). Among telomerase chemical activators, TA-65 is the most widely studied and has been shown to lead to elongation of short telomeres, rescue of associated DNA damage and improvement of some ageing-related parameters in mice (Bernardes de Jesus et al., 2011). Another well-studied telomerase activator is adeno-associated vectors (AAVs). AAV vectors provide a transient activation of telomerase in a non-integrative way and show a poor immunogenicity and an excellent safety profile, unable to replicate themselves. They had an increase in median lifespan and beneficial effects on health on mice (Bernardes de Jesus et al., 2012). Additionally, sex hormones capable of reactivate endogenous telomerase have been used for the treatment of telomeropathies, such as Danazol, to improve marrow failure syndromes and increase telomere length (Townsley et al., 2016, Guinobert et al., 2020).

In short, there are different treatments, some capable of increasing the expectancy life of patients, but they are not specific enough or effective enough for most of these diseases.

4. Aptamers

The field of biomedicine is being updated every day in order to discover new molecular therapies that can cover unresolved medical needs, such as telomeropathies. In recent years, many lines of research have focused on the design of new targeted molecular therapies. One of the therapeutic agents of great affinity and specificity that are being developed are aptamers.

Aptamers are synthetic single-stranded DNA or RNA sequences that adopt unique three-dimensional structures that allow them to recognize a specific target with high affinity. The term 'aptamer' was derived from the combination of the Latin word aptus ('to fit') and the Greek word meros ('part') (reviewed by (Zhuo et al., 2017)). Their potential uses include: disease diagnosis (Chen et al., 2017), new therapeutic agents, food hazard detection (Fadock and Manderville, 2017), biosensors (Miranda-Castro et al., 2016), toxin detection, drug carriers (Li et al., 2018), nanoparticle signaling, among others.

The concept of aptamers is similar to that of antibodies, which recognize a target in a very specific way, with high affinity. However, the use of aptamers has several advantages over the use of antibodies (Zhuo et al., 2017):

- They can be synthesized chemically *in vitro*, so the use of experimental animals is unnecessary, which lowers the cost.
- They can bind to non-immunogenic targets.
- They are smaller in size, allowing them to bind to inaccessible areas of the target.
- Their denaturation is reversible.
- They can be easily chemically modified.
- They are stable at room temperature.
- They are easily eliminated by the body through the kidneys, because of their low molecular weight, which in turn makes their toxicity low.

The aptamers selection is done by a process called as a SELEX (Systematic Evolution of Ligands by Exponential Enrichment). The starting point is an RNA or DNA library, where the aptamers with affinity to the ligand are selected and amplified by PCR for the next round. Many cycles are needed to select those with high affinity (Figure 20). This process was discovered in 1990, although it has undergone several improvements since then (review by (Mallikaratchy, 2017).

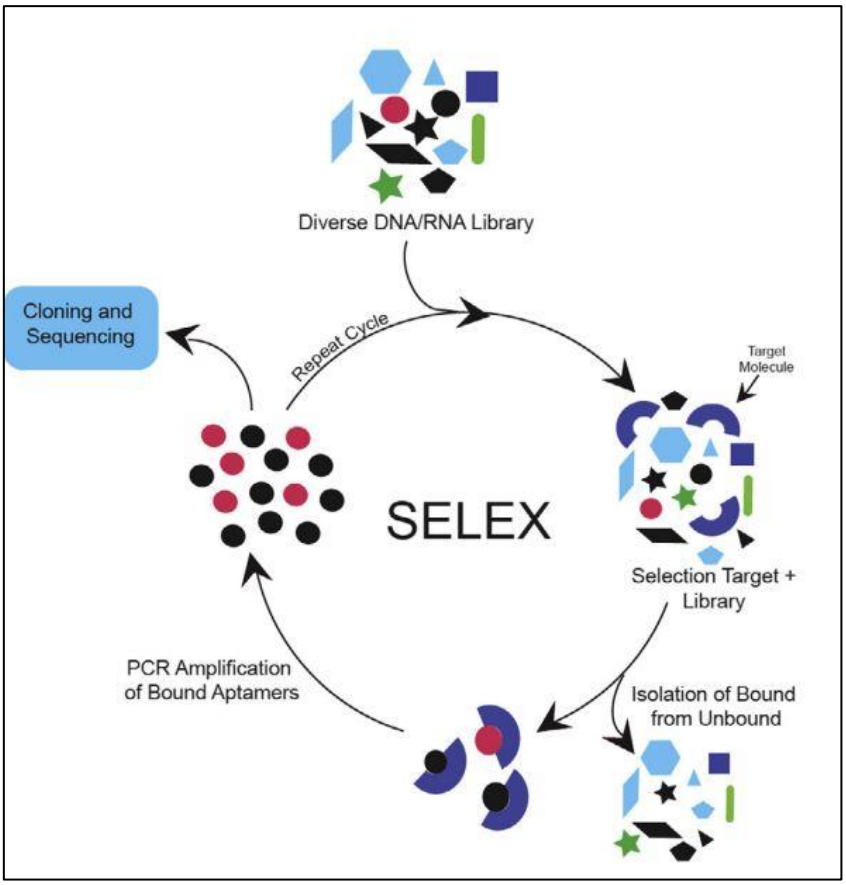

Figure 20. Schematic representation of the *in vitro* SELEX method. A DNA or RNA library is subjected to a target ligand and only the sequences that demonstrate affinity to the target are isolated. Then, bound sequences are collected and amplified by PCR for subsequent enrichment rounds. Multiple rounds are performed until a high affinity is found. *Adapted from (Wu and Kwon, 2016).*

A major advance was the discovery of automated SELEX methods, which are characterized by the ability to perform multiple SELEX cycles without supervision, intervention or manual handling, thus increasing efficiency. This design is based on a large scale of microfluids that are robotically controlled (reviewed by (Wu and Kwon, 2016).

Cell-SELEX is the biggest advantage of the SELEX methods so far, because the selection process can be performed without prior knowledge of the target protein, as it uses living cells with potential target proteins existing in them in native conformation (Figure 21). The result is aptamers capable of recognizing molecular targets in different cell types, for example, aptamers capable of differentiating cancer cells from normal cells (reviewed by (Zhong et al., 2020)).

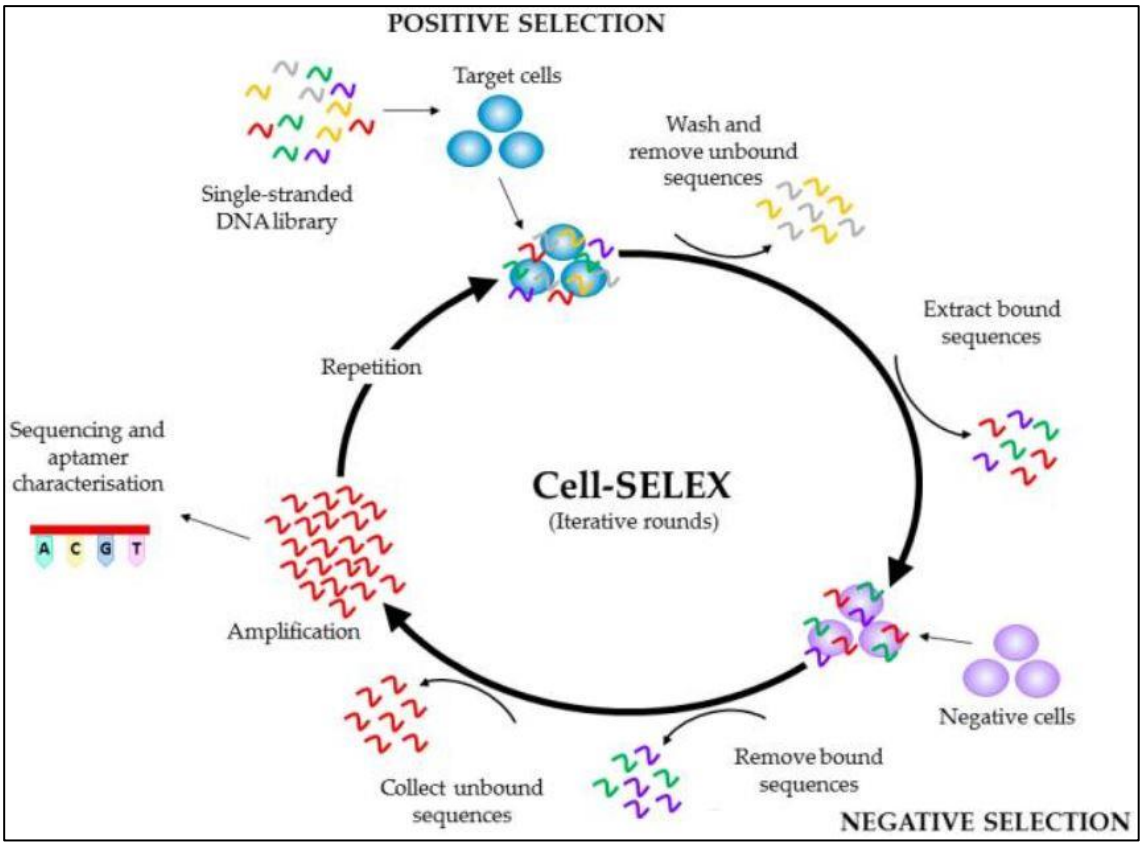

Figure 21. Schematic representation of Cell-SELEX methods. Initially, a DNA or RNA library is incubated with the target cells. Then, the cells are washed to remove unbound sequences, and the bound sequences are collected to be incubated with the control cells. Sequences attached to control cells are discarded, while the unbound sequences are amplified and used to start the next round of SELEX. *Adapted from (Hays et al., 2017).*

Normally the aptamers are modified to increase their *in vivo* half-life, because without any modification they have pharmacokinetic problems including metabolic instability and rapid renal filtration without unspecific protein binding (reviewed by (Kovacevic et al., 2018)). There are different types of chemical modifications, each with its advantages and disadvantages. Usually in the aptamers being developed, several of the following modifications are combined (reviewed by (Ni et al., 2017, Zhu and Chen, 2018, Kovacevic et al., 2018)):

- Modifications on the sugar ring: generally involve replacing the 2’ position with an O-methyl (OCH_3), fluoro (F) or amino (NH_2) groups (Figure 22). These modifications increase the half-life of serum aptamers, although NH_2 is not usually used because it is more difficult to synthesize chemically. The most commonly used is 2'-O-Methyl, because it is found naturally in the RNA,

increases Tm of RNA, increasing RNA:RNA or RNA:DNA stability, and reduces nucleases activity. In addition, these modifications do not decrease the binding affinity to the target.

- Modifications on the phosphodiester linkage: replaced phosphodiester linkage with phosphorothioate (Figure 22) or methylphosphonate analog is commonly used for aptamer modification. The modification of internucleotide linkage using phosphorothioate bond substitutes are substantially more stable towards hydrolysis by cellular degradation, because inhibit RNase A, RNase T1 and calf serum nucleases. Phosphorothioate bonds can be introduced between the last 3-5 nucleotides at the 5'- or 3'-end to inhibit exonuclease degradation or throughout the oligo to reduce endonucleases attack as well. However, modification of the whole structure may have less affinity with the target.

- Modifications on the 3´ end: 3'-biotin can resist the activity of 3'-exonuclease and the 3'-biotin-streptavidin conjugates slowed down the clearance rate of aptamers in blood circulation system *in vivo*. However, it is used more for purification applications. Another modification for aptamers therapy in 3´end is the capping with inverted thymidine, which could increase the stability and resistance of aptamers to 3'-exonuclease in human serum.

- Modifications on the bases: Locked nucleic acid (LNA) is an analog of ribonucleotide with a methylene linkage between 2'-O and 4'-C of the sugar ring (Figure 22). This modification shows longer lifetime *in vivo*, because improved plasma stability and maintained binding affinity, but the kidney and liver clearance is slower.

- PEGylation: is a method used to prolong aptamer lifetime. Normally, aptamers are pegylated with 40 kDa PEG unit (Figure 22), because it has a considerable half-live increase of up to 1 day in rodents and 10 days in human. The FDA has approved multiple PEG-modified biomedicines, but it may have adverse immune responses.

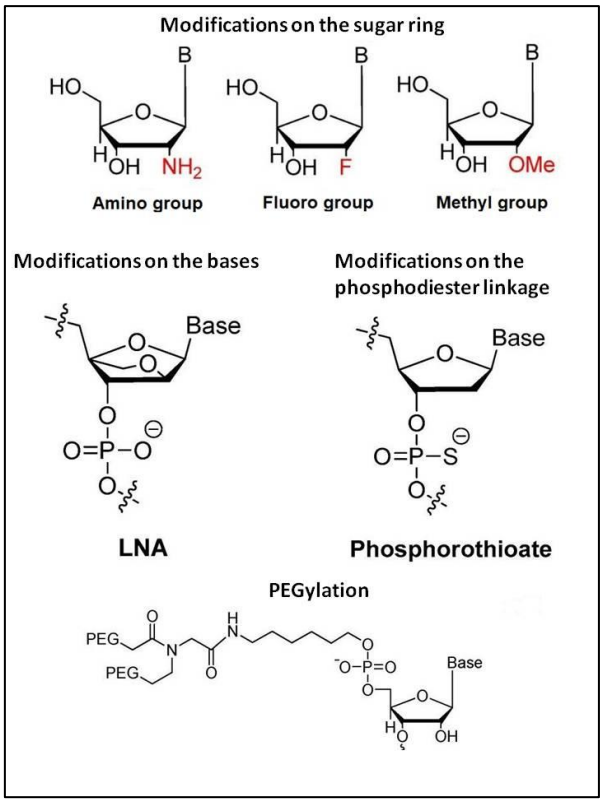

Figure 22. Common strategies in the chemical modifications of nucleic acid aptamers. *Adapted from (Ni et al., 2017).*

Although there are many aptamers in clinical stages of development, at this moment Pegaptanib (marketed as Macugen or Pfizer) is the only aptamer approved by the Food and Drug Administration for commercial use in 2014. This is a 27 nt RNA (2'-fluoro pyrimidines, 2'-O-methyl purines, 3'-inverted dT, 40kDa PEGylated) aptamer that targets the vascular endothelial growth factor (VEGF)-165, a VEGF isoform responsible for macular degeneration (Ng et al., 2006). For the treatment of macular degeneration there are two other aptamers, Zimura and Fovista, in clinical trial phase III, with similar modifications. In the field of coagulation, there are also various RNA and DNA aptamers (ARC1779, NU172 and ARC19499) in phase I and II of development. For cancer treatment, exist AS1411 (26-nt DNA PEGlyated) against nucleolin for acute myeloid leukemia (AML), metastatic renal carcinoma and advanced solid tumor treatment; and NOX-A12 (45-nt RNA PEGylated) against Chemokine ligand 12 for some myelomas and lymphomas treatment; both in clinical phase II. Finally, for chronic inflammatory diseases, there are two RNA PEGylated aptamers (NOX-E36 and NOX-H94), both in phase II (reviewed by (Zhou and Rossi, 2017)).

On the other hand, some aptamers have been developed in the field of telomerase, capable of sensing telomerase activity in cancer cell lines (Guo et al., 2020, Zhu et al., 2018, Zhou et al., 2019). The aim of these aptamers is to serve as biosensors that detect high telomerase activity, so that they can be used as tumor biomarkers. In addition, there is another aptamer designed against the RID2 domain of telomerase, which inhibits telomerase activity in a human cell line, so that it could be used in cancer treatment (Varshney et al., 2017).

5. The zebrafish as a vertebrate model

Zebrafish (*Danio rerio*) is one of the most widely used vertebrate animal models in research. It is a small, tropical freshwater teleost fish belonging to the Cyprinidae family, order Cypriniformes. Since it was first used in a scientific laboratory 40 years ago, its popularity in biomedical research has significantly increased due to their unquestionable advantages respect other vertebrate models, which include: (1) Extensive knowledge of the zebrafish genome and large genetic similarity to humans (Howe et al., 2013, Kettleborough et al., 2013). (2) High fecundity and large production of embryos (around 100 eggs/female/week) makes possible a statistically consistent study with a large number of individuals (Patton and Zon, 2001). (3) Low maintenance cost and small space needed. (4) A variety of genetic manipulation methods: transient gene knock down by using morpholinos (chemically-modified antisense oligonucleotides that transiently knockdown the gene expression) (Ekker, 2004), transient protein or RNA overexpression by injecting plasmids or RNA directly as well as chemical mutagenesis (Rohner et al., 2011) and generation of stable transgenic lines and targeted or generation of knock-ins by using CRISPR/Cas9 technology to single base-pair resolution (Li et al., 2016, Meng et al., 2008, Albadri et al., 2017, Ward et al., 2018). (5) High throughput small molecule screens, thanks to the small size of the larvae, which allows screening of a complete individual in 96-well plates as if they were cells (Murphey and Zon, 2006, Yoganantharjah and Gibert, 2017). (6) Embryos are transparent and their develop after fertilization is external and fast, only 36 hours are needed to full organ development (Figure 23). The ease access and transparency of the embryo are ideal for fluorescence live imageing, what allows an unprecedented resolution in a vertebrate model system even in a cellular level (Detrich, 2008).

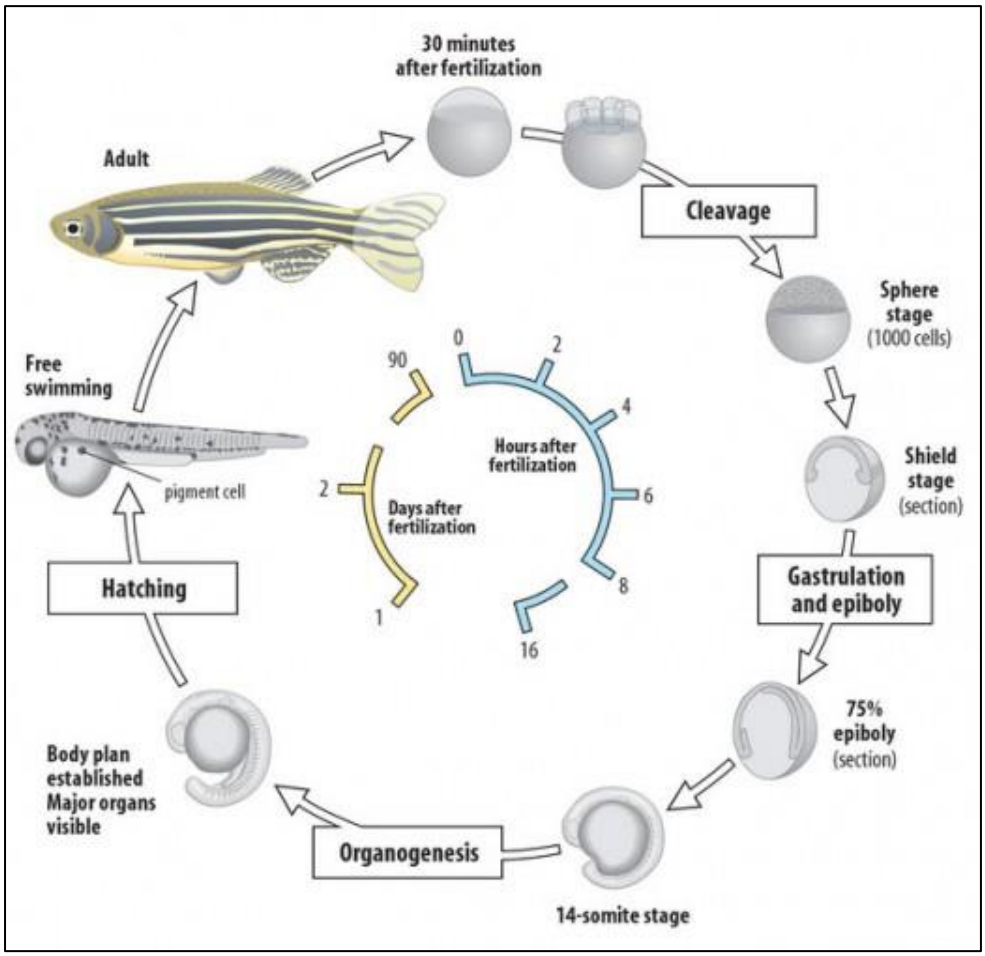

Figure 23. Schematic representation of zebrafish development. The development is from one-cell stage to adulthood, highlighting that up to 2 days post-fertilization (dpf) there is no pigmentation and the embryos are transparent. *Adapted from the website www.daniorerio.com*

All these advantages had led to the increased interest of scientists to use zebrafish as an animal model for biomedicine (Figure 24). Zebrafish has been proposed as an excellent vertebrate model for the study of vascular development (reviewed by (Hogan and Schulte-Merker, 2017), neurogenesis (reviewed by (Schmidt et al., 2013)), immune system (reviewed by (Renshaw and Trede, 2012, Garcia-Moreno et al., 2019)), regeneration ((Uribe et al., 2018, Cahill et al., 2017)), hematopoiesis (reviewed by (Gore et al., 2018)) and cancer research ((Wojciechowska et al., 2016, Idilli et al., 2017), among others. Recently, it has been discovered that zebrafish can also be a good model for behavioural studies, as Seebacher laboratory, among others, demonstrates in his sprint exercise training zebrafish model (Simmonds et al., 2019).

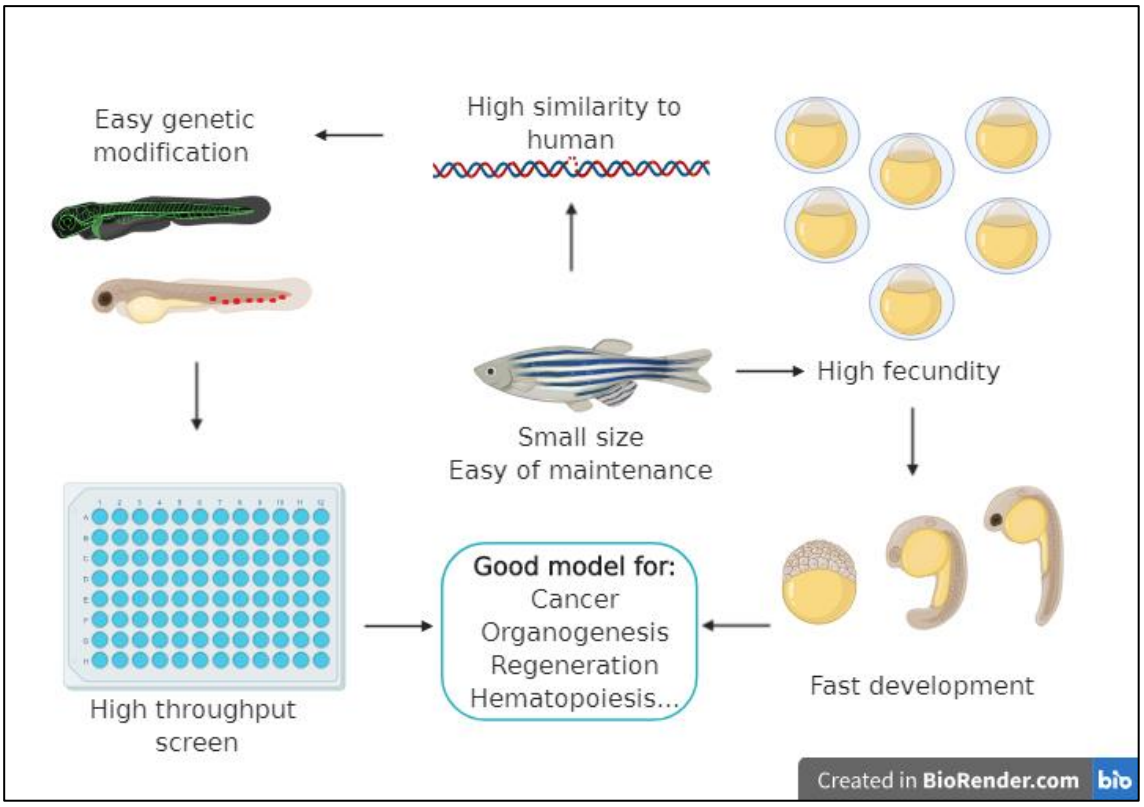

Figure 24. Zebrafish advantages respect other vertebrate models. *Created with BioRender*.

5.1. Telomeres and telomerase in zebrafish

Several studies show the value of zebrafish as a model in ageing, cancer and regeneration. All these processes have a clear implication of telomerase. Therefore, it is interesting to study whether the telomeric maintenance in zebrafish and humans is similar.

The zebrafish telomeric length is about 15-20 kb, similar to human length, which is 8-15 kb. However mouse telomere length is about 5 times more, 40–50 kb, which is the classical model for these studies (Varela et al., 2016, Anchelin et al., 2013). Regarding the main components of telomerase, it has been seen that there is a great similarity with humans. Zebrafish Tert secondary structure has more than 50% similarity to human TERT (Figure 25). In relation to the zebrafish telomerase RNA component, the bioinformatic identification showed a high conservation of the structure and function with the human *TERC* (Figure 26) (Xie et al., 2008), supporting the use of zebrafish as a model organism for the study of telomerase biology.

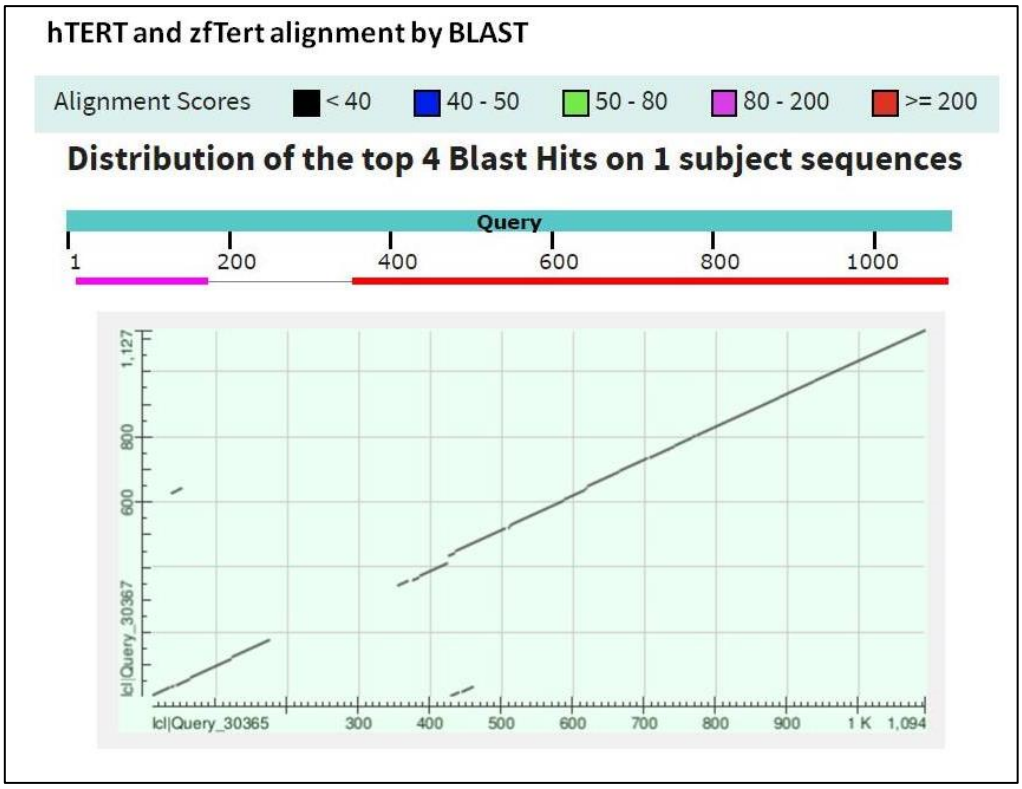

Figure 25. Bioinformatic analysis by BLAST between zebrafish Tert and human TERT. The alignment score is high at the amino terminal, then there is a gap, because human TERT is longer than zebrafish Tert, and the rest of the protein has a very high alignment score. *Adapted from https://blast.ncbi.nlm.nih.gov/Blast.cgi*

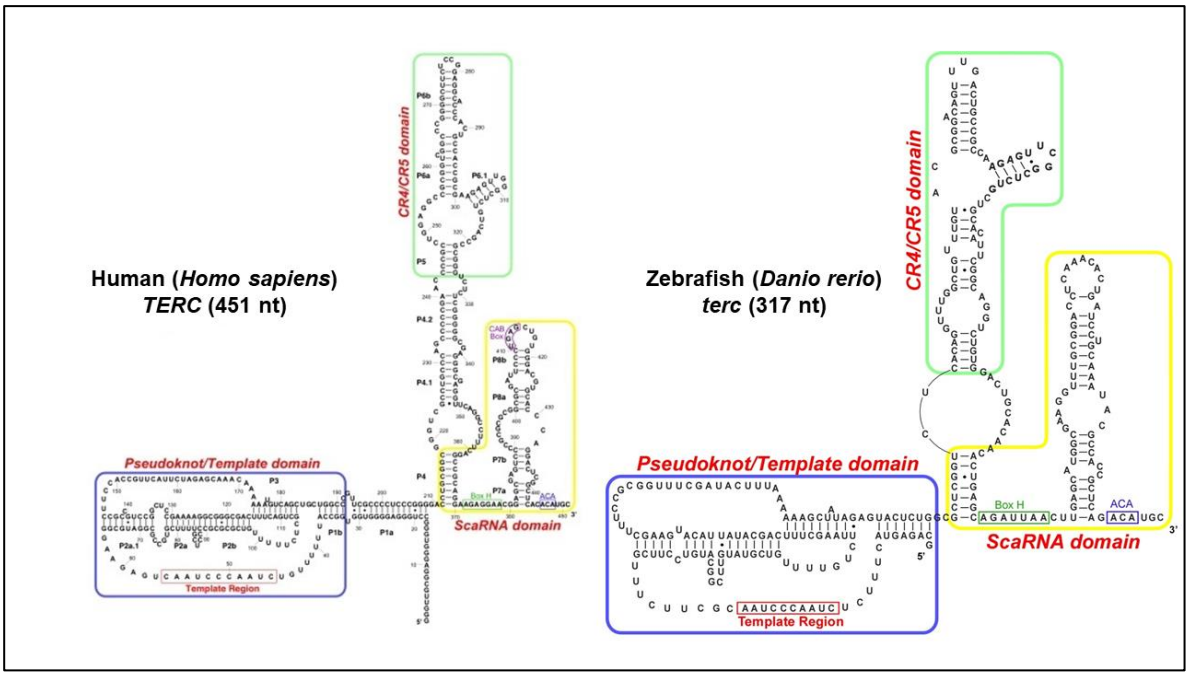

Figure 26. Secondary structure of human and zebrafish telomerase RNA component. Three structural domains (pseudoknot/template, CR4/CR5 and ScaRNA) are outlined and labeled. *Adapted from (Xie et al., 2008, Chen et al., 2000).*

In 2011, Dr. Cayuela's laboratory established the behaviour of telomere and telomerase during ageing and regeneration in zebrafish, concluding that the telomerase expression and telomere length are closely related throughout the fish's life cycle and that these two parameters can be used as biomarkers of ageing in zebrafish (Anchelin et al., 2011). In addition, it was found that telomerase is necessary for the zebrafish life, since a zebrafish with telomerase deficiency shows several premature ageing symptoms from the first generation, like premature infertility, gastrointestinal atrophy, increased inflammation, spinal curvature, sarcopaenia, loss of body mass and liver and retina degeneration. Importantly, the Tert mutant zebrafish line also reproduces the genetic anticipation phenomenon observed in humans with dyskeratosis congenita (Anchelin et al., 2013).

There are a few models of zebrafish DC (Pereboom et al., 2011, Zhang et al., 2012) and they all have in common hematopoietic defects, but there is no change in telomere length or telomerase activity in the early stages of DC pathogenesis, suggesting that the telomere maintenance do not contribute to DC until later in life. The phenotypic effect of these mutations could be explained by the extracurricular role of *TERC* in myelopoiesis (Alcaraz-Perez et al., 2014) (García-Castillo et al., unpublished) and other unidentified roles.

5.2. Hematopoiesis in the zebrafish embryo

Hematopoiesis is the formation of blood cellular components derived from hematopoietic stem cells (HSCs). This process occurs during embryonic development and throughout adulthood to produce and replenish the blood system. This is a vital process for invertebrate and vertebrate organisms. Like all vertebrates, zebrafish also experience different waves of hematopoiesis during embryogenesis: the primitive and definitive waves (reviewed by (Galloway and Zon, 2003, Jagannathan-Bogdan and Zon, 2013)).

The primary purpose of the primitive wave is to produce red blood cells and primitive myeloid cells that can facilitate tissue oxygenation as the embryo undergoes rapid growth. This wave is rapidly replaced by a "definitive" hematopoiesis at 26 hpf in zebrafish and involves HSCs, multipotent cells which can give rise to all blood lineages of the adult organism. The anatomical sites of hematopoiesis are different in zebrafish compared to mammals, although the molecular mechanisms behind hematopoiesis are highly conserved (reviewed by (Wattrus and Zon, 2018)).

Zebrafish primitive hematopoiesis occurs in two intraembryonic locations: the intermediate cell mass (ICM) blood islands and the anterior lateral mesoderm (ALM) (Figure 27). Primitive erythropoiesis is in the ICM, whereas myelopoiesis initiates in the ALM. It is analogous to the yolk sac mesodermal cells about 19 dpf in mammals and is responsible for generating most embryonic hematopoietic cells. At 24 h post-fertilization, circulation starts, and hematopoiesis shifts to the posterior blood island (PBI) for a brief period. The PBI serves as the functional equivalent to mammalian yolk sac (reviewed by (Perlin et al., 2017, Galloway and Zon, 2003)).

The definitive wave begins in the aorta-gonadmesonephros (AGM) at 36 hpf, giving rise to HSCs. There are three different HSC migration and colonization events that begin 2 days post-fertilization: AGM progenitor cells migrate to the caudal hematopoietic tissue (CHT), which is an intermediate site of blood development; the thymus, which is a site of lymphocyte maturation; and the developing kidney marrow, which is the larval and adult location for production of all hematopoietic cell types, comparable to mammalian bone marrow (Figure 27). In humans, the definitive wave continues in the fetal liver and placenta (about 35 dpf) before sowing the spleen, thymus and bone marrow (reviewed by (McGrath and Palis, 2005, Jagannathan-Bogdan and Zon, 2013)).

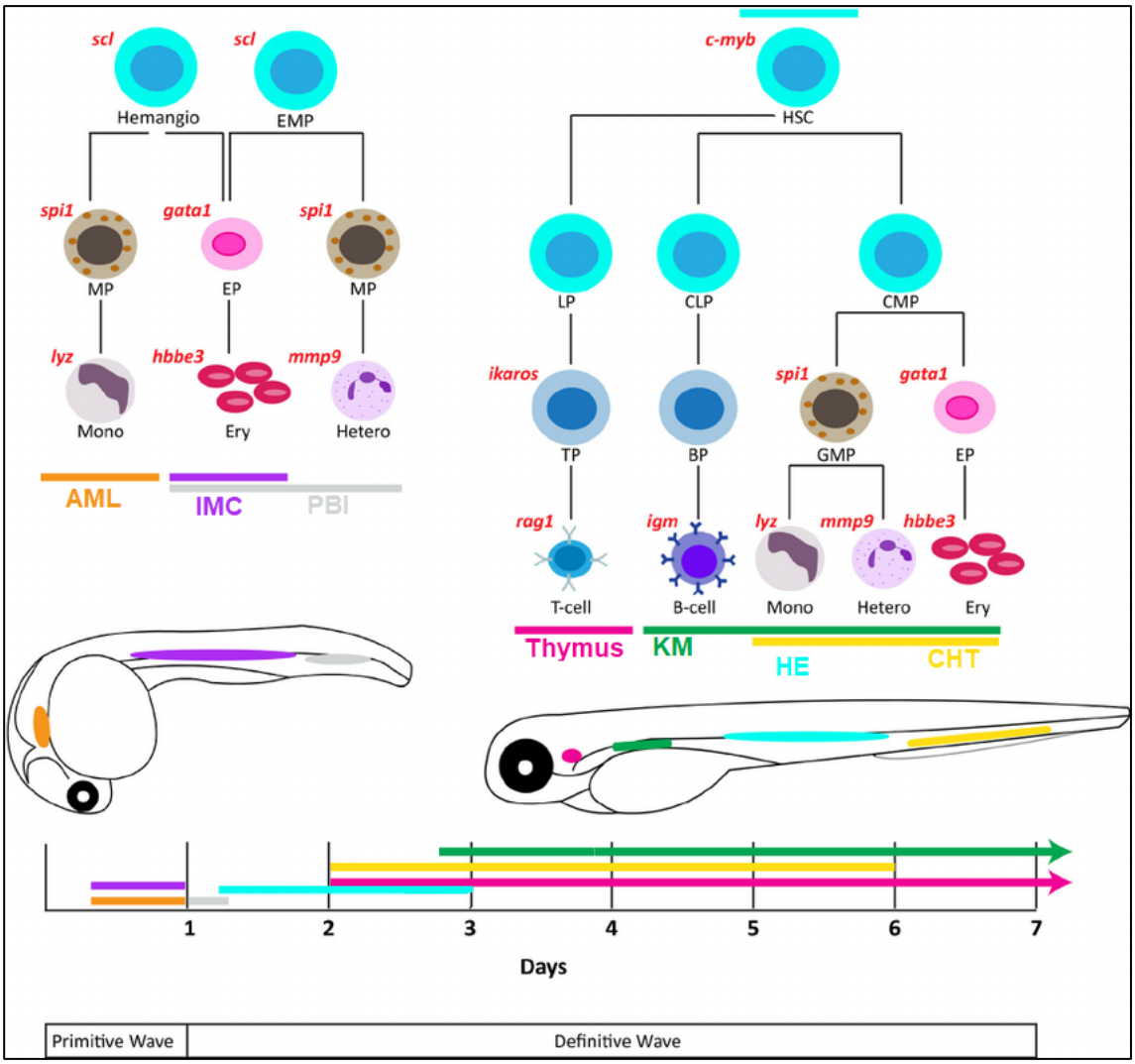

Figure 27. A schematic overview of zebrafish hematopoiesis. The primitive wave starts in two locations, the anterior lateral mesoderm (ALM) (orange), which generates primitive monocytes, and the intermediate cellular mass (ICM) (violet), which gives rise to mostly primitive erythrocytes before 24 hpf. Then, a transient wave occurs in the posterior blood island (PBI) where both neutrophils and erythrocytes are formed (grey). Definitive hematopoietic stem cells (HSCs) are initially formed by the sprouting of the hemogenic endothelium (HE) on the ventral wall of dorsal aorta (blue). At 48 hpf, some of these HSCs migrate to the caudal hematopoietic tissue (CHT) (yellow) to produce several cell lineages, and also to the thymus (purple), where T lymphocyte production occurs. Hours later, HSCs seed the developing kidney (green), the final site of definitive hematopoiesis where erythroid, B lymphocyte and myeloid production happen. The lineage-specific transcription factors that regulate this process are in red. Abbreviations: Hemangio: hemangioblast, GMP: granulocyte-monocyte progenitor, HSC: hematopoietic stem cell, CLP: common lymphoid progenitor, CMP: common myeloid progenitor, BP: B cell progenitor, EP: erythroid progenitor, TP: T

cell progenitor, Ery: erythrocyte, Hetero: heterophil, Mono: monocyte. *Adapted from (Rasighaemi et al., 2015).*

Zebrafish hematopoiesis has been much studied over the years, because the advantages of the zebrafish make it an ideal model for this. The transparency and external fertilization of the embryos make it possible to visualize by fluorescence the whole process (Table 1). They have a rapid initiation of hematopoiesis and conserved hematopoietic system with human/mammalian systems. Additionally, the oxygen diffusion through the skin, so they can survive without blood for several days allows to study the effects of loss of function of genes that cause embryonic lethality in mice due to hematopoietic defects. Finally, zebrafish can be used to perform transplants of kidney marrow that can be used in screening to find molecules capable of enhancing HSC development and engraftment and hematopoiesis. These properties show that the zebrafish is a versatile model for the study of hematopoiesis (Martin et al., 2011).

Transgenic line	Labeled cell type
Tg(cmyb:EGFP)	HSPCs, myeloid
Tg(−6.0itga2b:EGFP) Tg(cd41:EGFP)	HSPCs, thrombocytes
Tg(runx1:EGFP)	HSPCs, lateral plate mesoderm
Tg(runx1+23:EGFP)	HSPCs
TgBAC(gata2b:KalTA4)^{sd32} Tg(gata2b:GFP)	HSPCs
Tg(scl-α:d2EGFP)	HSPCs, intermediate cell mass
Tg(scl-β:d2EGFP)	HSPCs, intermediate cell mass
Tg(lck:GFP)	T cells, lymphocytes
TgBAC(ikaros:EGFP)	T Cells, lymphocytes
Tg(rag1:GFP)	T Cells
Tg(rag2:GFP)	T Cells
Tg(mhc2dab:GFP)^{sd6}	B Cells, dendritic cells
Tg(mpx:GFP)	Neutrophils
Tg(lyz:EGFP)	Neutrophils
Tg(mpeg1:EGFP)	Macrophages
Tg(gata1:GFP)	Erythrocytes
Tg(−20.7gata2:EGFP)^{la3} Tg(gata2a:GFP)	Erythrocytes

Table 1. Transgenic lines available to study hematopoiesis in zebrafish. Transgenic reporter lines labeling specific HSPC-derived cell lineages, amplifying the advantages of the zebrafish for *in vivo* imageing and lineage tracing. *Adapted from (Gore et al., 2018).*

OBJECTIVES

The specific objectives of the present work are:

1. Molecular characterization of the *TERC* domains responsible of its non-canonical hematopoietic function.
2. Development of aptamers derived from *TERC* to treat blood diseases.
3. Identification of *TERC* interactome.

CHAPTER I.
Molecular characterization of the *TERC* domains responsible of its non-canonical hematopoietic function.

Abstract

Telomerase is the enzyme responsible for the maintenance of telomeres and is composed mainly of a ribonucleoprotein component (TERT) and an RNA component (*TERC*). A growing number of studies attribute extracurricular functions to each of these components, beyond their role in telomeres. Previous studies from our laboratory have shown that *TERC* acts as a transcriptional factor in human and zebrafish models, capable of physically interact with a consensus DNA binding sequence found in promoters or enhancers of genes involved in myelopoiesis and with RNA polymerase II, both required for the initiation of transcription of these genes. Thus, a downregulation of *terc* in zebrafish results in decreased myelopoiesis, characterized by neutropenia and monocytopenia, while *terc* overexpression activates myelopoiesis and promotes neutrophilia. Furthermore, in this work, we have demonstrated the importance of CR4/CR5 *TERC* domain in myelopoiesis, since mutations in this domain decrease RNA polymerase II interaction and impaired myelopoiesis. Strikingly, induced pluripotent stem (iPS) cells derived from a DC patient with a CR4/CR5 mutation show reduced myelopoiesis, while those from a patient with a mutation in TERT differentiate as wild-type cells. These results will help to predict the predisposition to myelodysplastic syndrome of patients with CR4/CR5 *TERC* mutations for their appropriate stratification and personalized treatments.

1. Introduction

Telomerase is a ribonucleoprotein capable of synthesizing telomeric repeats at the end of eukaryotic chromosomes. It is composed of a catalytic subunit (telomerase reverse transcriptase, TERT) and an RNA component (*TERC*), in addition to several regulatory proteins (Hodes et al., 2002). *TERC* has three structural domains: the pseudoknot/core domain, the conserved regions 4 and 5 (CR4/CR5) and the ScaRNA domain. The pseudoknot domain contains the template sequence and interacts with telomerase holoenzyme, while the CR4/CR5 domain consists of P5, P6 and P6.1 regions connected as a three-way junction, being the P6.1 hairpin the most conserved region, because is the only one interacting with TERT. The ScaRNA domain is able to interact with telomerase-associated proteins, such as Dyskerin, GAR1, NOP10, TCAB1 and NHP2, being essential in biogenesis and regulation of telomerase holoenzyme (Zhang et al., 2011, Nguyen et al., 2018).

Telomerase is essential in proliferating tissues, such as germline cells, fetal tissues and adult stem cells, like HSCs (reviewed by (Srinivas et al., 2020)). Different mutations in the telomerase complex have been associated to a rare disorder, dyskeratosis congenita (DC) (Kirwan and Dokal, 2009, Dokal, 2011) and several human diseases, such as premature ageing, cancer, and idiopathic pulmonary fibrosis (Tsakiri et al., 2007). DC is an inherited ectodermal dysplasia syndrome that often occurs with the classic triad of nail dysplasia, skin pigmentation changes and oral leukoplakia, associated with a high risk of hematologic disease and cancer. The 90% of patients with DC developed at least a single lineage cytopenia by the fourth decade of life. Bone marrow failure is the principal cause of premature mortality, followed by pulmonary disease and cancer (reviewed by (Dokal, 2011, Mason and Bessler, 2011). All mutations identified in these patients are found in telomerase complex or in proteins involved in telomere maintenance, because telomere instability mainly affect the renewal capacity of HSCs (reviewed by (Vulliamy and Dokal, 2008, Dokal et al., 2011)). The incidence of hematological abnormalities, such as myelodysplastic syndrome (MDS), is higher in patients with *TERC* mutations than in patients with TERT mutations, and this observation cannot be explained only by telomere shortening (Vulliamy et al., 2011).

There is increasing evidence that telomerase complex is involved in several cell signaling pathways without apparent involvement of its well established function in telomere maintenance (reviewed by (Cong and Shay, 2008, Martínez and Blasco, 2011)). In the last twenty years, extracurricular roles have been discovered for both TERT and *TERC* subunits in cancer, inflammation, DNA damage and mitochondrial function, among others (Choi et al., 2008, Ding et al., 2013, Thompson and Wong, 2020, Ivanyi-Nagy et al., 2018, Liu et al., 2019).

Interestingly, our laboratory found a new *TERC* extracurricular role in myelopoiesis by regulating the expression of *CSF3* gene, which encodes a major granulopoiesis cytokine, and by maintaining an appropriate balance between *SPI1* and *GATA1*, which encodes master myeloid and erythroid transcription factors, respectively, in both zebrafish (Alcaraz-Perez et al., 2014) and human HL60 (neutrophil precursor) and U937 (monocyte precursor) cell lines (García-Castillo et al., unpublished). Thus, *terc* deficiency results in neutropenia and monocytopenia, while *terc* overexpression enforces myelopoiesis in zebrafish (Figure 16). Mechanistically, *TERC* controls myeloid gene expression through the interaction with a specific consensus sequences (*TERC*-binding sites) (Chu et al., 2011) present in the regulatory regions of myeloid genes and recruiting RNA polymerase II (RNA pol II) (Figure 18) (García-Castillo et al., unpublished). This mechanism is evolutionary conserved, since human neutrophil and monocyte progenitor cells with decreased *TERC* levels show a reduction in myeloid gene expression and RNA Pol II occupancy of those gene promoters. Therefore, *TERC* is a new type of long non-coding RNA with transcription factor functions.

In this work, we found that the CR4/CR5 domain of zebrafish *terc* mediates the interaction with RNA pol II and the mutation of this domain found in DC patients impairs this interaction and disables its noncanonical myelopoietic function. In addition, induced pluripotent stem (iPS) cells derived from a DC patient with a CR4/CR5 mutation shows reduced myelopoiesis, while those from a patient with a mutation in TERT differentiate as wild-type cells. Collectively, these results reveal a novel target for personalized medicine of DC patients.

2. Materials and methods

2.1. Ethics Statement

All experiments were performed according with the Guidelines of the European Union Council (Directive 2010/63/EU) and the Spanish RD 53/2013. Procedures were performed as have been approved by the Bioethical Committee of the University Hospital "Virgen de la Arrixaca" (Spain). Authorization from the Spanish National Embryo Ethical Committee was obtained to work with iPS cells.

2.2. Animals

Zebrafish (*Danio rerio* H., Cypriniformes, Cyprinidae) specimens were obtained from the Zebrafish International Resource Center (ZIRC) and mated, staged, raised and processed using standard procedures. Details of husbandry and environmental conditions are available on protocols.io (DOI:dx.doi.org/10.17504/protocols.io.mrjc54n) (Widrick et al., 2018). The transgenic line *Tg(mpx::eGFP)*^{i113} (*mpx*:*GFP* for simplicity) (Renshaw et al., 2006) was kindly provided by Dr. S. Renshaw.

2.3. Generation of *terc* mutant RNA molecules

Wild-type *terc* and template *(terc-template*^{M}*)* and CR4/CR5 (*terc-CR4/CR5*^{M}*)* mutants were synthesized and cloned in the *Eco*RV restriction site of pBluescript II SK (GeneScript). Template mutant has two nucleotide changes (C77U and A125C), which are present in MDS patients (Carroll and Ly, 2009), while CR4/CR5 mutant has a single nucleotide polymorphism (G194A), which is present in aplastic anemia (AA) patients (Carroll and Ly, 2009). For obtaining the ScaRNA mutant *terc* (*terc-ScaRNA*^{M}), the final part of the domain was removed by amplifying a DNA fragment using the wild-type *terc* as template and cloned in the *Bam*HI-*Eco*RI restriction sites of pBluescript II SK. All the mutations are shown in Figure 28.

2.4. RNA and morpholino injection

In vitro-transcribed RNA was obtained with mMESSAGE mMACHINE kit (Ambion) and polyadenylated, when necessary (not for *terc*), using the Poly(A) Tailing kit (Ambion) following manufacturer's instructions. Specific morpholinos (Gene Tools) were resuspended to 1mM in nuclease-free water (Table 2). Both RNAs and morpholinos were mixed in microinjection buffer (0.5x Tango buffer and 0.05 % phenol red solution) and microinjected into the yolk sac of one cell stage embryos using a microinjector Narishige IM300 (0.5-1 nl per embryo). The amount of *standard* and *tercMo* morpholino used was 4.8 pg/egg. The amount of injected wild-type or mutant *terc* is 200 pg/egg in all experimental groups.

Morpholino	Sequence (5'→3')	Recognized region	Dosis (Pg/egg)
standard (stdMo)	CCTCTTACCTCA GTTACAATTTATA	None	4.8
tercMo	AGCCGAACTCTT GGCGGCAGTCAAA	P6 and P6.1 loops in CR4/CR5 domain of *terc*	4.8

Table 2. Specific morpholinos used in this study and doses in zebrafish.

2.5. Neutrophil counts

Neutrophils in the CHT of *mpx*:GFP transgenic zebrafish embryos were counted in a M205-FA fluorescence microscope equipped with a DFC365FX camera (Leica).

2.6. Luciferase assay

pCVM-Renilla plasmid, containing Renilla luciferase (Promega) and the pGL3basic vector constructs containing the *csf3b* promoter region of zebrafish driven the firefly luciferase, were mixed along with indicated RNAs or morpholinos and injected into the yolk sac of the single cell stage embryos. After 48 hours, tail sections were obtained, pooled (10 larvae per pool), homogenized with a pellet pestle and centrifugate for 3 min at 13,000 rpm to remove cellular debris. The extracts were then assayed for firefly and Renilla luciferase activity using the Dual Luciferase Reporter Assay System (Promega) in a Luminometer Optocomp I (MGM Instruments). The results were normalized to the Renilla activity (Alcaraz-Perez et al., 2008).

2.7. RNA pulldown

RNA pulldown experiments were performed as described (Tsai et al., 2010), with some modifications. Biotin-labelled RNAs were *in vitro* transcribed using the Biotin 3´ End DNA Labeling Kit (Thermo Scientific), to incorporate 1-3 biotinylated ribonucleotides into the 3´ end of DNA/RNA strands, following manufacturer's instructions. Three micrograms of biotinylated RNA were heated to 70°C for 5 min and put on ice for 2 min. An equal volume of 2x RNA structure buffer (20 mM Tris pH 7, 0.2 M KCl, 20 mM $MgCl_2$) was added and then shifted to room temperature (RT) to allow RNA secondary structure formation. The folded RNAs were incubated for 1 hour at 4°C in rotation with 60 µl of washed Dynabeads MyOne Streptavidin C1 magnetic beads (Invitrogen). Larvae of 5dpf were anesthetized and the protein extract was obtained by homogenization in RIP buffer (25 mM Tris pH 7.4, 150 mM KCl, 0.5 mM DTT, 0.5% NP-40). The protein extract was centrifuged for 20 min at 4°C and the supernatant was pre-cleared 1h with 30 µl of beads at 4°C in rotation. Then 3 miligrams of pre-cleared protein were incubated with the biotinylated RNAs-beads complexes for 4h at 4°C in rotation. The complexes were magnet-captured, washed with RIP buffer five times at 4°C for 5 min and boiled in 2x Laemmli buffer (Sigma) at 90°C for 10 min for protein elution. The eluted proteins were subjected to polyacrylamide gel electrophoresis (PAGE) and wet transferred to a nylon membrane (GE Healthcare). The membranes were incubated for 1 h with TTBS (Tween-Tris-buffered saline) containing 5% (w/v) skimmed dried milk powder and immunoblotted using anti-phospho Serine 5 RNA polymerase II CTD repeat YSPTSPS mouse antibodies (dilution 1:1000) 16 h at 4°C (pS5 RNA pol II, ab5408, Abcam). The blots were then washed with TTBS and incubated for 1 h at room temperature with the secondary HRP-conjugated antibody (dilution 1:1000) in 5% (w/v) skimmed milk in TTBS. After repeated washes, the signal was detected with the enhanced chemiluminescence reagent and ChemiDoc XRS (Biorad).

2.8. *In vitro terc*-DNA binding assay

One hundred pb sense and antisense 3´ biotin-tagged-DNA probes of the zebrafish *csf3b* promoter region encompassing the *terc*-binding sites (SIGMA-Aldrich) were designed to measure the binding capacity of *terc* to these sequences. To perform the annealing, 25 µM of each probe were incubated in annealing buffer (10 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.2 mM EDTA) at 95°C for 4 min, 10 min at 70°C and they were slowly cooled down to room temperature for 20 min to allow annealing. Then, the dsDNA probes were bound to 10 µl of Dynabeads MyOne Streptavidin C1 magnetic beads (Invitrogen) for 15 min at room temperature (RT). Probe excess was removed by washing beads 2 times with annealing buffer for 5 min. After that, 50 ng of luciferase (Promega),

terc and *terc*-CR4-CR5^{M} RNAs were added and incubated at RT for 30 min in rotation. The beads-dsDNA-RNA complexes were washed 3 times at RT for 10 min and RNA was eluted by incubation in water at 95°C for 5 min. The eluted RNAs were retrotranscribed with SuperScript IV VILO Master Mix (Invitrogen) and subjected to quantitative-PCR (qPCR) for luciferase and *terc* detection. To be able to compare the Ct values of *terc* and luciferase, a previous normalization was made. Normalization consisted of a primers efficiency calibration curve starting from 1 ng/µl of RNA, and serial dilutions of 0.1 ng/µl, 0.01 ng/µl and 0.001 ng/µl. Then, *in vitro* reverse transcription and qPCR was carried out under the same conditions. Luciferase primers are more efficient than *terc* primers in 1.3 cycles.

Real-time PCR was performed with a StepOnePlus instrument (Applied Biosystems) using SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara). Reaction mixtures were incubated for 30 seconds (sec) at 95°C, followed by 40 cycles of 5 sec at 95°C, 20 sec at 60°C, and finally a melting curve protocol. The primers used are shown in Table 3. In all cases, each PCR was performed with triplicate samples and repeated, at least, with two independent samples.

Name	Species	Sequence (5´-3´)	Reference
Terc-Fq	zebrafish	GGTCTCACAGGTTTGGCTGT	(Alcaraz-Perez et al., 2014)
Terc-Rq	zebrafish	TGCAGGATCAGTGTTTGAGG	(Alcaraz-Perez et al., 2014)
lucFq	n/a	GGCTATGAAGAGATACGCCCT	
lucRq	n/a	TCAGCGTAAGTGATGTCCACCT	

Table 3. Primers used for RT-qPCR analysis.

2.9. HL60 cell culture

The human promyelocytic HL60 cell line was cultured in RPMI-1640 medium (Lonza) supplemented with 10% FCS, 2 mM glutamine and 1% penicillin-streptomycin. Cells were maintained at 37°C with 5% CO_2 and split before confluence every 72h.

2.10. *In vitro* transcription assay

Transcription assays were performed using as source of transcriptional machinery HL60 nuclear extracts or recombinant proteins. HL60 cells were washed twice in cold PBS, resuspended in nuclear solubilization buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.3% v/v Igepal) and incubated 30 min on ice. Nuclei were pelleted by centrifugation at 5000xg 4°C for 5 min, washed twice in nuclear solubilization buffer and lysed in CHAPS Lysis buffer (Merck-Millipore). *In vitro* transcription assays were performed in 60 µl of transcription buffer containing 10 mM Tris (pH 7.9), 10% glycerol, 50 mM KCl, 10 mM HEPES (pH 7.9), 1 mM DTT, 4 mM $MgCl_2$, 25 µg/mL BSA, 2 units/µl RNase inhibitor (RNaseOut, ThermoFisher). Briefly, 1 nM of pGL3basic *csf3b*-2Kb promoter construct was used as template and incubated with the indicated RNAs and 1 µg of HL60 nuclear protein extract for 20 min at 30°C to allow transcription complexes to form. Then nucleotides (0.625 mM final concentration) were added and the transcription reaction was incubated during 1h at 30°C. Template DNAs were digested by adding 3 µl of TURBO DNaseI (ThermoFisher) and incubation at 37°C for 30 min. Luciferase transcribed mRNA was isolated using 2.5 M Lithium Chloride precipitation, reverse transcribed (+RT) using SuperScript IV VILO Master Mix (Invitrogen) and subjected to qPCR using triplicates. The primers used are indicated in Table 2. Remaining traces of template construct in the purified mRNAs was monitored by performing a RT without reverse transcriptase (-RT). *In vitro* transcription efficiency was calculated using ΔCt method (+RT vs -RT).

In the case of recombinant proteins, *in vitro* transcription was performed as described (Yakovchuk et al., 2009), with some modifications. General transcription factors, all from SIGMA, were used as follows: TFIID native complex 1ng/µl, TFIIF 2 nM, TFIIB 10 nM and Pol II CTD 2 nM. Briefly, 1 nM of pGL3basic *csf3b*-2Kb promoter construct was used as template and incubated in transcription buffer with TFIID for 4 min at 30°C. The rest of transcriptional factors (TFIIF, TFIIB and Pol II) and the indicated RNAs were incubated together in a separate tube, in transcription buffer, at 30°C for 4 min. The content of both tubes was mixed, incubated for 20 min at 30°C to allow initiation complexes to form. Then, nucleotides were added (0.625 mM) to initiate transcription and the reaction was further incubated for 1 h at 30°C. Template DNA was digested by adding 3 µl of TURBO DNaseI (ThermoFisher) and incubation at 37°C for 30 min. As in the previous protocol, luciferase transcribed mRNAs were isolated using lithium chloride precipitation, reverse transcribed (+RT) using SuperScript IV VILO Master Mix (Invitrogen) and without reverse transcriptase (-RT), as a control. Finally, the samples were subjected to qPCR using triplicates. The primers used are indicated in Table 2. *In vitro* transcription efficiency was calculated using ΔCt method (+RT vs -RT).

2.11. iPS cell culture, differentiation towards hematopoietic lineage and colony-forming unit (CFU) assay

iPS cell lines were maintained undifferentiated in a 6mm plates treated with Matrigel (Corning) and mTeSR™ Plus medium (Stem Cell Technologies). Media was changed daily or every two days, and the cells were split weekly by dissociation with 200 U/ml of collagenase IV (Invitrogen). iPS cell culture were visualized daily by phase-contrast microscopy.

For hematopoietic differentiation, undifferentiated iPS cells at 70-80% confluence were treated with Matrigel (Corning) 24 hours before starting the differentiation. To generate the Embrionic Bodies (EBs), the iPS cells were treated with collagenase IV and scraped of the attachments. They were then transferred to 6 wells low-attachment plates (Corning) to allow EBs formation by incubation in differentiation medium consisting of KnockOut™ Dulbecco's modified Eagle's medium (ThermoFisher) supplemented with 20% non-heat-inactivated fetal bovine serum, 1% nonessential amino acids, 1 mM glutamine and 0.1 mM β-mercaptoethanol. The medium was changed the next day (day 1) with the same differentiation medium supplemented with hematopoietic cytokines: 300 ng/ml stem cell factor (R&D), 300 ng/ml FMS-like tyrosine kinase-3 ligand (R&D), 10 ng/ml IL-3 (R&D), 10 ng/ml IL-6 (R&D), 50 ng/ml granulocyte colony–stimulating factor (R&D) and 25 ng/ml bone morphogenetic protein-4 (Miltenyi) (Wang et al., 2004, Menendez et al., 2004, Wang et al., 2005). EBs were dissociated using collagenase B (Roche Diagnostic) for 2 hours at 37°C followed by 10 minutes incubation at 37°C with enzyme-free Cell Dissociation Buffer (Invitrogen) at day 15 and 21 of development. Single-cell suspension was obtained by gentle pipetting and passage through a 70-µm cell strainer (Becton Dickinson). The dissociated cells were stained with anti-CD34-fluorescein isothiocyanate, anti-CD31-phycoerythrin and anti-CD45-allophycocyanin (all from Becton Dickinson) antibodies and 7-actinomycin D. Live cells identified by 7-actinomycin D exclusion were analyzed using a FACSCanto II flow cytometer equipped with FACSDiva software (Becton Dickinson).

Hemogenic endothelial progenitors (HEPs) were identified as $CD31^{+}/CD45^{-}$. Immature (hematopoietic progenitor, HPs) and mature (hematopoietic) blood cells were identified as $CD45^{+}/CD34^{+}$ and $CD45^{+}/CD34^{-}$, respectively (Menendez et al., 2004, Bueno et al., 2009, Wang et al., 2005) .

CFU assays were performed by plating 100,000 cells from EBs at day 15 into methylcellulose H4434 culture medium (Stem Cell Technologies). Cells were incubated at 37°C in a 5% CO_2-humidified atmosphere and colonies counted at day 14 of the CFU assay using standard

morphological criteria (Bueno et al., 2009, Menendez et al., 2001, Catalina et al., 2009). The iPS cell workflow is depicted in Figure 33.

3. Results

3.1. *terc* mutants design

TERC is able to regulate the expression of myeloid genes acting as a long non-coding RNA with typical transcription factor functions, capable of recruiting RNA polymerase II to target gene promoters for improving myelopoiesis (García-Castillo et al., unpublished).

To gain further insights into the domains of the zebrafish *terc* molecule involved in its myelopoiesis function in order to stratify patients and anticipate the disease onset, a collection of mutants based in the mutations observed in DC patients (Ly et al., 2005) were obtained (Figure 28). First one has two point mutations, C77U and A125C, in pseudoknot-template domain and was named *terc-template*^{M}. Second one has a single point mutation G194A in CR4/CR5 domain and we named *terc-CR4/CR5*^{M}. Last one has a deletion of 68 nucleotides in ScaRNA domain of the 3´end of the molecule, after box H, called as *terc-ScaRNA*^{M} (Figure 28).

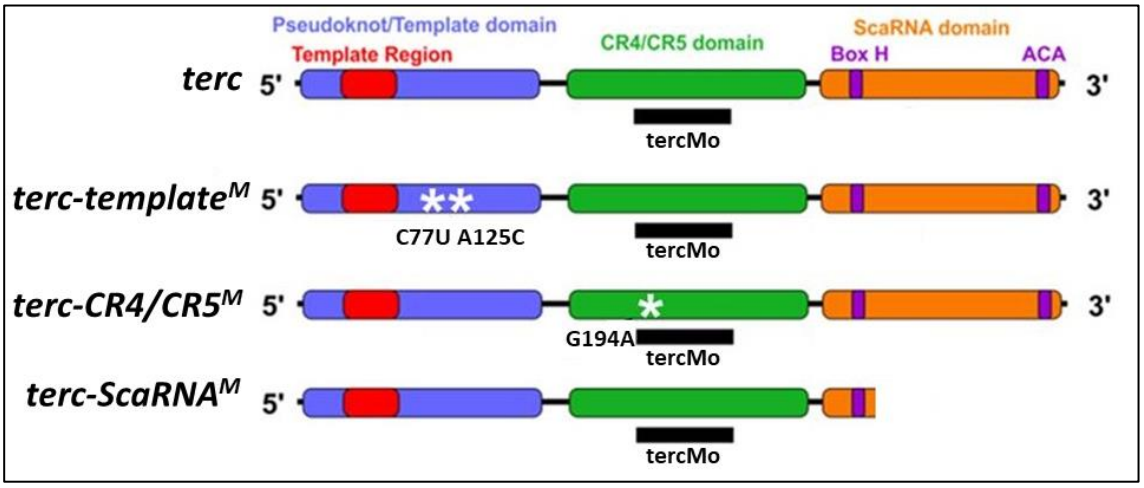

Figure 28. **Schematic representation of wild-type and mutant *terc* molecules**. Different domains, point mutations (white asterisks) and deletion of each mutant, and target site of *terc*Mo (black boxes) are shown.

3.2. CR4/CR5 domain is essential for the extracurricular role of *terc* in myelopoiesis

mpx:GFP zebrafish embryos, which have GFP-labeled neutrophils, were microinjected with the different *terc* mutants. *terc* morpholino, which has previously been shown to promote degradation of *terc* (Alcaraz-Perez et al., 2014), was used as a negative control, while wild-type *terc* was used as a positive control. The results showed that Template and ScaRNA *terc* mutants were both able to increase neutrophil number at similar levels than wild-type *terc*, while *terc-CR4/CR5*^{M} variant failed to do so (Figure 29a). These results were further confirmed by luciferase assays using a *csf3b* promoter reporter, which consisted in the 2 kb upstream the *csf3b* gene and contains a *terc* binding sequence (Alcaraz-Perez et al., 2014). While Template and ScaRNA *terc* mutants were able to increase luciferase activity as wild-type *terc*, *terc-CR4/CR5*^{M} was unable to do so (Figure 29b). These results indicate that CR4/CR5 domain mediates the extracurricular role of *terc* in myelopoiesis.

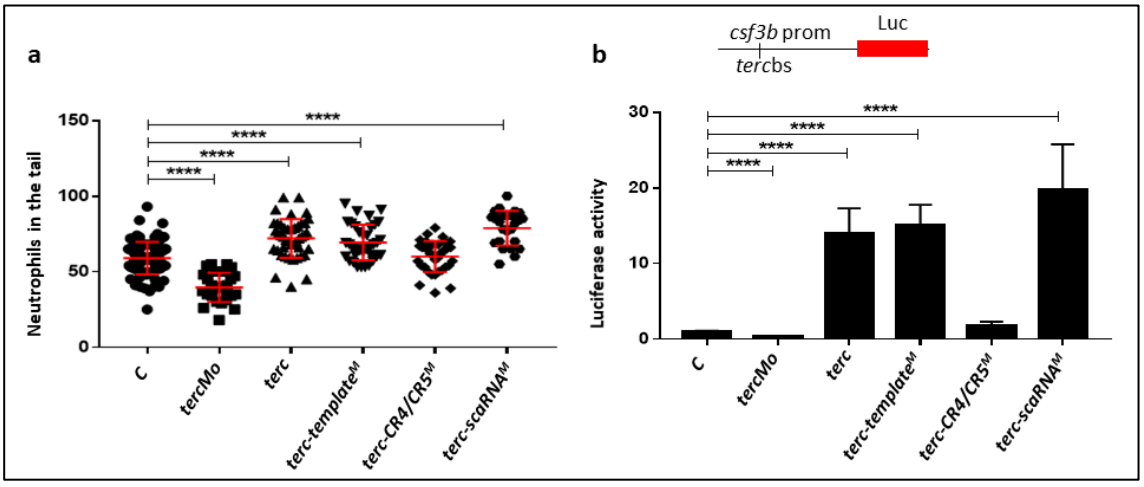

Figure 29. Effect of different *terc* mutations on myelopoiesis in zebrafish. (a) Neutrophil count in the CHT of 3-day *mpx*:GFP larvae after injection of *terc* RNAs or *terc*Mo. Each dot represents a larva and mean ± s.e.m is also shown. n = 96, 28, 44, 36, 36 and 24 larvae in control, *terc*Mo, *terc*, *terc-template*^{M}, *terc-CR4/CR5*^{M} and *terc-ScaRNA*^{M}, respectively. (b) Activity of *csf3b* promoter in the tail of 2-days larvae after injection of the luciferase reporter construct in combination with *terc*Mo or *terc*. Data are shown as mean + s.e.m (n= 3). *P< 0.05, **P< 0.01 and *** P< 0.001 for one-way analysis of variance (ANOVA) plus Bonferroni post-test.

3.3. RNApol II interaction is mainly mediated by CR4/CR5 domain of *terc*

The above results prompted us to study whether the ability of *terc* to bind to RNApol II was also mediated by its CR4/CR5 domain. To confirm this hypothesis, an RNA pulldown was performed with *terc* and *terc-CR4/CR5^{M}*, using *terc* antisense and GFP mRNAs as negative controls.

RNA pulldown procedure was based on biotinylating RNA, attaching it to magnetic streptavidin beads and incubating the complex with protein extract from 5 days old zebrafish larvae. Thus, proteins that were able to interact with these RNAs remained attached to magnetic beads and the rest were washed away. Then, specific interactors were eluted, subjected to SDS-PAGE and revealed with the pS5 RNA pol II antibody. The results showed that *terc-CR4/CR5^{M}* was able to interact with active RNA Pol II but with lower affinity than wild-type *terc* (Figure 30), suggesting that the CR4/CR5 domain of *terc* is involved in the interaction with RNA Pol II and that although the G194A mutation only reduced this interaction by 40%, it fully impaired its myelopoiesis activity.

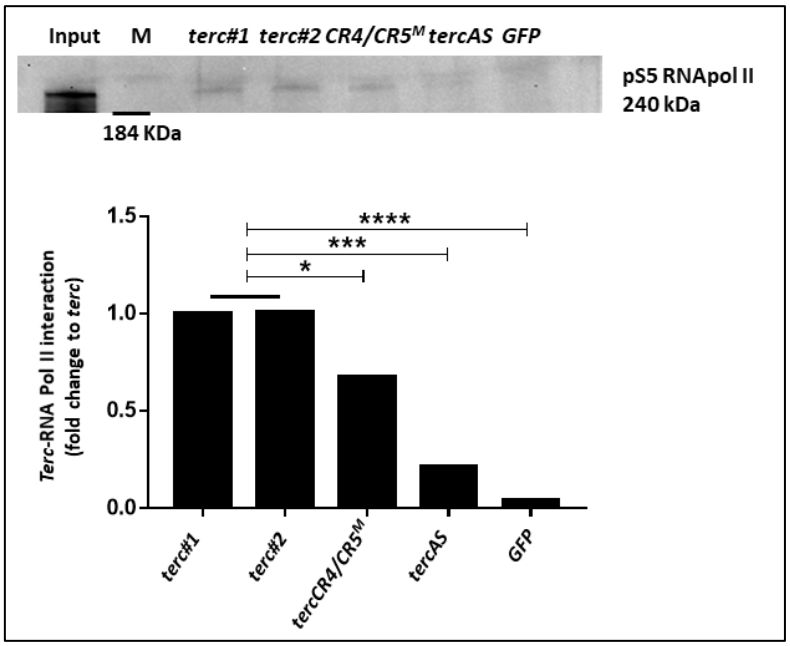

Figure 30. CR4/CR5 domain is involved in the interaction with RNA Pol II. Western blot of RNA pulldown eluates using anti-phospho-serine 5 RNA Pol II antibodies and quantification of the interaction. Data are shown as mean + s.e.m. (n=2). *P< 0.05, **P< 0.01 and *** P< 0.001 for one-way analysis of variance (ANOVA) plus Bonferroni post-test.

3.4. CR4/CR5 mutation does not affect *terc* DNA binding affinity

As RNA pulldown experiments demonstrated that the mutation in CR4/CR5 domain observed in DC patients impaired *terc* binding to RNA Pol II, we next checked if the ability to bind DNA was also affected by this mutation. To test the DNA binding capacity of *terc* and *terc-CR4/CR5*^{M}, an *in vitro* DNA binding assay has been performed using a 100 bp biotinylated probe of *csf3b* promoter containing *terc* binding site. Unexpectedly, *terc-CR4/CR5*^{M} was able to bind to *csf3b* promoter with higher affinity than wild-type *terc* (Figure 31). Taken together, these results indicate that the mutation of the CR4/CR5 domain of DC patients impairs the extracurricular role of *terc* in myelopoiesis due to its reduced binding affinity to RNA Pol II despite having intact its DNA binding affinity.

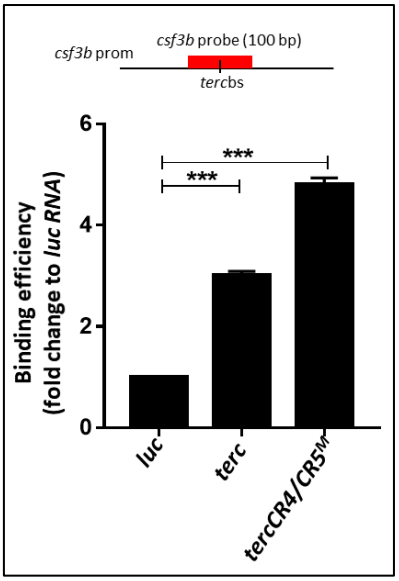

Figure 31. CR4/CR5 mutation does not affect *terc* DNA binding affinity. RT-qPCR of *in vitro csf3b* promoter-binding assay eluates. Data are shown as mean + s.e.m. (n=3). **P*< 0.05, ***P*< 0.01 and *** *P*< 0.001 for one-way analysis of variance (ANOVA) plus Bonferroni post-test.

3.5. CR4/CR5 mutation impairs the ability of *terc* to increase *csf3b* transcription

To further confirm the role of *terc* in the regulation of myelopoiesis, an *in vitro* transcription assays using either nuclear extracts from the HL60 cell line or recombinant transcription factors, as sources of transcriptional machinery, and *csf3b* promoter driven the luciferase reporter as template, were performed. Results show that while wild-type *terc* increased the transcription efficiency of *csf3b* promoter, *terc-CR4/CR5*^{M} failed to do so (Figure 32). This result further confirms that CR4/CR5 domain is essential for *terc* myelopoietic function.

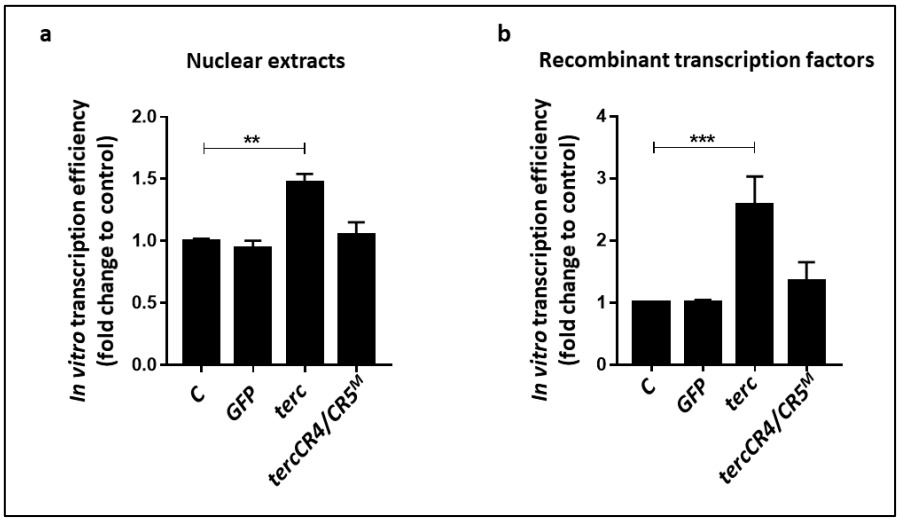

Figure 32. CR4/CR5 mutation impairs the ability of *terc* to increase *csf3b* transcription. *In vitro* transcription efficiency of *csf3b* promoter when 25 nM of indicated RNAs (GFP, *terc, terc-CR4/CR5*^{M}) were added to the reaction using either HL60 nuclear extracts (**a**) or recombinant transcription factors (**b**) as sources of the transcriptional machinery. Data are shown as mean + s.e.m. (n=3) **P*< 0.05, ***P*< 0.01 and *** *P*< 0.001 for one-way ANOVA plus Bonferroni post-test.

3.6. DC patient-derived iPS cells harboring a mutation in CR4/CR5 domain of *TERC* show impaired myelopoiesis

All the results obtained so far indicates that CR4/CR5 domain of *terc* is crucial for the transcriptional activation of *csf3b* and myelopoiesis in zebrafish through its physical interaction with RNA Pol II. To investigate whether this mechanism is conserved in human, two iPS cell lines derived from DC patients were used for myeloid differentiation experiments. One of them has a heterozygous mutation in CR4/CR5 domain of *TERC* (G319A) (Boyraz et al., 2016), exactly the same mutation that we have analyzed in all our zebrafish studies, while the other iPS cell line has a heterozygous pathogenic missense point mutation in TERT (A716V) (S. Agarwal, unpublished), as an additional control of *TERC* unique function in myelopoiesis. As a control of the experiment, an iPS cell line derived from a healthy donor (HD) was also included.

The workflow to induce hematopoietic differentiation is shown in Figure 33.

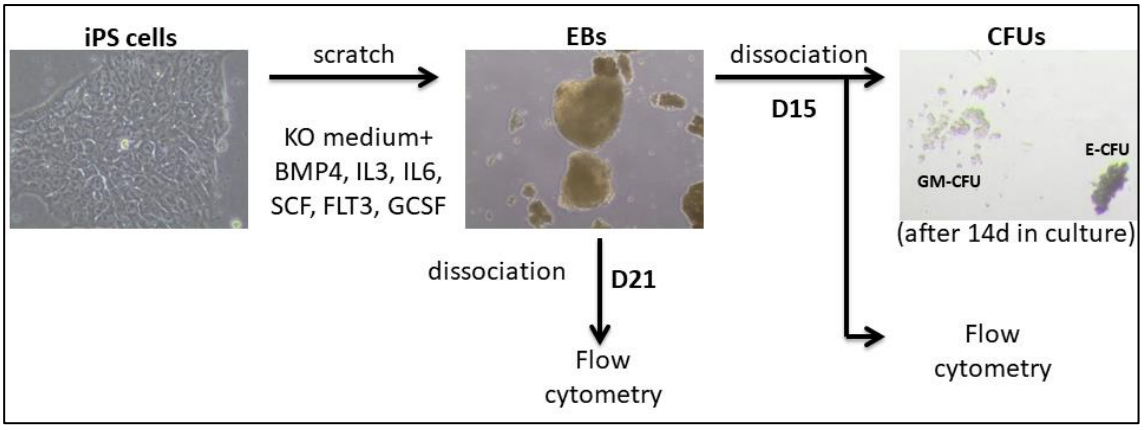

Figure 33. iPS cells workflow. EBs were generated from iPS cells in culture by scratching and adding medium supplemented with hematopoietic cytokines (BMP4, IL3, IL6, SCF, FLT3 and GCSF). After 15 days of EBs culture, half of the EBs were dissociated and used in CFUs assays and analysis by flow cytometry to confirm hematopoietic differentiation. On day 21, remaining EBs were dissociated to study the complete differentiation by flow cytometry. CFUs composition was analysed after 14 days of culture and percentage of granulocytic/monocytic-CFUs (GM-CFUs) versus erythroid-CFUs (E-CFUs) was calculated.

Consistently with our findings in zebrafish, while both HD and *TERT* mutant iPS cells were able to generate about a 70% of GM-CFUs and a 30% E-CFUs, the iPS cells harboring the G319A mutation in the CR4/CR5 domain of *TERC* generated a much higher percentage of E-CFUs and fewer

GM-CFUs (Figure 34a). This indicates that *TERC* mutation hinders myelopoiesis but does not affect erythropoiesis in a TERT independent manner. In addition, as the *TERC* G319A is in heterozygosis, it seems that the levels of the wild-type allele of *TERC* are not able to sustain an appropriate expression of myeloid genes. As expected, fewer colonies were obtained in *TERT* and *TERC* mutants than in HD control (Figure 34b), which might reflect reduced proliferation capacity due to a defect in telomere maintenance upon differentiation (Boyraz et al., 2016).

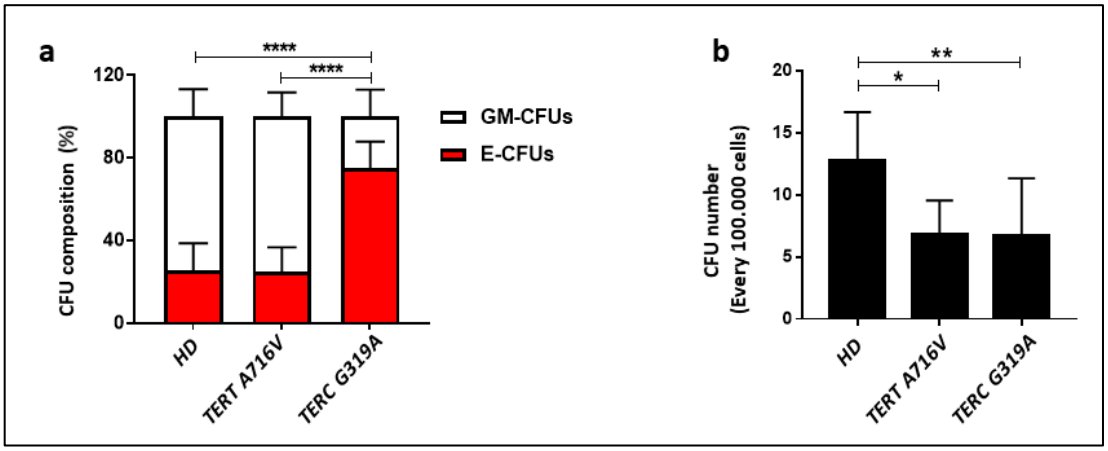

Figure 34. DC patient-derived iPS cells harboring a mutation in CR4/CR5 domain of *TERC* show impaired myelopoiesis. (a) Composition of GM-CFUs (white) and E-CFUs (red) at day 14, using EBs derived from indicated iPS cell lines. (b) Number of colonies per 100,000 EB cells. The data are shown as mean + s.e.m. (n=7) **P*< 0.05, ***P*< 0.01 and **** *P*< 0.0001 for two-way ANOVA in (a) and one-way ANOVA in (b) plus Dunnet´s post-test.

EBs hematopoietic cell composition was also analyzed by flow cytometry using pluripotency (CD34), endothelial (CD31) and blood (CD45) markers to differentiate between hemato-endothelial progenitor cells (HEPs, $CD45^-/CD31^+/CD34^+$), hematopoietic progenitors (HPs, $CD45^+/CD34^+$) and mature blood cells ($CD45^+/CD34^-$) (Figure 35a). No differences were observed among the 3 cell lines at day 15 (Figure 35b), indicating that the CFUs assay started with a very similar number of HEPs and HPs, being these populations the ones that best correlates with the CFU clonogeneicity (Ramos-Mejia et al., 2010). Furthermore, EB cell composition at day 21 was also similar between HD and *TERT* and *TERC* mutants (Figure 35c), except in percentage of mature blood cells, where *TERC* mutant showed a small increase in relation to the HD and *TERT* mutant. Therefore, CR4/CR5 *TERC* mutation reduces the ability of iPS cells to generate GM colonies, i.e. mature myeloid cells, without affecting the differentiation of EBs.

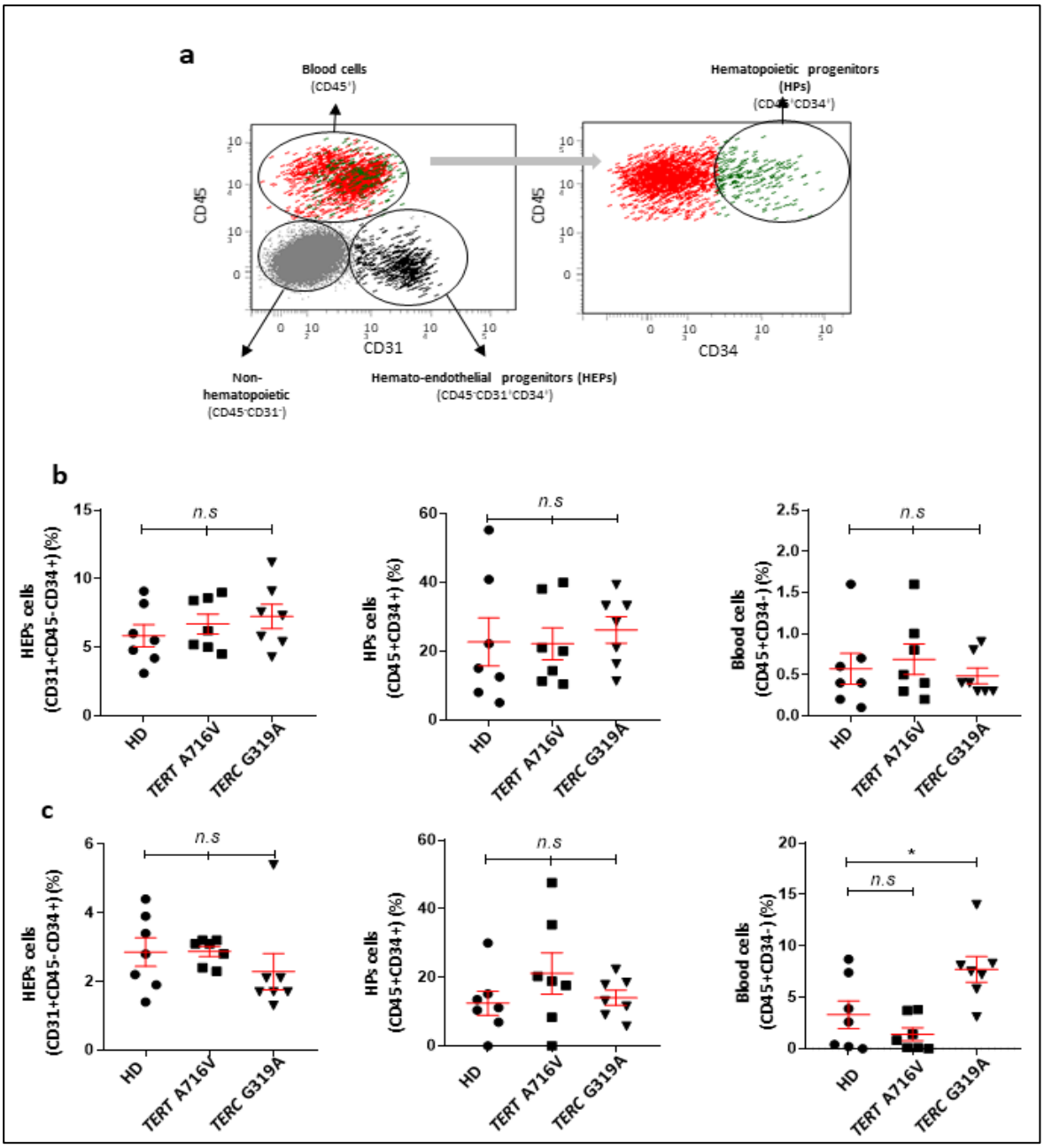

Figure 35. Hemogenic progenitor differentiation is unaffected by TERT and *TERC* mutations. (a) Representative dot plots showing different hematopoietic cell populations in EBs by flow cytometry. $CD31^+$ cells are hemogenic progenitors (HEPs, bipotential precursor of hematopoietic and endothelial cells), $CD45^+/CD34^+$ cells are hematopoietic progenitor cells (HPs) and $CD45^+/CD34^-$ cells are mature blood cells. (b, c) Quantitation of cell populations in EBs by flow cytometry at day 15 (b) and 21 (c). Data are shown as mean + s.e.m. (n=7), **P*< 0.05, ***P*< 0.01 and *** *P*< 0.001 for one-way ANOVA plus Dunnet´s post-test in all the panels. ns, not significant.

4. Discussion

Telomerase RNA component plays an essential role in zebrafish myelopoiesis by regulating the expression of myeloid genes independently of TERT and telomere length (Alcaraz-Perez et al., 2014). This role is conserved in humans, because reduced *TERC* levels resulted in decreased myeloid gene expression in human neutrophil and monocyte progenitor cell lines without reducing either telomerase activity or telomere length (García-Castillo et al., unpublished). Mechanistically, both human and zebrafish *TERC* physically interacts *in vitro* and *in vivo* with RNA Pol II, the main component of the transcription machinery, and with consensus sequences found in myeloid genes, suggesting that *TERC* attracts the transcriptional machinery to its target genes increasing their expression and promoting myelopoiesis (García-Castillo et al., unpublished). Therefore, *TERC* behaves like a typical transcription factor, despite being a lncRNA.

In the present work, we wanted to identify the domains of *TERC* involved in this novel function. For this purpose, we analyzed the impact of different mutations in each domain found in DC patients and it was observed that the CR4/CR5 is responsible for regulating myelopoiesis in both zebrafish and humans. Notably, although a mutation of the CR4/CR5 domain fully impaired its myelopoietic activity, it did not affect *terc*-DNA binding activity but rather the interaction with RNA Pol II. This indicates that *terc* function in myelopoiesis is not only related to *terc* levels, but also to a fully competent structure of the molecule, especially of the CR4/CR5 domain that allows a productive interaction with RNA Pol II. Interestingly, modeling of three-dimensional structure of wild-type *terc* and *terc-CR4/CR5*^{M} showed that the point mutation G194A of the CR4/CR5 domain resulted in a dramatic alteration of the structure of the molecule, especially of CR4/CR5 and template domains (Figure 36). The alteration observed in the template domain may also explains the impaired telomerase activity of DC patient harboring this mutation, being this fact perhaps responsible for uncovering the extracurricular role of *TERC* reported in our study using the unique advantages of the zebrafish model.

As CR4/CR5 domain is frequently mutated in DC patients (reviewed by (Brummendorf and Balabanov, 2006)), we used an iPS cell line derived from a DC patient with one of the most common mutation observed in CR4/CR5 domain (G319A) (Boyraz et al., 2016), which is equivalent to the G194A of zebrafish *terc*, and one harboring a missense point mutation in *TERT* (A716V), both of them heterozygous. Results confirmed the data obtained in zebrafish, since CR4/CR5 mutation caused defective myelopoiesis, while TERT mutation had no effect in differentiation. These results also confirms that the novel function of the CR4/CR5 domain of *TERC* in the regulation of myelopoiesis is fully independent of TERT and is consistent with clinical data showing that DC

patients with *TERC* mutations have a higher probability to develop hematological abnormalities than patients with *TERT* mutations (Vulliamy and Dokal, 2008).

In summary, we report here that CR4/CR5 domain of *TERC* is responsible for regulation of myelopoiesis independently of TERT in human and zebrafish. Zebrafish *terc* perform this extracurricular function by recruiting RNA Pol II to the promoter of the master gene regulator of neutrophil differentiation *csf3b* through the binding to its consensus binding sites, which assures an efficient RNA Pol II occupancy around the transcription start site of *csf3b*, its appropriate expression and, therefore, robust myelopoiesis. This mechanism is impaired in DC patients with a CR4/CR5 mutation, since it prevents a robust interaction with RNA Pol II, despite not altering its DNA binding affinity, that would result in decreased expression of myeloid genes and eventually a poor myelopoiesis (Figure 37). Our results provide new molecular knowledge about DC pathogenesis and could serve as a basis for designing new strategies for therapeutic intervention, particularly in DC patients harboring mutations that affect the CR4/CR5 domain of *TERC*. In addition, this study may help to better stratify DC patients for anticipation to symptom onset and personalized medicine.

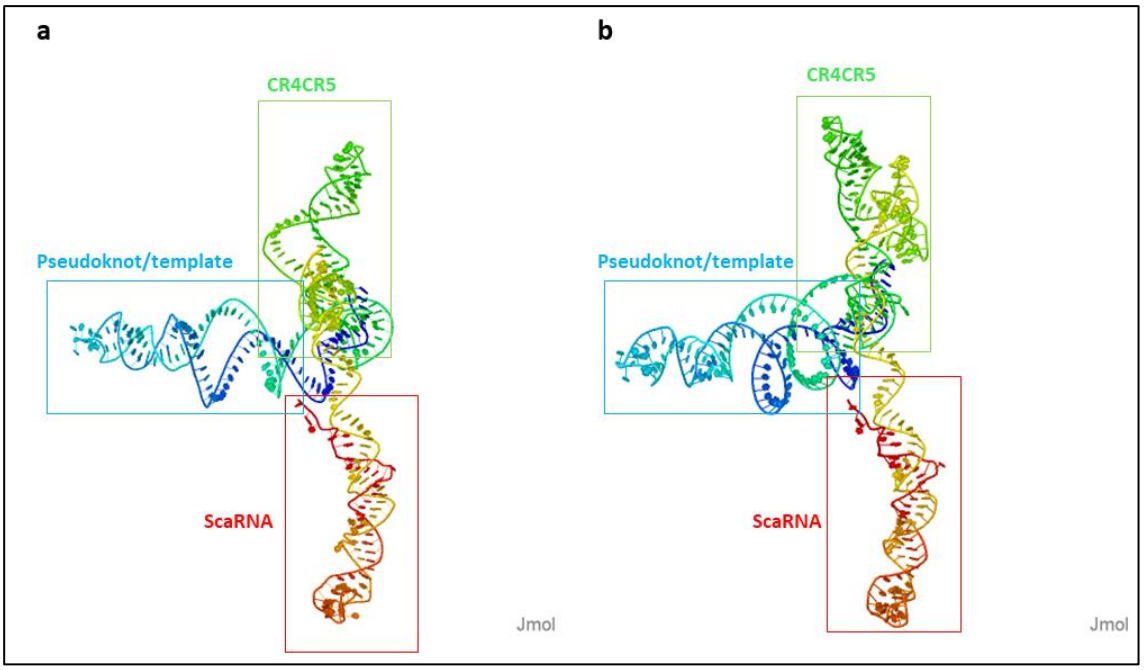

Figure 36. Prediction of *terc* and *terc-CR4/CR5^{M}* mutant (G319A) tertiary RNA structure. The processing of the structure was done with Jmol application of RNA Composer system (http://rnacomposer.cs.put.poznan.pl/), an open-source Java viewer for chemical structures in 3D (Antczak et al., 2016, Biesiada et al., 2016, Popenda et al., 2012, Rybarczyk et al., 2015). Different domains of the molecule are shown in different colors, starting 5' in dark blue, followed by green, yellow, orange and ending 3' in red. (a) *terc* wild-type structure. (b) *terc-CR4/CR5^{M}* structure.

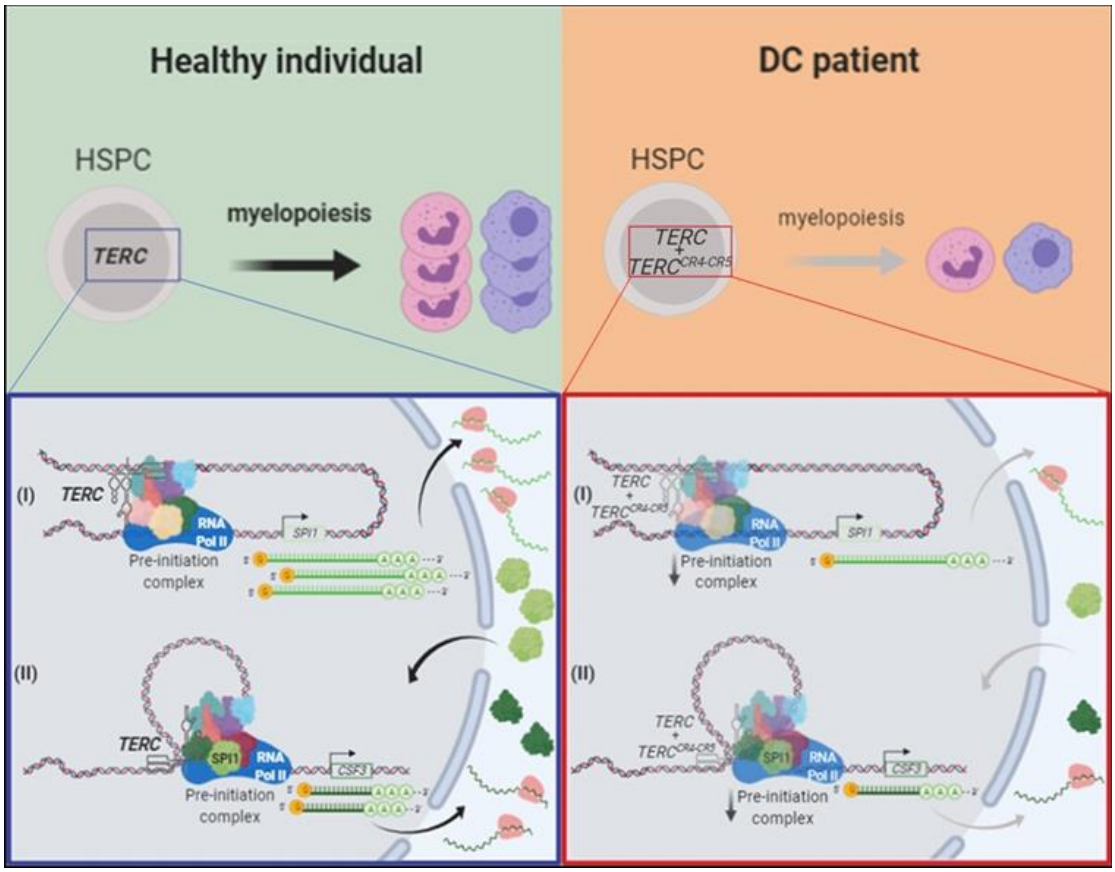

Figure 37. *TERC* regulation model of myeloid gene expression. Under normal conditions, *TERC* can regulate the expression of myelopoiesis genes (*SPI1* and *CSF3*) in zebrafish and humans. Telomerase RNA binds DNA through its consensus binding sites and with RNA Pol II, facilitating its recruitment to myelopoietic gene promoters, ensuring robust myelopoiesis. DC patients with a mutation in CR4/CR5 domain of *TERC*, possibly due to a change in RNA structure, do not properly recruit RNA Pol II to promoters and fail to regulate the expression of myeloid genes, resulting in an alteration in myelopoiesis that causes a drop in myeloid cell production. CR4/CR5 mutations will anticipate the development of MDS and signal the need for precision, personalized treatment. Pink cells are neutrophils and purple cells are macrophages. Dark green proteins are GCSF cytokine and light green protein are master transcriptional factor *SPI1*.

CHAPTER II.
Development of aptamers derived from *TERC* to treat blood diseases

Contenido inhibido autorizado por Comisión General de Doctorado de fecha 16 de Septiembre de 2020.

Chapter III.
Identification of *TERC* interactome.

Contenido inhibido autorizado por Comisión General de Doctorado de fecha 16 de Septiembre de 2020.

CONCLUSIONS

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

1. The CR4/CR5 *TERC* domain in essential in myelopoiesis, since the mutations of DC patients found in this domain decrease RNA polymerase II interaction and impaired myelopoiesis in zebrafish, despite they do not affect the DNA binding affinity of *terc*. Similarly, iPS cells derived from a DC patient with a CR4/CR5 mutation show deficient myelopoiesis, while those from a patient with a mutation in TERT differentiate as wild type cells.

2. Aptamers derived from the CR4/CR5 domain of *TERC* stimulate myelopoiesis without affecting erythropoiesis in zebrafish. They function as full *terc*; that is, they increased the expression of master myeloid genes independently of endogenous *terc*, by binding to its DNA target sequences present in the regulatory regions of these genes and to RNA Pol II, enforcing their transcription. However, the aptamers harbouring the CR4/CR5 mutations found in DC patients failed to perform all these functions.

3. Aptamers rescue neutropenia of preclinical zebrafish models of DC and PN. In addition, human aptamers derived from the CR4/CR5 domain of *TERC* increase myelopoiesis in human iPS cells, demonstrating their therapeutic potential to treat neutropenia caused by different genetic alterations.

4. Zebrafish *terc* interacts with 96 proteins involved in key biological processes, such as protein folding, degradation (ubiquitination) and translation, carbon and lipid metabolism, nonsense-mediated mRNA decay, mitochondrial biogenesis and cell cycle.

5. Zebrafish *terc* harbouring the mutations of the CR4/CR5 domain found in DC patients shows a similar interactome as wild type *terc*, despite these mutations impair both telomerase activity and *TERC*-dependent regulation of myelopoiesis in human, supporting the notion that DC is caused by *TERC* haploinsufficiency rather than a dominant effect.

6. The interactome of *terc* paves the way for future functional studies aimed at further illuminate the non-canonical roles of *terc* that could help to improving diagnosis and treatment of patients with *TERC* mutations by revealing novel prognosis biomarkers and therapeutic targets.

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RESUMEN EN CASTELLANO

1. Introducción

La información genética en los organismos eucariotas se almacena en los cromosomas, formados por DNA y proteínas, constituyendo una compleja estructura. En los extremos de los cromosomas se localizan los telómeros, secuencias cortas repetidas en tándem altamente conservadas, con una longitud variable entre especies, que protegen la integridad de los cromosomas (Greider, 1996, Greider, 1998, Srinivas y otros, 2020).

Los telómeros se acortan tras cada división celular debido al "problema de la replicación de los extremos" (Ohki y otros, 2001), donde la DNA polimerasa no puede replicar la cadena en dirección 3´→5´completamente. En ausencia de un mecanismo compensatorio, tras un número determinado de divisiones celulares, las células acabarían perdiendo su material génico y se produciría una muerte celular programada o una senescencia replicativa (Turner y otros, 2019).

Los organismos eucariotas han solventado este problema mediante la telomerasa, una DNA polimerasa dependiente de RNA con actividad retrotranscriptasa que añade repeticiones teloméricas al final de los cromosomas, haciendo posible que la longitud de los telómeros se mantenga para mantener la supervivencia celular (Blackburn, 2005). La telomerasa es un complejo ribonucleoproteico compuesto por una subunidad catalítica con actividad retrotranscriptasa (telomerase reverse transcriptase, TERT), un componente de RNA (telomerase RNA component, *TERC*) que actúa como molde para la síntesis de telómeros y una serie de proteínas accesorias encargadas de regular la biogénesis, localización subcelular y las funciones *in vivo* (Wyatt y otros, 2010, Podlevsky y Chen, 2012).

Mientras que TERT se encuentra muy bien conservado a lo largo de las especies, la conservación de *TERC* es mucho más divergente. *TERC* presenta longitudes muy variadas (revisado por (Musgrove y otros, 2018)), sin embargo, mantiene la organización de los dominios. En concreto, en humano tiene una extensión de 451 nucleótidos de longitud estructurados en 3 dominios. El dominio "Pseudoknot-Template" consiste en un gran bucle que contiene la secuencia molde, una hélice P1 y una región P2/P3, que interactúa con TERT. El dominio "CR4/CR5" consiste en regiones P4, P5, P6 y P6.1, siendo la horquilla P6.1 es la región más conservada, porque es la única parte que interactúa con TERT, siendo crítica para la asociación de TERT y para la actividad catalítica de la telomerasa. Finalmente, el dominio "ScaRNA" tiene una región P7 y P8 (también denominada dominio CR7), con un motivo H/ACA, esencial en la biogénesis, localización, procesamiento y acumulación de *TERC* (Zhang y otros, 2011, Nguyen y otros, 2018).

Al margen de su función en la síntesis de los telómeros, cada vez son más los estudios que describen otras funciones "extracurriculares" de la telomerasa, tanto de TERT, como de *TERC* (Choi y otros, 2008, Santos y otros, 2004, Martínez y Blasco, 2011, Liu y otros, 2019). En concreto, nuestro laboratorio ha demostrado que *TERC* desempeña un papel extracurricular en la mielopoyesis. La deficiencia de *terc* induce neutropenia y monocitopenia en el pez cebra, independientemente de la longitud de los telómeros y de la actividad de la telomerasa (Alcaraz-Perez y otros, 2014). *TERC* es capaz de unirse al DNA a través de una secuencia consenso (Chu y otros, 2011) y a la RNA Polimerasa II (RNA Pol II), reclutándola a los promotores de los genes mieloides, aumentando la expresión de *csf3b* y *spi1b* en pez cebra o de sus homólogos humanos *CSF2* y *SPI1*, respectivamente y, por tanto, asegurando una mielopoyesis robusta (García-Castillo, y otros, manuscrito en revisión).

Los telómeros están implicados directamente en la senescencia celular, jugando un papel fundamental en los procesos de envejecimiento. A las enfermedades asociadas con el acortamiento de los telómeros se les conoce como telomeropatías o síndromes teloméricos. La primera telomeropatía detectada fue la Disqueratosis Congénita (DC). La DC se produce por mutaciones en algún componente del complejo telomerasa o en los genes de las proteínas asociadas al telómero. Los pacientes tienen un raro trastorno multisistémico hereditario. La prevalencia es de aproximadamente 1 de cada 100.000 personas y la muerte se produce a una edad media de 16 años. Las manifestaciones clínicas de la DC generalmente aparecen durante la infancia e incluyen anomalías de pigmentación reticular de la piel, leucoplasia oral y uñas distróficas. Los síntomas se acompañan de un espectro de otras enfermedades, como inmunodeficiencia, complicaciones pulmonares, enfermedades hematológicas y cáncer. La insuficiencia de la médula ósea es la principal causa de mortalidad prematura, seguida de las enfermedades pulmonares y el cáncer (revisado por (Dokal, 2011, Mason y Bessler, 2011). Estudios sobre familias con DC revelan que, aunque todas sufren acortamiento telomérico, hay una gran diversidad en la sintomatología. En concreto, los pacientes con mutaciones en *TERC* mostraron una mayor probabilidad de presentar anormalidades hematológicas y cáncer que los pacientes con mutaciones TERT (Vulliamy y otros, 2011) lo que no puede explicarse solo por acortamiento telomérico, apoyando una función extracurricular de *TERC*.

Otros síndromes teloméricos humanos son la anemia aplásica (AA, un estado de insuficiencia de la médula ósea caracterizado por bajos recuentos sanguíneos e insuficiencia de células hematopoyéticas) y el síndrome mielodisplásico (MDS, otra insuficiencia de la médula ósea donde las células no maduran adecuadamente), entre otros (revisado por ((Martinez y Blasco, 2017, Dokal, 2011). El tratamiento actual para estas telomeropatías se basa en aumentar la actividad telomérica con distintas aproximaciones o en el trasplante de órganos como médula ósea, hígado y

pulmón, en última estancia (Townsley y otros, 2016, Guinobert y otros, 2020). Sin embargo, se sigue trabajando en un tratamiento más efectivo para estas enfermedades.

Los aptámeros son novedosos agentes terapéuticos, que consisten en secuencias sintéticas de DNA o RNA monocatenarias, que adoptan estructuras tridimensionales únicas que les permiten reconocer un objetivo específico con alta afinidad (revisado por (Zhuo y otros, 2017)). Aunque el mecanismo de actuación es similar al de los anticuerpos, presentan ventajas frente a ellos, como la síntesis química (evita el uso de animales de experimentación), bajo coste de producción, fácil modificación química, pequeño tamaño, elevada estabilidad y baja toxicidad. Sus usos potenciales incluyen: diagnóstico de enfermedades (Chen y otros, 2017), detección de peligros alimentarios (Fadock y Manderville, 2017), biosensores (Miranda-Castro y otros, 2016), detección de toxinas, portadores de fármacos (Li y otros, 2018) y señalización de nanopartículas, entre otros.

El pez cebra (*Danio rerio*) es uno de los modelos animales más utilizados en investigación, debido a sus indudables ventajas respecto a otros modelos de vertebrados, como el amplio conocimiento de su genoma y gran similitud genética con los humanos (Howe y otros, 2013, Kettleborough y otros, 2013). A esto hay que sumarle otras ventajas como la fertilización externa de los embriones trasparentes y su elevada fecundidad (Patton y Zon, 2001), el bajo coste y poco espacio necesario para su mantenimiento y la facilidad de manipulación genética y generación de líneas transgénicas mediante el uso de tecnología CRISPR/Cas9 (Li y otros, 2016, Meng y otros, 2008, Albadri y otros, 2017, Ward y otros, 2018), entre otras.

Estas ventajas han llevado al pez cebra a ser propuesto como un excelente modelo para diferentes estudios biomédicos, como el de la hematopoyesis, debido a que la transparencia y fertilización externa de los embriones permite visualizar *in vivo* el desarrollo de la hematopoyesis.

2. Objetivos

- Caracterización molecular de los dominios *TERC* responsables de su función hematopoyética.
- Desarrollo de aptámeros derivados de *TERC* para tratar enfermedades hematológicas.
- Identificación del interactoma de *TERC*.

3. Resultados y discusión

3.1. Caracterización molecular de los dominios *TERC* responsables de su función hematopoyética no canónica

Estudios previos de nuestro laboratorio han demostrado que *TERC* actúa regulando la mielopoyesis en modelos de pez cebra y líneas celulares humanas. De esta manera, en el modelo de pez cebra, una disminución de los niveles de *terc* da lugar a una disminución de la mielopoyesis, caracterizada por una neutropenia y monocitopenia, mientras que la sobreexpresión de *terc* activa la mielopoyesis y promueve la neutrofilia. El estudio molecular del mecanismo ha revelado que *TERC* actúa como un factor de transcripción en líneas celulares humanas y en pez cebra, capaz de interactuar físicamente con una determinada secuencia de DNA conservada, que se encuentra en los promotores o "enhancers" de genes clave en la regulación de la mielopoyesis y con la RNA pol II. Ambas interacciones son necesarias para el inicio de la transcripción de estos genes, ya que las mutaciones de la secuencia en el DNA o el bloqueo de la interacción *TERC*/ RNA pol II disminuye la expresión de los genes mielopoyéticos.

En este estudio se quiso profundizar en la función de los dominios de *TERC* en la mielopoyesis a fin de poder clasificar a los pacientes de DC y anticipar la aparición de la enfermedad. Para ello se obtuvieron un conjunto de mutantes de la molécula de *terc* de pez cebra, basados en mutaciones observadas en pacientes con DC (Ly y otros, 2005) en diferentes dominios. El primer mutante portaba dos mutaciones puntuales, C77U y A125C, en el dominio "pseudoknot-template" (*terc-template*^{M}), el segundo una única mutación G194A en el dominio CR4/CR5 (*terc-CR4/CR5*^{M}) y el último consistía en una deleción de 68 nucleótidos en el dominio del ScaRNA del extremo 3' de la molécula (*terc-ScaRNA*^{M}). La microinyección de estos mutantes en el pez cebra aumentó el número de neutrófilos de manera similar a la molécula silvestre, excepto en el caso del mutante en CR4/CR5. Estos resultados se confirmaron, además, mediante ensayos de luciferasa con el promotor de *csf3b*, donde *terc-CR4/CR5*^{M} fue el único incapaz de aumentar la actividad luciferasa.

Para confirmar que el dominio CR4/CR5 media la función extracurricular de *terc* en la mielopoyesis, se midió la interacción de *terc-CR4/CR5*^{M} con la RNA Pol II y su capacidad de unión al DNA *in vitro*. Los resultados indicaron que la mutación del dominio CR4/CR5, conservada en pacientes con DC, disminuyó la afinidad de *TERC* por la RNA Pol II, a pesar de tener intacta su afinidad de unión al DNA.

Para investigar si este mecanismo se conserva en el humano, se utilizaron dos líneas de células madre pluripotentes inducidas (iPS) derivadas de pacientes con DC. Una de ellas tenía una mutación en heterocigosis en el dominio CR4/CR5 de *TERC* (G319A) (Boyraz y otros, 2016), exactamente la misma mutación que se ha analizado en todos nuestros estudios con pez cebra, mientras que la otra línea celular iPS tenía una mutación en heterocigosis cambio de sentido en TERT (A716V) (S. Agarwal, sin publicar). Estas iPS fueron sometidas a experimentos de diferenciación mieloide. Como control del experimento, se incluyó una línea celular iPS derivada de un donante sano (HD). De manera consistente con nuestros hallazgos en el pez cebra, la mutación en el dominio CR4/CR5 de *TERC* redujo la capacidad de las células iPS para generar colonias mieloides maduras, sin afectar la eritropoyesis, mientras que el mutante de TERT se comportó como el control HD.

En resumen, el dominio CR4/CR5 de *TERC* fue responsable de la regulación de la mielopoyesis independientemente de TERT, en pez cebra y humano. *terc* realiza esta función extracurricular reclutando la RNA Pol II al promotor del gen *csf3b*, clave en la diferenciación de neutrófilos, a través de la unión a determinadas secuencias consenso del DNA, asegurando una mielopoyesis robusta. Este mecanismo se ve afectado en los pacientes con DC con una mutación CR4/CR5, ya que impediría una interacción fuerte con la RNA Pol II, a pesar de no alterar su afinidad de unión al DNA, lo que daría lugar a una disminución de la expresión de los genes mieloides y, en última instancia, a una mielopoyesis pobre.

Nuestros resultados proporcionan nuevos mecanismos moleculares sobre la patogénesis de la DC y en particular en los pacientes con DC que albergan mutaciones que afectan al dominio CR4/CR5 de *TERC*, podrían servir de base para diseñar nuevas estrategias para una intervención terapéutica. Además, este conocimiento ayudaría a estratificar mejor a los pacientes con DC anticipándonos a la aparición de los síntomas y avanzando hacia una medicina personalizada.

3.2. Desarrollo de aptámeros derivados de *TERC* para tratar enfermedades de la sangre

El RNA de telomerasa tiene una función muy importante en la mielopoyesis al regular la expresión de los genes mieloides. El dominio CR4/CR5 de *TERC* es esencial en esta función extracurricular, no viéndose implicados otros dominios.

Sobre la base de estos conocimientos, diseñamos una estrategia terapéutica hipotetizando que el dominio CR4/CR5 de *terc* pudiera realizar la función mielopoyética por él mismo. Para ello se sintetizaron varios aptámeros basados en el dominio CR4/CR5 de *terc* de pez cebra. Los aptámeros son oligonucleótidos o péptidos de cadena simple, capaces de reconocer específicamente y con gran

afinidad las moléculas diana. Son pequeños, baratos, fáciles de sintetizar y no presentan inmunogenicidad *in vivo*. Esto los convierte en candidatos ideales para aplicaciones terapéuticas (Zhu y Chen, 2018).

Se diseñaron los siguientes aptámeros para ser probados preclínicamente en el modelo de pez cebra:

i) *CR4CR5*: Aptámero basado en el dominio CR4/CR5 completo de *terc* con 79 nucleótidos.

ii) *AA*: Aptámero basado en la horquilla P6.1 de dominio CR4/CR5 con 17 nucleótidos. Esta horquilla fue seleccionada porque la mutación G305A en los humanos (G194A en el pez cebra) produce AA (Carroll y Ly, 2009). Como control, se diseñó otro aptámero con misma secuencia, pero que contenía la mutación de los pacientes con DC. Interesantemente, utilizando un programa de predicción de estructuras secundarias/*terc*iarias de RNA, observamos que la mutación G194A alteraba drásticamente su estructura, sugiriendo la importancia de la conservación de la estructura terciaria de *terc* para realizar su función en la mielopoyesis y anticipando su relevancia en la DC.

iii) *MDS*: Este aptámero consta de parte de los subdominios P4.2 y P5 del dominio CR4/CR5 y tiene una longitud de 44 nucleótidos. En el subdominio P5 hay dos mutaciones (G322A y G323T en humanos y G206U y A208U en el pez cebra) que se encuentran en pacientes con DC que desarrollan MDS (Carroll y Ly, 2009). Como control se diseñó otro aptámero con las mutaciones de los pacientes. Sorprendentemente, en este caso, las mutaciones no alteraron fuertemente la estructura si la comparamos con el aptámero sin mutaciones.

iv) *CR7*: Se trata de un aptámero control. Se diseñó basándose en el dominio "ScaRNA", que no está implicado en la mielopoyesis. Consiste en la horquilla CR7, que contiene la horquilla P8 y tiene 46 nucleótidos de largo. Mutaciones en este dominio no tienen un efecto en la mielopoyesis.

Los estudios funcionales en el modelo de pez cebra permitieron determinar que los aptámeros *CR4CR5, AA* y *MDS* eran capaces de aumentar el número de neutrófilos. Además, tanto *CR4CR5*, como *AA,* fueron capaces de aumentar los niveles de transcrito de los genes mieloides *spi1b* y *csf3b*, de forma similar a *terc*. El aptámero *MDS* fue descartado, ya que no activaba los genes mieloides como lo hacía la molécula entera de *terc* y preferimos, por tanto, focalizarnos en los aptámeros *AA* y *CR4CR5*.

La especificidad de los aptámeros fue estudiada en diferentes líneas transgénicas de pez cebra con las diferentes células sanguíneas marcadas con proteínas fluorescentes, donde se observó que los aptámeros aumentan el número de monocitos, pero no afectan al de eritrocitos. Este

resultado demostraba la especificidad de acción de los aptámeros, lo que es extremadamente interesante para su uso terapéutico.

Mecanísticamente, los aptámeros funcionaban como la molécula de *terc* completa; es decir, i) aumentaban la expresión de los genes reguladores de la mielopoyesis, independientemente de la expresión de *terc* endógena y de la actividad telomerasa. ii) se unían a secuencias de DNA de unión a *terc* que se encuentran en las secuencias reguladoras de estos genes diana e iii) interaccionaban con la RNA Pol II, reclutándola a los promotores o "enhancers", favoreciendo así la transcripción. Es importante destacar que los aptámeros que albergan mutaciones en CR4/CR5 conservadas en los pacientes con DC no pudieron realizar todas estas funciones.

Los modelos preclínicos de neutropenias en pez cebra, como son las enfermedades de DC (deficiencia de *terc*) y poikiloderma con neutropenia (PN, deficiencia de usb1) demostraron el potencial terapéutico de los aptámeros. Además, los aptámeros humanos equivalentes a *AA*, aumentaron la expresión de genes mieloides promoviendo la mielopoyesis en las células madre pluripotentes inducidas humanas. Por tanto, en este estudio se han desarrollado dos herramientas terapéuticas con gran potencial para usar en pacientes con DC, PN u otras neutropenias.

3.3. Identificación del interactoma de *TERC*

Se ha demostrado que *TERC* desempeña un papel extracurricular en varios procesos biológicos, más allá del mantenimiento telomérico (Alcaraz-Pérez y otros, 2014, Liu y otros, 2019). Hemos desvelado en este trabajo como *terc* ejerce su función en la mielopoyesis. Sin embargo, no descartamos que puedan intervenir más proteínas o tener más funciones, más allá del telómero. Con el fin de conocer y clarificar cómo actúa y en qué otros procesos podría estar implicado *terc*, se realizó un experimento *ex vivo* de purificación de interactores. Para ello, extractos de proteínas de larvas de pez cebra de 5 días de edad se incubaron con *terc* biotinilado, unido a bolas de estreptavidina. Las proteínas eluídas fueron analizadas por cromatografía líquida (LC) seguida de espectrometría de masas en tándem (MS/MS). Como control de las interacciones inespecíficas, se utilizó el RNAm de *terc* antisentido y el de GFP.

Nuestros resultados mostraron que *terc* fue capaz de unirse a 96 proteínas implicadas en varios procesos biológicos de gran importancia, como el plegamiento, la degradación (ubiquitinación) y la traducción de proteínas, el metabolismo del carbono y los lípidos, la degradación de RNA mensajero mediada por mutaciones terminadoras, el metabolismo de RNA, la biogénesis mitocondrial y el ciclo celular. Curiosamente, no se detectó a Tert como un interactor,

tal vez porque las bolas de estreptavidina bloquearon la interacción o afectaron parcialmente la estructura. Todo ello nos sugiere que se pueden estar perdiendo otros interactores, aunque nos permite afirmar que los interactores identificados no lo hacen a través de Tert, lo que es interesante para nuestros estudios focalizados *terc*.

Con el objetivo de estudiar si este interactoma se ve alterado en los pacientes de DC se repitió el procedimiento utilizando las dos versiones mutantes de *terc* en el dominio CR4/CR5. Por un lado, el mutante *tercP5*, que posee dos mutaciones conservadas en pacientes con DC en la horquilla P5 (G206U y A208U), característica de los pacientes con DC que presentan síndrome mielodisplásico (Carroll y Ly, 2009). Por otro lado, el mutante *tercP6.1*, que posee una única mutación (G194A) en la horquilla P6.1, característica de los pacientes con DC que presentan anemia aplásica (Carroll y Ly, 2009).

El análisis del proteoma de *tercP5* y *tercP6*.1 mostró un interactoma completamente idéntico al identificado con la molécula de *terc* silvestre, indicando que estas mutaciones no confieren una ganancia de función, concordando con estudios previos de pacientes de DC, donde la haploinsuficiencia de *terc* es la responsable de la enfermedad (Mason y Bessler, 2011). Sin embargo, se observó que estas mutaciones dieron lugar a una menor cantidad de interactores, cuestión que no pudo ser resuelta, ya que este análisis proteómico no es un método cuantitativo. Para la cuantificación de los interactores, sería necesario un etiquetado isotópico estable por proteómica basada en aminoácidos (SILAC) de estas muestras.

En general, el análisis del proteoma de *terc* es un gran avance para descifrar todas las funciones de este RNA más allá de la biología telomérica. La caracterización de estas interacciones podría ayudar a mejorar el diagnóstico y el tratamiento de los pacientes con mutaciones en *TERC*.

4. Conclusiones

De los resultados obtenidos en este trabajo se pueden obtener las siguientes conclusiones:

1. El dominio CR4/CR5 de *TERC* es esencial en la mielopoyesis, ya que mutaciones de pacientes con DC encontradas en este dominio disminuyen su interacción con la RNA Pol II y perjudican la mielopoyesis en el pez cebra, a pesar de que no afectan la afinidad de unión al DNA de *terc*. De manera similar, las células iPS derivadas de un paciente con DC con una mutación en CR4/CR5 muestran una mielopoyesis deficiente, mientras que las de un paciente con una mutación en TERT se diferencian como las células control.

2. Los aptámeros derivados del dominio CR4/CR5 de *terc* estimulan la mielopoyesis sin afectar la eritropoyesis en el pez cebra. Funcionan como la molécula de *terc* completa; es decir, aumentan la expresión de los principales genes mieloides independientemente del *terc* endógeno, al unirse a sus secuencias diana de DNA presentes en las regiones reguladoras de estos genes y a la RNA Pol II, reforzando su transcripción. Sin embargo, los aptámeros que albergaban las mutaciones en CR4/CR5 encontradas en los pacientes con DC no cumplieron todas estas funciones.

3. Los aptámeros rescatan la neutropenia en modelos preclínicos de pez cebra de DC y PN. Además, los aptámeros humanos derivados del dominio CR4/CR5 de *TERC* aumentan la mielopoyesis en las células iPS humanas, demostrando su potencial terapéutico para tratar la neutropenia causada por diferentes alteraciones genéticas.

4. *terc* interactúa con 96 proteínas implicadas en procesos biológicos clave, como el plegamiento, degradación (ubiquitinación) y traducción de proteínas, el metabolismo del carbono y lípidos, la degradación de RNA mensajero mediada por mutaciones terminadoras, la biogénesis mitocondrial y el ciclo celular.

5. La molécula de *terc* con mutaciones en el dominio CR4/CR5 encontradas en pacientes con DC, muestra un interactoma similar a *terc*, a pesar de que estas mutaciones perjudican tanto la actividad de la telomerasa, como la regulación de la mielopoyesis en el ser humano, lo que apoya la noción de que la DC es causada por la haploinsuficiencia de *TERC*, sin tener un efecto dominante.

6. El interactoma de *terc* prepara el camino para futuros estudios funcionales destinados a aclarar aún más los papeles no canónicos de *terc* que podrían ayudar a mejorar el diagnóstico y el tratamiento de los pacientes con mutaciones en *TERC* al revelar nuevos biomarcadores de pronóstico y objetivos terapéuticos.