Telomeres and telomerase: importance in human cancer, the premature

aging syndrome Dyskeratosis congenita and frailty

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A thesis submitted to McGill University in partial fulfillment of the

requirements of the degree of

Doctor of Philosophy

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À mon blinge

ACKNOWLEDGMENTS

The world of scientific research is unbelievably exciting and gratifying, and I could not imagine myself doing anything else. I could have never been a scientist however if it was not for the people I met throughout my life, the scientists and the non-scientists, the ones I met in the past, and the ones who are still surrounding and supporting me.

First, I would like to thank my thesis supervisor, Dr. Chantal Autexier for her support, comprehension and guidance. She is a very complete person, a great mentor and motivator, and I was very lucky to find in you an honest and understanding person with whom I can discuss freely of everything.

I would also like to thank Dr. Stéphane Richard for his extreme generosity, his guidance and precious advice regarding my career. My experience at the LDI would not have been the same without you and your lab. Many thanks to the members of my advisory committee: Dr. Elaine C. Davis, Dr. Peter Siegel and Dr. Stefano Stifani for your advices, support, patience and encouragements.

Finally, I would like to thank the Cole Foundation, the McGill Department of Anatomy and Cell Biology and the Fonds de la Recherche en Santé du Québec (FRSQ) for precious financial support.

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Je tiens à remercier spécialement mes collègues et amis : Fred, Mathieu et Olivier. C'est grâce à vous que mon séjour au LDI a été agréable. Je suis heureuse de vous avoir eu auprès de moi et vous allez me manquer. Un grand merci à ma coloc de lab Catherine, à ta disponibilité, ton grand sens de l'organisation et à nos discussions. Merci aux mentors que j'ai eu la chance de rencontrer au cours de ma carrière : Dr. Georges Lévesque, Dr. Madeleine Carreau, Nathalie Rouleau, Dr. Roger Bossé

À ma famille, maman, papa, Jean-Simon, Marie-Josée, grandmaman Dodo pour votre amour, votre support inconditionnel et votre générosité. À Marie-Pier et Anne-Marie, votre amitié est très précieuse.

À Paul, pour ton amour, ton support, ta générosité, ta compréhension. Merci d'avoir partagé mon fardeau depuis le début, merci pour les visites au labo au milieu de la nuit, les détours en voiture, merci d'avoir fait semblant de travailler pour m'accompagner et d'avoir dormi sur le divan du centre de recherche. Merci surtout d'avoir tout partagé avec moi sans compter pour m'aider à réussir dans ce que je fais. C'est grâce à toi que je me suis rendue jusqu'ici, et grâce à toi que je peux continuer d'avancer aujourd'hui encore.

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ABSTRACT

Telomeres and telomerase stand at a junction of cellular processes that govern aging, cancer and disease. Premature aging syndromes and age-related diseases are characterized by short telomeres which compromise cell function and viability, whereas cancer cells are able to reactivate telomerase or alternative lengthening of telomeres (ALT) mechanisms to maintain their telomeres and become immortal.

Telomeres and telomerase represent very attractive targets for the development of anticancer therapies. However, there is concern that these therapies may lead to cell resistance, including the reactivation of telomerase or ALT in telomerase-positive cells. Here we show that telomeric recombination can be promoted by telomere-induced dysfunction, an anticancer strategy currently in development, despite the presence of an active telomerase. Our results highlight an important potential mechanism of cancer cell resistance, but might also help to understand the interplay between telomerase and the ALT pathway in the cell.

Defective telomere maintenance is associated with premature or accelerated-aging disease. Mutations in almost every component of the telomerase holoenzyme have been found to be implicated in Dyskeratosis

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congenita (DC), revealing the importance of a functional telomerase for stem cell maintenance and cell proliferative potential. We found that the telomerase component dyskerin is sumoylated on highly conserved lysines and that lysine-to-arginine dyskerin mutants reproduce the phenotype observed in DC. Our findings identify that impaired posttranslational modifications can lead to DC, and importantly point to new possibilities in the treatment of DC.

Finally, we investigated the complex relationship between telomere maintenance, frailty and cardiovascular disease. We examined the feasability of measuring telomere length as a predictor of morbidity in elderly patients undergoing cardiac surgery. Our preliminary data do not identify telomere length as a predictor of surgery outcomes. Our findings also suggest that telomere length measurement in an

epidemiological/clinical context must be interpreted with great caution.

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RÉSUMÉ

Les télomeres et la télomérase se rencontrent à la jonction de processus cellulaires qui régulent le vieillissement, le cancer et certaines maladies. Plusieurs maladies de vieillissement précoce ou maladies associées au vieillissement sont caractérisées par la présence de courts télomères, qui compromettent la viabilité et la fonction de la cellule, alors que les cellules cancéreuses sont capables de réactiver la télomérase ou un mécanisme alternatif d'élongation des télomères (ALT) pour maintenir leurs télomères et devenir immortelles.

Les télomères et la télomérase représentent des cibles très attrayantes pour le développement de thérapies anti-cancer. Il existe toutefois certaines possibilités que ces thérapies conduisent au développement d'une résistance chez la cellule. Ces mécanismes de résistance peuvent inclure la réactivation de l'enzyme télomérase ou réactivation du mécanisme ALT dans les cellules télomérase-positives.

Nous démontrons que la recombination des télomères peut être induite par une dysfonction des télomères, une stratégie anti-cancer présentement en développement, et cela malgré la présence de télomérase dans la cellule. Nos résultats mettent l'emphase sur un mécanisme potentiel de résistance, et pourraient aussi permettre de

mieux comprendre comment le mécanisme ALT et la télomérase sont régulés dans la cellule.

Un maintien déficient des télomères est associé aux maladies de vieillissement précoce ou accéléré. Des mutations dans la majorité des composants de l'enzyme télomérase ont été retrouvées chez des patients atteints de Dyskératose congénitale (DC), révélant l'importance d'une télomérase fonctionnelle pour la fonction des cellules souches et le potentiel réplicatif des cellules. Nous avons identifié que la protéine dyskérine est sumoylée sur des lysines hautement conservées et que la présence de protéines mutantes dans la cellule imite les phénotypes associés à la maladie DC. Nos résultats démontrent qu'un défaut de modifications post-traductionnelles peut conduire à la maladie DC mais surtout, identifient de nouvelles possibilités pour le traitement des patients atteints de DC.

Finalement, nous avons investigué la relation complexe qui existe entre le maintien des télomères, la fragilité et les maladies cardiovasculaires. Nous avons examiné le potentiel de mesurer les télomères pour prédire la mortalité et morbidité chez des patients âgés sur le point de subir une chirurgie cardiaque. Nos résultats préliminaires indiquent que la taille des télomères ne peut servir à prédire l'issue d'une

chirurgie cardiaque. Nos résultats suggèrent que l'utilisation de la taille des télomères comme marqueur dans un contexte épidémiologique ou clinique doit être étudiée et interprétée avec précaution.

Preface

This Ph.D. thesis is written in accordance with the guidelines for the thesis preparation of the Faculty of Graduate and Postdoctoral Studies of McGill University. A brief introduction and comprehensive review of the literature are presented in Chapter 1. The description of the Material and Methods used for this work follows in Chapter 2. Chapters 3, 4 and 5 present the results obtained. Because each chapter focuses on a different theme, they are preceded by a brief preface that helps to situate them in a broader research context. The results presented in Chapter 3 were previously published as a manuscript. Chapter 4 and 5 describes the results of two manuscripts currently in preparation. The results presented in Chapter 5 originate from a collaborative project with a clinical aspect and broader area of research, but only the molecular part of the project and experimentation is presented. A detailed discussion is presented in Chapter 6 and the references appear at the end of the thesis.

Published or manuscripts in preparation included in this thesis: **Brault, M.E.** and Autexier, C. (2011) ALTered telomeres in response to telomere dysfunction. Cell cycle 10 (22) :3807-9.

Brault, M.E. and Autexier, C. (2011) Telomeric recombination induced by dysfunctional telomeres. Molecular Biology of the Cell 22:179-88. (Chapters 1, 2, 3 and 6)

Brault, M.E., Lauzon, C. and Autexier, C. (2011) Sumoylation regulates DKC1 trafficking to maintain human telomerase RNA stability and promote telomere length maintenance. To be submitted to Nature Cell Biology.

Brault, M.E., Afilalo, J., Kwan, R., Ohayon, S., Boivin, J.F., Autexier, C. (2011) Telomere length as a biomarker to predict morbidity and mortality in elderly patients undergoing invasive cardiac surgery. To be submitted to The American Journal of Cardiology.

In additional to the data presented in the thesis, the student has contributed to the following published work:

Marie-Egyptienne, D.T., **Brault, M.E.**, Nimmo, G.A., Londono-Vallejo, J.A., Autexier, C. (2009) Growth defects in mouse telomerase RNA-deficient cells expressing a template-mutated mouse telomerase RNA. Cancer Letters 275:266-76.

The first two authors contributed equally to this work.

Marie-Egyptienne, D.T., **Brault, M.E.**, Zhu,S., Autexier, C. (2008) Telomerase inhibition in a mouse cell line with long telomeres leads to rapid telomerase reactivation. Experimental Cell Research 314:668-75.

Brault, M.E., D'Souza, Y. and Autexier, C. (2007) Telomerase structure, function and evolution. In: Origin and Evolution of Telomeres. Lubo Tomaska and Jozef Nosek, eds. Eureka.com and Kluwer Academic/Plenum Publishers, New York, NY. The first two authors contributed equally to this work.

Contribution of authors

The candidate performed nearly all the experimental work presented in this thesis and wrote all the manuscripts. The candidate proposed and formulated the hypotheses, rationale and design of the research projects described in Chapter 3 and 4. The TRAP assays (Figure 4-6, 4-7) in Chapter 4 were performed by Catherine Lauzon, which is the second author of the manuscript in preparation. The data presented in Chapter 5 result from a project originally proposed by Dr. Jonathan Afilalo and designed and performed by the thesis candidate. All studies were done under the supervision and with the support of Dr. Chantal Autexier.

Contribution to original knowledge

The work presented in this thesis focuses on the role of telomeres and telomerase in cancer, age-related diseases and aging. In addition, it explores the role of posttranslational modifications in telomerase function and disease. Finally, it analyzes the feasibility of utilizing telomere length as a biomarker to predict the outcomes of surgery. The major contributions to original knowledge are summarized below :

- Telomeric recombination can be induced by telomere dysfunction in telomerase-positive cells.
- 2. Sumoylation of dyskerin, a component of the telomerase holoenzyme, could be important for telomerase function.
- Defects in dyskerin sumoylation can potentially cause the disease Dyskeratosis congenita.
- Circulating leukocytes telomere length cannot always predict telomere length of every tissue.
- The utilization of telomere length as a biomarker cannot be applied to all population of individuals and all types of disease or condition.

List Abbreviation

APB	ALT-associated PML bodies
ALT	alternative lengthening of telomeres
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3 related
ATRX	α -thalassemia/mental retardation syndrome X-linked
a.u.f.	arbitrary unit of fluorescence
CAB	Cajal bodies motif
CO-FISH	chromosome orientation fluorescence hybridization
CR4/CR5	conserved regions 4/5
CR7	conserved region 7
CVD	cardiovascular disease
Daxx	death-domain–associated protein
DC	dyskeratosis congenita
DDR	DNA-damage response
CTE	C-terminal extension motif
DKC1	dyskerin
D-loop	displacement loop
DNMT	DNA methyltransferase
ECTR	extra-chromosomal telomeric repeats
ERCC1	excision repair cross-complementing 1
FBS	fetal bovine serum
FISH	fluorescence in situ hybridization
GAR	Glycine/Arginine rich domain
y-H2AX	phosphorylated histone H2AX
HCSM	hydrophobic cluster sumoylation motif
hTERT	human telomerase catalytic subunit
Htr	human telomerase RNA
dn-hTERT	dominant negative hTERT

HA	hemagglutinin tag
HR	homologous recombination
IC	internal PCR control
ΙκΒα	nuclear factor- κB inhibitor α
Kb	kilobases
kDa	kilo Dalton
MEF	mouse embryonic fibroblast
MuA-hTR	hTR with point mutation in the template domain
NBS	Nijmegen breakage syndrome
NF-κB	nuclear factor-кВ
NHEJ	non homologous end joining
NLS	nuclear localization signal
NOP10	Nucleolar protein 10
nt	nucleotides
NTE	N-terminal extension
OB fold	oligonucleotide binding fold
PBS	phosphate-buffered saline
PD	population doubling
PFGE	pulse field gel electrophoresis
2D-PFGE	two dimensional pulse field gel electrophoresis
PML bodies	promyelocytic leukemia bodies
PNA	peptide nucleic acid
PUA	pseudouridine synthase and archaeosine-specific
	transglycosylases domain
Q-FISH	quantitative fluorescence in situ hybridization
RanGAP1	Ran GTPase-activating protein 1
RAP1	Repressor/Activator Protein 1
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RID1/2	RNA binding domain 1 and 2

RNP	ribonucleoprotein
RPA	replication protein A
RT	reverse transcriptase
RTL	relative telomere length
RRL	rabbit reticulocyte lysate
scaRNA	small Cajal bodies RNA
shRNA	short hairpin RNA
snRNA	small nuclear RNA
snoRNA	small nucleolar RNA
STE	stem terminus element
STS	Society of Thoracic Surgeons
TCAB1	telomerase Cajal body protein1
TIN2	TRF2- and TRF1-Interacting Nuclear protein 2
T-motif	telomerase-specific motif
T-SCE	telomeric sister chromatid exchanges
TIF	telomere dysfunction induced foci
TRAP	telomeric repeat amplification protocol
TRF	telomeric restriction fragment
TRFI/2	telomeric repeat binding factor 1/2
TRFH	TRF homology region
TruA/B	catalytic pseudouridine synthase domain
UBC9	ubiquitin-conjugating enzyme 9
WRN	Werner
wt-hTR	wild-type hTR
ZBTB1	zinc finger and BTB domain protein 1

Chapter 1

Literature review

1.1 Telomeres and Telomerase : the guardians of genomic integrity

In 2009, the Nobel Prize in Physiology or Medicine was awarded to Elizabeth H. Blackburn, Carol W. Greider and Jack W. Szostak for their discovery of "how chromosomes are protected by telomeres and telomerase". Their work provided the answer to a long-standing fundamental question in biology: why native chromosome ends are not mistaken from double-strand DNA breaks by the cellular DNA repair machinery? The answer came in 1982 when Blackburn and Szostak demonstrated that repeated sequences called telomeres are protecting chromosome ends to maintain genomic stability, and that their function is evolutionary conserved. Three years later, Greider and Blackburn identified a unique ribonucleoprotein (RNP) enzyme, telomerase, able to add de novo telomere repeats onto chromosome ends. Subsequent research showed that telomeres shorten with successive cell division and contribute to the onset of replicative senescence, underlying a critical role for telomeres in cell aging. Inversely, increased telomerase activity and telomere lengthening is associated with cellular immortality, connecting telomeres and telomerase to cancer. Many efforts have been made to reactivate telomerase and delay cellular senescence of human cells, not only to prevent cellular aging but also perhaps to cure premature and

accelerated aging diseases. On the other hand, attempts to inhibit telomerase in cancer cells are made with the hope of stopping their growth and triggering their death. Telomere and telomerase therapeutics however face many challenges including the potential cancer-promoting role of telomerase in the fight against cellular aging, and the reactivation of telomerase or other mechanisms of resistance when cancer cells are targeted. The Nobel laureates' discoveries have inspired many new ideas in the fields of cancer, aging, stem cell biology and human disease but thirty years later, much remains to be discovered.

1.1.1 Chromosome ends are naturally protected

The existence of special structures at the end of chromosomes was proposed even before the elucidation of the double helix nature of DNA by Francis Crick and James Watson in 1953 (Watson and Crick, 1953). Cytogenetic experiments by Hermann Muller (Nobel Prize 1945) and Barbara McClintock (Nobel Prize 1983) showed that broken chromosomes result in chromosomal instability whereas the naturally occurring chromosome ends are protected (McClintock, 1939; Muller, 1938). The structures, named telomeres (from the Greek telos "end" and meros "part") by Muller, were predicted to confer to the cell the ability to distinguish native chromosome ends from DNA double-strand breaks. However, the molecular nature of telomeres was unknown.

1.1.2 Chromosomes shorten with cellular division

The discovery of DNA structure by Watson and Crick (Nobel Prize 1962) and existence of DNA polymerases by Kornberg (Nobel Prize 1959) raised more questions about chromosome ends. Conventional DNA polymerases replicate DNA only in the 5' to 3' direction and require short RNA primers for initiation. Hence, while synthesis of the leading strand in the 5'-3' direction results in complete strand replication, synthesis of the lagging strand and removal of the terminal RNA primer results in DNA loss at the 5'end (Figure 1-1). Olovnikov and Watson proposed that this phenomenon, referred to as "the end-replication problem", would lead to a newly synthesized DNA molecule with a single-strand tail and to shortening of chromosomes with successive divisions (Olovnikov, 1973; Watson, 1972). Olovnikov proposed a link between telomeric DNA shortening and the Hayflick limit (Olovnikov, 1971, 1973), which refers to the maximum number of cell divisions that human cells can undergo when cultured in vitro (Hayflick and Moorhead, 1961). Cells that reach the

Hayflick limit enter into a state that has been named "replicative



Figure 1-1: The end-replication problem.

The telomere G-rich strand is assembled by leading strand synthesis while the telomere C-rich strand is assembled by lagging strand synthesis. RNA primers (in blue) are removed after replication and leave gaps that are filled using the adjacent Okazaki fragments as primers. Removal of the most distal RNA primer results in a terminal gap that cannot be filled because of the absence of an Okazaki fragment in 5'. The newly synthesized molecule inherits a single-strand tail or 3' overhang.

1.1.3 Repeated DNA sequences, or telomeres, can confer genomic

stability

In the following years, several hypotheses were made to explain how telomeres might confer chromosome protection. Some models suggested that chromosomes contain palindromic sequences that generate hairpin structures able to circumvent the end-replication problem

(Bateman, 1975; Cavalier-Smith, 1974). In 1978, Blackburn and Gall reported that the telomeres of ribosomal DNA (rDNA) molecules of the ciliated protozoan Tetrahymena thermophila contain between 20 to 70 tandem repeats of the hexanucleotide CCCCAA (Blackburn and Gall, 1978). Later, Blackburn and Szostak collaborated to show that ribosomal telomeric DNA from Tetrahymena can be ligated to the ends of a linearized yeast plasmid and provide chromosomal stability (Szostak and Blackburn, 1982). Subsequent studies showed that yeast telomeres contain the tandem repeat sequence C₁₋₃A and that telomere sequences of both Tetrahymena and yeast could be extended in yeast (Shampay et al., 1984). Considering the restricted homology between Tetrahymena and yeast telomeric repeats, Shampay et al. suggested that addition of telomeric DNA was unlikely a result of recombination or transposition events (Shampay et al., 1984). Rather, they proposed the existence of a terminal transferase-like activity, able to synthesize telomeric repeats onto chromosome ends de novo.

1.1.4 Telomerase : a novel DNA polymerase that requires a protein and a RNA component for activity

In 1985, Greider and Blackburn demonstrated the existence of the putative transferase-like enzyme in Tetrahymena cell extracts, by showing that Tetrahymena TTGGGG repeats can be added to a synthetic DNA oligonucleotide primer (TTGGGG)₄ used to initiate synthesis. Moreover, they showed that the enzymatic activity is evolutionary conserved in an experiment where Tetrahymena TTGGGG repeats could be added to a yeast GTGGG primer (Greider and Blackburn, 1985). Two years later, Greider and Blackburn isolated the enzyme responsible for telomeric repeat addition, and showed that it is a RNP complex dependent on both RNA and protein components for activity (Greider and Blackburn, 1987). They named the novel RNA-dependent DNA polymerase "telomerase" and suggested that the RNA component was involved in determining the sequence of telomeric repeats added onto telomeres. In 1989, cloning and characterization of the gene encoding the Tetrahymena telomerase RNA showed that it contains the sequence CAACCCCAA, and thus the template for the synthesis of TTGGGG telomeric repeats (Greider and Blackburn, 1989). Although most initial observations were made in the

organism Tetrahymena, we now know that telomere maintenance by the enzyme telomerase is highly conserved among a wide variety of eukaryotic organisms.

1.2 Telomeres possess a capping function

Mammals and all other vertebrate telomeres are very similar to Tetrahymena telomeres and consist of tandem repeats of TTAGGG sequences, which end in a G-strand 3' overhang (Moyzis et al., 1988) (Figure 1-2). As a general rule, telomeres are composed of G- and Cstrands because the telomere strand that ends in 3' is rich in guanosine while the complementary 5' strand is rich in cytosine. Telomeric repeats are bound by a complex of six telomere-specific proteins, called the shelterin complex, which functions in protecting chromosomes and controlling telomere length (Palm and de Lange, 2008). The shelterin complex helps to form and maintain a large duplex lariat structure called the telomere-loop (t-loop). Together, (1) telomeric DNA itself, (2) the shelterin complex and (3) the t-loop mediate the protective function of telomeres also known as telomere capping (Figure 1-2).



Figure 1-2: Mammalian telomeres.

Mammalian telomeres are composed of TTAGGG repeats that end in a 3' overhang. The shelterin complex proteins TRF1, TRF2, POT1, TIN2, TPP1 and RAP1 (text in bold) bind telomeric sequences in a specific manner. The 3'-overhang can fold and invade the duplex DNA in cis, displace the G-rich strand and form the t-loop structure. A displacement loop (D-loop) is formed at the site of invasion. Telomerase access to its substrate, the single-stranded telomere terminus, is regulated by shelterin. The MRE11/RAD50/NBS1 (MRN) complex, tankyrase, Bloom's syndrome (BLM) and other proteins also interact with telomeres. Reprinted by permission from Macmillan Publishers Ltd: Nature 447: 924-31, © 2007.

1.2.1 Telomeric DNA

1.2.1.1 A notion of critical length

The length of telomeres varies from species to species, ranging from 30 base pairs (bp) in ciliates to 150 kilobases (kb) in rats and some strains of Mus musculus (Kipling and Cooke, 1990). Human telomeres span from 10 to 15kb at birth and gradually shorten with age or disease (de Lange et al., 1990; Hastie et al., 1990). The critical telomere length required for proper telomere function is under debate. Human telomeres become fusogenic upon loss of 77bp (12.8 TTAGGG repeats) (Capper et al., 2007) but transfection experiments reveal that as little as 400bp are sufficient to seed the formation of new telomeres (Barnett et al., 1993; Farr et al., 1991; Hanish et al., 1994). Hemann et al. demonstrated that it is the shortest telomere of a cellular population, and not the average telomere length, that induces telomere dysfunction (Hemann et al., 2001).

1.2.1.2 Telomeres end in a 3' overhang

In eukaryotes, telomeres end in a G-rich single-strand tail called the 3' overhang. The 3' overhang is unlikely generated during DNA replication because it would not explain the presence of a 3' overhang at the telomere end assembled by leading-strand DNA synthesis (Figure 1-1). The current accepted model is that the 3' overhang is generated by nuclease resection of the C-strand (Makarov et al., 1997). The last bases of the 3' end of human telomeres are highly variable and appear to be generated randomly in the sequence TTAGGG when telomerase is not present (Sfeir et al., 2005). In contrast, the 5' end of human telomeres

reliably ends with the sequence ATC-5' (Sfeir et al., 2005). However, it is changed to a random position within the AATCCC sequence when the POT1 shelterin protein is inhibited, suggestive of a role for POT1 in determining the structure of the 5' end (Hockemeyer et al., 2005).

1.2.1.3 The t-loop structure provides architectural protection

The 3' overhang is crucial for the function of telomeres by allowing the formation of the t-loop (Griffith et al., 1999). T-loops are formed through strand invasion of the 3' single-strand overhang into the adjacent duplex telomeric DNA (Figure 1-2). The 3' overhang base pairs with the Crich telomeric strand, displacing the original G-strand to create a displacement loop (D-loop). While a 3' overhang with a single TTAGGG repeat is sufficient for t-loop formation *in vitro*, 3' overhang of mammalian telomeres have been found to vary from 50 to 500 nucleotides (nt) (Wright et al., 1997). Although they have a broad size distribution, the size distribution of t-loops is proportional to the size range of telomeres (Griffith et al., 1999; Wang et al., 2004). It has been proposed that the t-loop structure confers protection physically, by hiding telomere termini to prevent degradation and initiation of DNA repair (Griffith et al., 1999).

1.2.2 Structure of the shelterin complex

In mammalian cells, the t-loop is formed and maintained by a complex of six telomere-specific proteins called the shelterin complex (Palm and de Lange, 2008). In addition to having t-loop formation activity, shelterin represses DNA repair factors, thereby enabling the cell to distinguish telomeres from DNA breaks, and also regulates the access of telomerase to telomeres. The six shelterin proteins specifically localize to telomeres and are abundant at the telomeres. Until recently, known shelterin functions were limited to telomeres. However, a non-telomeric role for the human ortholog of the yeast Repressor/Activator Protein 1 (RAP1) shelterin protein has been found in transcriptional regulation and NF κ B signalling (Kabir et al., 2010). A recent study also reported the binding of the two shelterin components Telomeric Repeat binding Factor 1 and 2 (TRF1 and TRF2) to extratelomeric sites (Simonet et al., 2011).

1.2.2.1 TRF1, TRF2 and POT1 confer telomeric specificity

The specificity of shelterin for telomeric DNA is mediated by three of its proteins: TRF1, TRF2 and Protection Of Telomeres 1 (POT1) which all bind TTAGGG repeats specifically (Baumann and Cech, 2001; Bilaud et al., 1997; Broccoli et al., 1997b; Zhong et al., 1992). TRF1 and TRF2

each contain a carboxy-terminal DNA-binding domain of the SANT/Myblike type which specifically binds the double-strand DNA sequence 5'-YTAGGGTTR-3' where single-base changes in the AGGGTT core are not tolerated (Bianchi et al., 1999). In addition to the SANT/Myb DNA-bindingdomain, they also share a TRF homology (TRFH) domain which is involved in the dimerization of the protein. The TRFH domain is connected to the DNA binding-domain by a non-conserved flexible hinge domain (Bianchi et al., 1997; Broccoli et al., 1997a). TRF1 and TRF2 N-termini differ: TRF1 N-terminus contains acidic amino acids while TRF2 contains a basic Gly/Arg-rich (GAR) domain. Human POT1 contains two oligonucleotide/oligosaccharide-binding (OB) folds, and sequence analysis suggests the presence of a third OB-fold (Theobald and Wuttke, 2004). POT1 is highly specific for the single-strand DNA sequence 5'-(T)TAGGGTTAG-3' which it can bind either at the 3' end or within a longer single-strand region (Lei et al., 2004; Loayza et al., 2004).

1.2.2.2 TIN2, TPP1 and RAP1 assemble and stabilize shelterin

TRF1 and TRF2 do not interact directly and they are connected together by the TRF2- and TRF1-Interacting Nuclear protein 2 (TIN2) which stabilizes TRF2 on telomeres (Ye et al., 2004a). TIN2 binds to the

TRF1 TRFH domain through its C-terminal FxLxP motif, and to the TRF2 hinge domain through a region in its N-terminus (Chen et al., 2008). TIN2 also recruits TPP1 (previously called TINT1, PTOP or PIP1) using a third protein interaction site located in its N-terminus (O'Connor et al., 2006). TIN2 plays a central role in the stability of shelterin by binding to TRF1, TRF2 and TPP1, although it is not known if TIN2 binds them simultaneously or alternates between them. Consistent with a role in stabilizing shelterin, TIN2 mutants profoundly destabilize TRF1 and TRF2 at telomeres and induce a DNA damage response (Kim et al., 2004) and disruption of the TIN2 gene results in early embryonic lethality in mice (Chiang et al., 2004). POT1 C-terminus is in turn connected to TIN2 through the POT1 interaction domain of TPP1. Surprisingly, POT1 recruitment to telomeres is mediated by its interaction with TPP1 and not its OB-fold DNA binding domains (Liu et al., 2004). Finally, RAP1 is an essential partner of TRF2 and it is found in a 1:1 stoichiometry with TRF2 (Zhu et al., 2000). It contains three domains, a BRCT domain, a Myb domain and linked by a coiled region, a RAP1 carboxy-terminal (RCT) domain which binds to TRF2 hinge domain. The mammalian RAP1 Myb domain lacks DNA-binding activity, RAP1 is thus dependent on TRF2 for telomere binding (Li and de Lange, 2003).
1.2.3 How are human chromosomes protected by shelterin

1.2.3.1 Shelterin proteins possess t-loop formation activity *in vitro*

It is presumed that shelterin protects telomeres by affecting their architecture and by forming the t-loop. This idea is consistent with the ability of TRF2 to form t-loop-like structures *in vitro* from duplex TTAGGG repeat model substrates with 3' overhangs (Stansel et al., 2001). TRF2 also possesses topoisomerase activity *in vitro* that could promote unwinding and strand invasion (Amiard et al., 2007). TRF1 in turn possess *in vitro* looping, bending and pairing of telomeric repeats activity that could assist in the folding of telomeres into a t-loop (Bianchi et al., 1997; Bianchi et al., 1999; Griffith et al., 1998). The mechanism of t-loop formation of shelterin *in vivo* remains to be determined. The t-loop is expected to assist the repression of unwanted DNA repair activities by inhibiting the access of DNA repair proteins to telomeres.

1.2.3.2 Telomeres are recognized by the ATM and ATR DNA repair pathways

The shelterin complex and t-loop inhibits DNA damage responses at the telomeres by repressing the two major pathways involved in the cellular response to DNA damage : the ataxia telangiectasia mutated (ATM) and the ATM and Rad3 related (ATR) pathways (Denchi and de Lange, 2007). ATM is activated by double-strand breaks but resection of the double-strand break during repair promotes the activation of ATR which controls the response to single-strand breaks (Jazayeri et al., 2006; Myers and Cortez, 2006). In response to DNA damage, the ATM and ATR kinases phosphorylate a set of downstream targets to activate DNA repair, cell cycle checkpoints, apoptosis or senescence (Matsuoka et al., 2007). Since telomeres are composed of both double-strand and single-strand DNA, they must be able to repress both the ATM and the ATR pathways.

1.2.3.3 Telomere uncapping induces a DNA-damage response

In mammalian cells, TRF2 inhibition through the expression of a dominant negative TRF2 variant lacking the Myb domain and the N-terminal basic domain (TRF2 $\Delta B \Delta M$) or RNA interference results in telomere

deprotection, cleavage of the 3' overhang by the nuclease excision repair cross-complementing 1 (ERCC1)/XPF1 and telomere end-to-end fusions by the non-homologous end-joining (NHEJ) pathway (Smogorzewska et al., 2002; van Steensel et al., 1998; Zhu et al., 2003). Deletion of TRF2 is lethal, a consequence of the permanent activation of DNA damage responses (Celli and de Lange, 2005). Depending on the cell type, inhibition of TRF2 results in the activation of either p53 or pRb, two tumor suppressors, and leads to premature senescence. Senescence induced by uncapped telomeres is biochemically and cytologically indistinguishable from replicative senescence, or p53- and ATM-dependent apoptosis (Smogorzewska and de Lange, 2002; van Steensel et al., 1998). In contrast, overexpression of TRF2 delays cellular senescence and acts by protecting critically short telomeres (Karlseder et al., 2002).

Following telomere uncapping or natural erosion, many doublestrand breaks response and repair factors or posttranslationally modified proteins are recruited to the telomeres including ATM-Ser1981, H2AX-Ser139, p53 binding protein 1 (53BP1), mediator of DNA damage checkpoint 1 (MDC1), RAD17-Ser645, the MRE11/RAD50/NBS1 (MRN) complex and RIF1, forming the so-called Telomere Dysfunction-Induced

Foci (TIF) (d'Adda di Fagagna et al., 2003; Dimitrova and de Lange, 2006;

Silverman et al., 2004; Takai et al., 2003).

1.2.3.4 Repression of ATM by TRF2

The DNA damage response and NHEJ following TRF2 inhibition is completely abrogated in ATM^{-/-} cells (Denchi and de Lange, 2007), suggestive of a role for TRF2 in the inhibition of ATM. The role of TRF2 in repressing ATM is supported by the autophosphorylation of ATM on Ser1981 and phosphorylation of Chk2, an ATM target, following TRF2 inhibition (d'Adda di Fagagna et al., 2003). It is not clear however whether TRF2 blocks the ATM pathway by preventing the access of the ATM pathway sensor Mre11 (Petrini and Stracker, 2003) onto telomeres or whether TRF2 represses ATM or Mre11 directly. It is known, however that TRF2 can bind ATM and block its autophosphorylation, even at nontelomeric sites of DNA damage (Karlseder et al., 2004).

1.2.3.5 Repression of ATR by POT1

While humans have a single POT1 gene, mice possess two POT1 orthologs, POT1a and POT1b (Hockemeyer et al., 2006). POT1a acts to repress the DNA damage response and Knockdown of POT1a in mouse cells induces TIFs and aberrant homologous recombination (He et al., 2006; Wu et al., 2006). The formation of TIFs in mouse embryonic fibroblasts (MEFs) following inhibition of POT1a or TPP1, is diminished when ATR is inhibited, confirming the role of POT1a in the repression of ATR (Denchi and de Lange, 2007). It has been shown that POT1a inhibits ATR activation by competing with replication protein A (RPA), a partner of ATR for DNA damage sensing, for the binding to single-strand telomeric DNA (Gong and de Lange, 2010). In contrast, POT1b but not POT1a controls telomeres 5'-end resection (Hockemeyer et al., 2006) but both POT1a and POT1b repress aberrant telomere recombination (Palm et al., 2009).

1.2.3.6 The role of shelterin in telomere length homeostasis

The role of shelterin is not limited to telomere protection, as it also participates in regulating telomere length homeostasis through a negative feedback mechanism. The abundance of shelterin proteins increases with the number of TTAGGG repeats, and can thus be used to control telomere length. Expression of a dominant negative TRF1 that lacks the N-terminal acidic domain (TRF1⁶⁶⁻⁴³⁹) induces telomere elongation while overexpression of TRF1 induces telomere shortening. Telomere elongation and telomere shortening are progressive, suggesting that TRF1 controls telomerase-dependent telomere maintenance. Accordingly, tethering experiments confirm the cis-acting effect of TRF1 on telomerase

(Ancelin et al., 2002). In turn, ATM and the MRN complex promote telomere lengthening by negatively regulating TRF1 binding to telomeres (Wu et al., 2007). Similarly, tankyrase 1 can bind to and PARsylate TRF1 thereby inhibiting its binding to telomeres *in vitro*. TRF1 PARsylation by tankyrase 1 is inhibited by TIN2 (Donigian and de Lange, 2007; Ye and de Lange, 2004).

TIN2 and TPP1 can also act as negative regulators of telomeres however, the crucial player in cis-inhibition of telomerase is POT1 (Kim et al., 1999; Loayza and De Lange, 2003; Ye et al., 2004b). POT1 is believed to act as a terminal transducer of telomere length information at the telomere terminus, which is the substrate of telomerase. Diminution of POT1 or expression of a POT1∆OB mutant, which lacks single-stranded DNA-binding activity, leads to telomerase-dependent telomere elongation (Loayza and De Lange, 2003; Ye et al., 2004b). In vitro experiments demonstrated that POT1 and telomerase compete for occupancy of 3' overhangs, suggesting that POT1 inhibits the access of telomerase to its substrate (Kelleher et al., 2005; Lei et al., 2005). However, other studies have demonstrated that POT1 and TPP1 are also telomerase processivity factors *in vitro* (Wang et al., 2007; Xin et al., 2007).

1.3 Telomerase : a specialized reverse transcriptase

In most organisms, the solution to the end replication problem is the enzyme telomerase. Telomerase is a specialized RNA-dependent DNA polymerase able to catalyze the addition of de novo telomeric repeats onto telomeres. The complementary strand is then synthesized by conventional DNA polymerase. Telomerase is active in unicellular organisms and is required for long-term survival of the population. While the human telomerase RNA subunit (hTR) is ubiguitously expressed in humans, the expression of the human telomerase reverse transcriptase (hTERT) is the limiting factor for telomerase activity. In most multicellular organisms including humans, TERT is repressed in the majority of somatic cells. In humans, low levels of telomerase are observed in highly proliferative tissues such as some intestinal crypt cells, basal cells of the epidermis, endothelial cells and hair follicles. Telomerase is also highly expressed in stem cells and germ cells, and in most cancer cells.

The RNP is composed of a reverse transcriptase, TERT, and an RNA subunit, TR (Feng et al., 1995; Greider and Blackburn, 1985, 1987; Lingner et al., 1997). As the name suggests, TERT contains the seven conserved reverse transcriptase (RT) motifs which allow the reverse transcription of an RNA template. Unlike other reverse transcriptases, the

template region is provided by the RNA component of telomerase itself, a unique feature of telomerase. In human cells, the RNA subunit of telomerase contains the template region 3'-AAUCCCAAUC-5' that serves both for annealing of the template with the 3' overhang and the addition of TTAGGG repeats. The telomerase enzyme is also unique in its ability to translocate on its substrate to perform multiple cycles of template addition, a feature named "repeat addition processivity". The ability of telomerase to catalyze the addition of each nucleotide of the template before translocating or dissociating is named "nucleotide addition processivity" (Greider, 1991).

1.3.1 Telomerase is a processive enzyme

Processive elongation by telomerase involves a recognition step, followed by multiple elongation-translocation-elongation cycles. First, the telomeric DNA substrate is recognized by the telomerase RNP, and the 3' end of the substrate is base-paired with the RNA template of hTR while the 5' end of the substate is postulated to interact with a telomerase anchor site(s). Next, nucleotide addition occurs at the 3' end of the substrate until the 5' end of the template is reached, followed by translocation/repositioning of telomerase on the newly synthesized DNA.

Another round of elongation by nucleotide addition can be initiated (Figure

1-3).



Figure 1-3:Telomere synthesis by telomerase.

Telomere synthesis involves three basic steps : a) telomerase recognizes its substrate and anneals to the 3' end of the template region of hTR helped by the anchor site(s), b) telomerase adds nucleotides one by one to the 3' end of the substrate until the 5' end of the template is reached and c) telomerase translocates and repositions on its substrate to initiate in d) a second round of nucleotide addition. Reprinted by permission from Landes Bioscience : Annual Review of Biochemistry 75, 493-517, © 2006.

1.3.2 Telomerase reverse transcriptase : a catalytic subunit

The telomerase catalytic subunit is widely conserved among organisms. All TERT family members share the seven defined RT motifs: 1, 2, A, B', C, D and E in their central region. TERT RT domain is also characterized by a large insertion between the RT motifs A and B'. RTs have been compared to a right hand with fingers (motifs 1 to B'), palm (motifs C to E) and thumb (C-terminal extension, CTE) domain, with the active site sitting in the palm. The high-resolution protein structure of the beetle *Tribolium castaneum* catalytic subunit revealed that TERT shares the same right-hand structure as other RTs. The insertion between A and B' is thus referred to as insertion in fingers domain (IFD) (Cristofari and Lingner, 2003; Lue et al., 2003). The CTE motif is essential for enzymatic activity in human and is required for telomerase processivity (Bachand and Autexier, 2001; Huard et al., 2003). In addition to the central RT domain and CTE, TERT also contains a long N-terminal extension (NTE). The N-terminal region of TERT contains two RNA binding domains: a high affinity RNA binding domain corresponding to the most C-terminal end of the NTE and motifs CP. QFP and T and a low affinity RNA-binding domain RID1/N-GQ. The two domains mediate the interaction between hTERT and hTR. The C-terminus of TERT is poorly conserved compared to the

N-terminus and RT domain. Despite weak conservation, mutations in the C-terminus of hTERT impair nucleotide addition processivity (Huard et al., 2003). Importantly, the addition of a hemagglutin (HA) tag on the C-terminus of hTERT impairs telomerase activity *in vivo* but not *in vitro*, suggesting that this region is involved in the localization of hTERT to telomeres (Counter et al., 1998).

1.3.3 Telomerase RNA : a box H/ACA RNA

Unlike TERT, TR is not conserved among species with respect to both size and sequence. The size of the RNA varies from 147 nucleotides in ciliates (Greider and Blackburn, 1989; McCormick-Graham and Romero, 1995; Romero and Blackburn, 1991) to 2030 nucleotides in yeast (Gunisova et al., 2009; Kachouri-Lafond et al., 2009). In addition, TRs are not transcribed by the same RNA polymerases: ciliate TR is transcribed by RNA polymerase III (Greider and Blackburn, 1989) while the yeast and vertebrate TRs are transcribed by RNA polymerase II (Feng et al., 1995; Singer and Gottschling, 1994). The proposed secondary structures of TR from most species share a conserved core: a large loop containing the template, a 5' template boundary element, a pseudoknot, a loop-closing helix, and a stem terminus element (STE) (figure 1-4). These regions are

essential for telomere synthesis. Larger vertebrate and yeast telomerase RNAs contain additional domains that serve in species-specific protein interaction, telomerase biogenesis, localization and accumulation (Cristofari et al., 2007; Fu and Collins, 2003; Jady et al., 2004; Lukowiak et al., 2001; Mitchell et al., 1999a; Theimer et al., 2007).



Figure 1-4: Secondary structure of hTR and known protein components

The core domain and the conserved region (CR)4/CR5 domain bind hTERT (blue) independently. The H/ACA scaRNA domain binds the RNP complex : NHP2, nucleolar protein 10 (NOP10), GAR1 and Dyskerin. TCAB1 (purple) binds both Dyskerin (green) and the CAB box located in the CR7 region. Reprinted by permission from the National Academy of Sciences : PNAS Aug.15 © 2011 (Zhang et al., 2011). Human TR (hTR) contains three major functional domains : the core domain (template/pseudoknot), the conserved region (CR)4/CR5 domain (STE), and the H/ACA small Cajal body-specific (sca)RNA domain (Ly et al., 2003). The core domain and CR4/CR5 domain bind hTERT independently and are the only domains required with hTERT to reconstitute telomerase activity *in vitro* (Autexier et al., 1996; Mitchell and Collins, 2000). The scaRNA domain is implicated in the biogenesis and regulation of telomerase *in vivo* (Mitchell et al., 1999a). The scaRNA domain binds to the four H/ACA box RNPs: GAR1, dyskerin, NOP10 and NHP2. It also contains a conserved Cajal body-specific localization signal (CAB box) (Jady et al., 2004).

1.3.3.1 Pseudoknot/core domain

The core domain of hTR is located at the 5' end of the RNA and can be divided in three regions: the template region, the P2/P3 pseudoknot (with the four helices P2a.1, P2a, P2b and P3) and the P1 helix. The four helices in the P2/P3 pseudoknot are essential for telomerase activity (Ly et al., 2003). The P2b/P3 region contains almost all highly conserved nucleotides found in the hTR core domain. Consistent with a conserved role for the P2b/P3 domain, most disease causing mutations that have been found in the telomerase RNA are located in the pseudoknot/core domain, mostly in the P2b/P3 pseudoknot (Armanios et al., 2007; Ly et al., 2005; Marrone et al., 2007; Vulliamy et al., 2001; Vulliamy et al., 2004).

1.3.3.2 STE region (CR4/CR5)

The STE domain is also conserved across organisms. In hTR the STE is CR4/CR5 and it can interact with TERT independently of the core region. CR4/CR5 contains a highly conserved hairpin, p6.1 which is critical for TERT binding and telomerase activity (Chen et al., 2002). The P6.1 hairpin contains two conserved pseudouridines (Kim et al., 2010). P6.1 pseudouridylation increases P6.1 stability compared to unmodified P6.1 and slightly attenuates telomerase activity while increasing processivity *in vitro* (Kim et al., 2010).

1.3.3.3 H/ACA scaRNA domain

The H/ACA scaRNA domain is implicated in the biogenesis and regulation of the telomerase holoenzyme. The hTR scaRNA domain includes a telomerase specific CR7 hairpin domain, which in turn contains

a CAB-box. ScaRNAs associate with box H/ACA RNPs to catalyse pseudouridylation of small nuclear (sn)RNAs. The four H/ACA RNPs that bind to H/ACA scaRNAs: GAR1, dyskerin, NOP10 and NHP2, also bind hTR. In addition to the box H- and ACA-conserved motifs, telomerase and scaRNAs share a third conserved CAB-box motif which acts as Cajal body-specific localization signal (Chen et al., 2000; Jady et al., 2004; Mitchell et al., 1999a; Richard et al., 2003) The H/ACA and CR7 are dispensable for the reconstitution of human telomerase activity in vitro, however they are essential in accumulation, processing and localization of TRs. The recently identified telomerase Cajal body 1 (TCAB1)/WDR79 protein binds the CAB-box of scaRNAs and hTR to localize them to the Cajal bodies (Tycowski et al., 2009; Venteicher et al., 2009). Most importantly, TCAB1 depletion by RNAi prevents the localization of TR to Cajal bodies, disrupts telomere-telomerase association and abrogates telomere synthesis by telomerase (Venteicher et al., 2009). Surprisingly, TCAB1 also binds dyskerin directly, in a RNA-independent manner (Venteicher et al., 2009). It has also been suggested that hTR binds two full sets of H/ACA RNP proteins but only one TCAB1 (Egan and Collins, 2010).

1.4 Telomerase-associated proteins

TERT and TR constitute the catalytic core of the enzyme and are sufficient to reconstitute telomerase activity *in vitro*. However, additional proteins are associated with the complex and participate in the assembly, localization, regulation and enzymatic activity of telomerase (Collins, 2006).

Mass spectometric analysis of affinity-purified telomerase has identified dyskerin, NHP2, NOP10, pontin/reptin and TCAB1 as essential components of telomerase *in vivo* (Fu and Collins, 2007). Dyskerin, NHP2, GAR1 and NOP10 are implicated in telomerase stability, accumulation, maturation and localization of hTR (Dragon et al., 2000; Fu and Collins, 2007; Mitchell et al., 1999a; Pogacic et al., 2000). Amino acid substitutions in DKC1 reduce hTR accumulation and cause the X-linked form of dykeratosis congenita (DC), a bone marrow failure syndrome (Wong and Collins, 2003). The ATPases pontin (RUVBL1) and reptin (RUVBL2) are responsible for telomerase accumulation and assembly (Venteicher et al., 2008) and TCAB1 is required for telomerase recruitement to Cajal bodies (Venteicher and Artandi, 2009).

The Ku heterodimer (Ku70/Ku80), a major player in nonhomologous end joining, directly interacts with TERT (Chai et al., 2002)

and TR (Ting et al., 2005). Ku binding to yeast TR (TLC1) increases its abundance and induces telomere lengthening, whether it has the same role in human is still unknown (Zappulla et al., 2011). The ubiquitin ligase MKRN1 mediates the ubiquitination of hTERT to promote its degradation. Overexpression of MKRN1 in telomerase-positive cells decreases telomerase activity and induces telomere shortening (Kim et al., 2005). hPIF1 interacts with telomerase and causes telomere shortening (Mateyak and Zakian, 2006). In the yeast, Pif1p removes telomerase from telomeres by unwinding the RNA/DNA hybrid formed between TLC1 and the substrate DNA (Boule et al., 2005; Boule and Zakian, 2007). Nucleolin, a major nucleolar phosphoprotein, directly interacts with hTERT and plays an important role in the nucleolar localization of telomerase (Khurts et al., 2004).

1.5 Trafficking of telomerase : Cajal bodies deliver telomerase to telomeres

Telomerase trafficking appears to be regulated in a cell cycledependent manner (Tomlinson et al., 2006). In G2 and G1, hTERT and hTR are found in separate intranuclear structures, where hTR is found in Cajal bodies and hTERT is found in discrete nucleoplasmic structures. In

early S-phase, TERT is brought inside the nucleolus while hTR stays within Cajal bodies which become localized to the periphery of the nucleolus. In mid-S phase, hTR and hTERT colocalize to Cajal bodies before being delivered to telomeres. The access of hTR and hTERT to telomeres is restricted to the S-phase, when telomere synthesis occurs. The accumulation of hTR in Cajal bodies is CAB-box and TCAB1dependent and TCAB1 depletion or CAB-box mutations lead to telomerase mislocalization and deficient telomere synthesis (Venteicher et al., 2009). Moreover, missense mutations in TCAB1 have been found in patients with dyskeratosis congenita, linking impaired telomerase trafficking with the disease (Zhong et al., 2011). Finally, trafficking of hTR from Cajal bodies to telomeres depends on hTERT, suggesting that the assembly of a functional telomerase complex must occur before being transported to telomeres (Tomlinson et al., 2008).

1.6 Alternative lengthening of telomeres

For most cells, reactivation of the enzyme telomerase is the solution to the end replication problem. However, a subset of cells are able to maintain their telomere in the absence of telomerase, and utilize a mechanism referred to as the alternative lengthening of telomeres (ALT) mechanism (Bryan et al., 1995; Bryan and Reddel, 1997). So far, ALT has only been observed in abnormal conditions such as in cancer cells, immortalized cell lines or tumors and is thus considered a backup pathway for telomerase that results from a loss of normal function.

1.6.1 The telomeres in ALT cells are long and heterogeneous

In addition to a lack of telomerase activity, human tumors or immortalized cells utilizing ALT are characterized by exceptionally long and heterogeneous telomeres, ranging from <2 kb to >50 kb, with an average length of 20kb (Bryan et al., 1997; Murnane et al., 1994). The heterogeneity in telomere length is present in individual cells, with telomeres ranging from undetectable, or very short, to extremely long (Perrem et al., 2001). The telomere length dynamics of ALT cells is characterized by rapid shortening and lengthening events, suggesting the involvement of recombination events (Murnane et al., 1994).

1.6.2 ALT-associated PML bodies

Another unusual feature of ALT cells is the presence of ALTassociated promyelocytic leukemia (PML) bodies (APBs) (Yeager et al., 1999). PML bodies are dynamic nuclear structures involved in diverse

cellular functions including maintenance of genomic stability, senescence, apoptosis, protein refolding, degradation, transcriptional regulation and chromatin modification (Bernardi and Pandolfi, 2007). In addition to the standard promyelocytic leukemia (PML) body components, the PML protein and Sp100, APBs contain a high number of repair and recombination proteins, telomeric DNA, and telomere-specific proteins (Draskovic et al., 2009; Yeager et al., 1999). It has been speculated that APBs play a direct role in ALT by bringing chomosome ends together with the recombination proteins RAD51 and RPA (Draskovic et al., 2009).

1.6.3 Extrachromosomal telomeric DNA

One of the most striking features of human ALT cells is the presence of telomeric sequences that are not associated with chromosomes, referred to as extrachromosomal telomeric repeat (ECTR) DNA. ECTR DNA can take many forms, the most common being doublestrand telomeric circles (t-circles) (Cesare and Griffith, 2004; Wang et al., 2004) or partially single-stranded circles (C- and G-circles) (Henson et al., 2009; Nabetani and Ishikawa, 2009). It can also be double-stranded linear (Ogino et al., 1998; Tokutake et al., 1998) or take the form of highly branched structures (Nabetani and Ishikawa, 2009). T-circles are likely the products of t-loop junction resolution, resulting in a free t-circle and a truncated telomere. Recent observations however suggest they are not specific to ALT cells, but rather the consequence of a trimming mechanism also operating in normal cells to negatively regulate telomere length (Pickett et al., 2009; Pickett et al., 2011). Contrary to t-circles, the presence of partially single-stranded C-circles seems to be highly ALT specific. The appearance of C-circles correlates with the onset of ALT and their levels are proportional to ALT activity (Henson et al., 2009).

1.6.4 ALT is a recombination-based mechanism

The telomere length dynamics of human ALT cells suggested that homologous recombination (HR)–mediated events were involved. This idea has been supported by experiments in which a plasmid tag is exponentially copied from one telomere to another with increased population doublings (Dunham et al., 2000). While the idea that ALT involves recombination events is well accepted, the exact mechanism is uncertain.

1.6.4.1 Recombination-mediated lengthening using a copy template

The aforementioned tag experiment is consistent with a model of telomeric recombination involving the recombination-based synthesis of new telomeric sequences using adjacent telomere sequences as a template. This model is expected to result in a net increase in telomeric DNA. Many templates can be used for recombination-mediated telomere synthesis: an adjacent chromosomal telomere, the telomere of the sister chromatid, the same telomere via telomere-loop formation or even linear or circular extrachromosomal DNA (Cesare and Reddel, 2010) (Figure 1-5).



Figure 1-5: Copy templates used for alternative lengthening of telomeremediated telomere lengthening

The templates can be: a) the same telomere through t-loop formation , b) the telomere of the sister chromatid, c) linear extrachromosomal telomeric DNA or d) circular extrachromosomal telomeric DNA. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews. Genetics 11(5):319-30, © 2010.

1.6.4.2 Unequal telomere sister chromatid exchanges

Another model is based on the observation that telomere sister chromatid exchanges (T-SCE) are more frequent in ALT cells than telomere-positive and normal cells. These exchanges can be visualized by a strand-specific fluorescence in situ hybdridization (FISH) technique named chromosome orientation (CO)-FISH (Bailey et al., 2001; Bailey et al., 2004b). Unequal T-SCEs are expected to result in one daughter cell with long telomeres and one daughter cell with short telomeres. Because the T-SCE model does not involve any net synthesis of telomeric DNA, the proliferation of a subpopulation of cells is expected to occur at the expense of another subpopulation of "donor" cells (Bailey et al., 2004a) (Figure 1-6).



Figure 1-6: telomere sister chromatid exchanges and recombinationmediated synthesis

a) T-SCEs and b) recombination-mediated synthesis of telomeric DNA using the adjacent chromosomal telomere. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews. Genetics 11(5):319-30, © 2010.

1.6.5 Non-ALT recombination-based mechanisms

There have been several reports of telomerase-negative cancer

cells that do not have all the characteristics typically associated with ALT

cells (Cerone et al., 2005a; Fasching et al., 2005; Marciniak et al., 2005). For instance, a number of glioblastoma multiforme tumors and some types of soft tissue sarcomas that have neither detectable telomerase nor the usual phenotypic characteristics of ALT (Costa et al., 2006; Hakin-Smith et al., 2003), highlighting the potential for complex and varied mechanisms of telomere maintenance. The occurrence of telomere maintenance by recombination via more than one mechanism was first described in yeast (Chen et al., 2001; Teng and Zakian, 1999). In the absence of telomerase, type I survivors emerge with short telomeres and amplified subtelomeric sequences, while type II survivors maintain elongated telomeres. Recently, telomere maintenance without significant telomere elongation was also observed in telomerase-negative primary and tumor mouse cells with short telomeres, telomerase-positive mouse cells with short telomeres, and lymphocytes from dyskeratosis congenita patients with short telomeres due to a mutation in hTERT (Morrish and Greider, 2009). Telomere maintenance in these cells was found to be effected by subtelomeric or telomeric recombination, providing evidence that several recombination-based mechanisms can contribute to telomere maintenance in mammalian cells.

1.7 Telomerase in cancer

Telomere maintenance, either by telomerase or alternative lengthening of telomere mechanisms, is essential for indefinite cellular proliferation. Thus, it appears that telomere shortening in normal human cells acts as a protective barrier against the development of cancer by limiting cell divisions. The first evidence that telomeres determine the proliferative potential of human cells comes from the experimental reintroduction of hTERT in telomerase-negative cells. Normal cells overexpressing hTERT activate telomerase activity, maintenance or elongation of telomeres and most importantly extended or indefinite lifespan without oncogenic transformation (Bodnar et al., 1998; Jiang et al., 1999; Morales et al., 1999). In contrast, expression of a dominantnegative inactive TERT in tumor cells induces progressive telomere shortening, crisis and cell death (Hahn et al., 1999; Zhang et al., 1999). Consistent with telomere maintenance being a prerequisite for cancer development, 85%-90% cancer cells reactivate telomerase, whereas 10-15% activate the ALT mechanism (Kim et al., 1994; Shay and Bacchetti, 1997).

1.7.1 The two-stage M1/M2 model of sensescence

Telomerase or ALT activation in cells is sufficient to elongate telomeres and allow the bypass of both mortality 1 (M1) and M2 stages. In normal cells, telomere shortening eventually leads to replicative senescence (M1) triggered by a p53-dependent DNA damage response (Allsopp and Harley, 1995; Smogorzewska and de Lange, 2002). Mutations in checkpoint components or viral transformation can allow cells to bypass this senescence checkpoint and enter a state known as crisis (M2). Further telomere shortening however leads to genomic instability, chromosomal fusion, breakage-fusion-bridge cycles, and eventually cell death (Ducray et al., 1999). Occasionally, rare survivors are able to escape cell death by acquiring a telomere maintenance mechanism (TMM) to become immortalized (Artandi and DePinho, 2000; Ishikawa, 1997). Both M1 and M2 are potent tumor-suppressors, although some chromosome rearrangements derived from the genomic instability in M2 might contribute to the acquisition of a TMM (Duncan and Reddel, 1997).





1.7.2 Anti-telomerase cancer therapies

Given the fundamental role of telomere length maintenance in cellular immortalization, anti-telomerase and anti-telomere strategies represent promising approaches for the development of anticancer therapies. A promising anti-telomerase approach consists of inhibiting telomerase activity, which leads to telomere shortening and growth arrest or apoptosis. However, one limitation of telomerase inhibition is the lag phase associated with the time required for telomeres to shorten sufficiently to observe anti-proliferative effects. A consequence of this lag phase is that telomerase inhibition results in apoptosis of human cells with short telomeres while cells with long telomeres have time to adapt (Hahn et al., 1999; Zhang et al., 1999) . Hence, there is concern that telomerase inhibition in cancer cells could lead to telomere maintenance by alternative mechanisms including recombination. Alternative telomere maintenance mechanisms are observed after telomerase inhibition with a dominantnegative hTERT (Bechter et al., 2004) or genetic deletion of telomerase (Chang et al., 2003; Hande et al., 1999; Morrish and Greider, 2009; Niida et al., 2000).

1.7.2.1 Targeting telomere integrity

An alternative to anti-telomerase strategies is to target telomere integrity, or telomere capping function. It has been suggested that alterations in telomere capping might facilitate the activation of ALT in human cells, most likely by allowing HR reactions at telomeres (Cesare and Reddel, 2008). Studies in *S. cerevisiae* and *Kluyveromyces* *lactis* revealed increased telomeric recombination after induction of telomere dysfunction through mutation or deletion of telomere-capping proteins (Grandin et al., 2001; Iyer et al., 2005; Teng et al., 2000). In addition, mutant telomerase RNA expression and the resulting incorporation of mutated telomeric repeats in *K. lactis* leads to increased recombination and elongated telomeres (Underwood et al., 2004). More recently, studies in yeast revealed that loss of the double-strand telomeric DNA-binding protein RAP1 or telomeric mutations that disrupt the binding of RAP1 can lead to recombinational telomere maintenance (Bechard et al., 2009; Sfeir et al., 2010). Finally, elevated levels of T-SCEs and formation of t-circles are observed in mice after POT1a deletion (Wu et al., 2006).

1.8 Telomeres and Telomerase in aging-related diseases

1.8.1 Dyskeratosis congenita : an aging-related disease

Dyskeratosis congenita (DC) is a rare and fatal inherited progressive disorder clinically characterized by a triad of nail dystrophy, reticular skin pigmentation and oral leukoplakia (Cole, 1930; Engman, 1926; Zinsser, 1906). DC patients have a predisposition to develop pulmonary fibrosis and a variety of malignancies but in 70% of cases, bone marrow failure is the primary cause of early mortality (Dokal, 2000). There is currently no cure for DC. Some treatments are used to reduce the symptoms associated with the disease and help with the condition of patients . Short-term treatments include anabolic streroids, granulocyte colony stimulating factors, granulocyte-macrophage colony stimulating factors and erythropoietin which can improve the hematopoietic function of patients (Erduran et al., 2003). The only long-term treatment is stem cell transplantation from a matched sibling donor. However, the success rates of transplantation are limited due to the high rate of pulmonary and vascular complications, probably due to pre-existing pulmonary disease in some patients.

1.8.1.1 Dyskeratosis congenita is a disease of telomere maintenance

Abnormally short telomeres are a hallmark of DC. The disease becomes more severe, with an onset of the disease at younger ages, in successive generations. This trait is called disease anticipation. The telomere shortening phenotype initially suggested that DC was a disease of telomere maintenance. Confirming the hypothesis, mutations in almost all telomerase holoenzyme components have been identified including: dyskerin (Heiss et al., 1998), hTR (Vulliamy et al., 2001), hTERT

(Armanios et al., 2005), NOP10 (Walne et al., 2007), NHP2 (Vulliamy et al., 2008) and TCAB1 (Zhong et al., 2011). The shelterin protein TIN2 has also been found to be mutated (Walne et al., 2008). Interestingly, POT1b deletion in mice with a telomerase-deficient background recapitulates DC, although no mutations in POT1 have been found in patients (Hockemeyer et al., 2008). Dyskeratosis congenita is characterized by defective maintenance of hematopoietic and dermatological systems and most importantly stem cells, all of which require telomerase activity. The mutations found in the different components of telomerase have been shown to reduce TR levels and telomerase activity (Armanios et al., 2005; Cerone et al., 2005b; Marrone et al., 2004), or telomerase trafficking (Zhong et al., 2011), leading to short telomeres and ultimately proliferative defects in important tissues. DC is genetically heterogeneous and X-linked recessive, autosomal dominant and autosomal recessive inheritances have been identified.

1.8.1.2 The X-linked form of DC is the most common and severe form of the disease

The X-linked form of DC is the most common and tends to be associated with a more severe phenotype and earlier onset of the disease.

X-linked DC is caused by mutations in the *DKC1* gene in Xq28 which encodes the highly conserved protein dyskerin (DKC1) (Heiss et al., 1998). Human DKC1 have several homologs including yeast Cbf5p, rat NAP57, Drosophila NOP60B and mouse DKC1.

1.8.2 DKC1

DC mutations are spread throughout the DKC1 protein which contains a catalytic pseudouridine synthase (TruB) domain, a pseudouridine synthase and archaeosine-specific transglycosylases (PUA) domain, and two nuclear localization signals at the N- and Cterminus. The TruB domain is composed of two motifs, TruB I and TruB II, which participate in eukaryotic ribosomal RNA processing. The PUA domain is a putative RNA-binding domain.

1.8.2.1 DKC1 is a member of box H/ACA RNPs

The 514 amino acid protein is an essential nucleolar protein required for the biogenesis and function of box H/ACA small nucleolar (sno), small-Cajal-body specific (sca) and telomerase RNPs. Dyskerin binds the three specific proteins NHP2, Gar1 and NOP10 to form the core of H/ACA RNPs. The RNP complex associates with RNA components
containing the H- and ACA-box conserved motifs (Kiss et al., 2010). The canonical structure of box H/ACA RNA is composed of a 5' hairpin followed by a single-strand H box (ANANNA) and a 3' hairpin followed by a single-strand ACA box (Henras et al., 2004). Box H/ACA sno- and sca-RNP complexes catalyze the site-specific pseudouridylation of ribosomal (rRNAs) and spliceosomal RNAs (snRNAs) respectively while the telomerase RNP participates in telomeric DNA synthesis. Although defects in pseudouridylation and ribosome synthesis have been observed in mouse models of DC (Mochizuki et al., 2004; Ruggero et al., 2003), such defects are not observed in patient-derived mutant DKC1 fibroblasts or lymphoblastoid cell lines (Mitchell et al., 1999a; Wong and Collins, 2006).

1.8.2.2 DKC1 mutations are found outside the RNP and RNA binding sites

Several DKC1 mutations have been identified and most of them are grouped in two major clusters : amino acids 31-72 and 314-420. Interestingly, the two clusters of mutations are located outside the catalytic TruB domain. Modeling of the clusters of mutations on the archaeal H/ACA RNP complex revealed that the mutations cluster in a single domain that however do not overlap with the binding sites of GAR1, NOP10 and NHP2 (Li and Ye, 2006). Accordingly, almost all DKC1 mutations do not affect the formation of the DKC1/NOP10/NHP2 pre-RNP complex *in vitro* (Trahan et al., 2010). Additionally, DKC1 mutation clusters do not locate to RNA binding sites in a model structure, and DKC1 mutations do not alter the association of the pre-RNP complex with hTR H/ACA motif (Li and Ye, 2006; Trahan et al., 2010). These results suggest important yet unknown roles for these clusters in dyskerin function. Apart from the most common mutation A353V which accounts for approximately 40% of X-linked DC cases, most DKC1 mutations are unique for one family.

1.8.2.3 Decrease DKC1 reduces hTR levels

In patients with X-linked DC, the levels of hTR are reduced without any significant impairment in rRNA processing and pseudouridylation (Mitchell et al., 1999b). Dyskerin binding to the H/ACA motif of hTR is required for the accumulation of hTR and mutations in dyskerin leads to reduction of hTR levels. Importantly, a decrease in dyskerin protein levels is sufficient to cause DC, and a recently identified family with X-linked DC and intact DKC1 sequence but decreased dyskerin protein level showed reduced levels of hTR and short telomeres (Parry et al., 2011; Zeng et al., 2012).

1.8.3 Sumoylation of H/ACA RNPs

Sumoylation is a post-translational modification which results in the covalent attachment of a small ubiquitin-like modifier (SUMO) protein on target proteins. SUMO transfer is catalyzed by a cascade of enzymes involving ATP, an E1 SUMO-activating enzyme (SAE1/SAE2), an E2 conjugating enzyme, ubiquitin conjugating enzyme 9 (UBC9) and an E3 SUMO ligase. Sumovation takes place on target lysine residues usually found in the consensus sumovlation motif ΨKXE where Ψ represent a hydrophobic amino acid. In humans, there are three different functional SUMO isoforms: SUMO1, SUMO2 and SUMO3. SUMO2 and SUMO3 share only ~50% homology to SUMO1 but they share ~97% homology to each other and hence are often referred to as SUMO2/3. Similarly to ubiquitin, SUMO2/3 have the ability to form polymeric chains through a conserved lysine (K11) (Ulrich, 2008). SUMO1 lacks this residue and thus the ability to form chains, although it can terminate and cap poly-SUMO2/3 chains (Matic et al., 2008). SUMO modification is involved in many cellular processes including protein-protein interaction, transcriptional regulation,

cellular localization and DNA damage. Recently, a role for sumoylation in the biogenesis and function of RNPs has been reported (Westman et al., 2010). Most importantly, sumoylation of the core box C/D RNP protein Nop58 was found to be important for its binding to snoRNAs and for their localization to the nucleolus.

1.9 Telomeres and Telomerase in aging

1.9.1 Telomeres as biomarkers for aging

Telomere shortening is associated with human aging, and premature aging-diseases are characterized by short telomeres, supporting the idea that telomere length may directly determine human longevity. In addition to progressive replication-mediated and pathologyrelated shortening, telomeres also shorten in response to DNA damage, mainly induced by oxidative or inflammatory stress. The contribution of oxidative stress to telomere shortening is predicted to be higher than the contribution of the end-replication problem (von Zglinicki et al., 2000a). The shortening rate is thus highly variable among individual, depending on the cell type, genetic background, environmental factors and lifestyle. Consequently, telomere length is emerging as a candidate biomarker of aging, aging-related diseases, stress, cancer and many other diseases.

1.9.2 Telomere length association with aging

The available evidences suggest that telomere length at birth is mainly determined by genetic factors. Paternal inheritance has been suggested, and there is a positive correlation between the age of fathers and telomere length in children, suggesting a vertical transmission of telomere length (Njajou et al., 2007; Unryn et al., 2005). However, classic twin studies suggest that variation of longevity is mostly influenced by nongenetic factors (Christensen et al., 2006; Herskind et al., 1996). The study of telomere length in 306 pairs of elderly male twins revealed that environmental factors play a fundamental role in telomere maintenance with little genetic effect (Huda et al., 2007). The association between telomere length and mortality was also assessed in 143 unrelated individuals over the age of 60 years, and individuals with shorter telomeres had poorer survival and increase mortality attributable to cardiovascular disease and infectious disease (Cawthon et al., 2003).

1.9.3 Telomere length association with disease and lifestyle

Correlations between short telomeres and many diseases have been reported. Adult and childhood obesity have been associated with shorter telomeres (Buxton et al., 2011; Zannolli et al., 2008). Diabetic patients have shorter telomeres than non-diabetic controls (Uziel et al., 2007). Correlations between stress (Epel et al., 2004), tobacco smoking (Valdes et al., 2005), physical activity (Puterman et al., 2010), low socioeconomic status (Cherkas et al., 2006) and even intimate partner violence (IPV) exposure and duration (Humphreys et al., 2011) have been identified.

1.9.4 Telomere association with cardiovascular disease

In addition to cancer, cardiovascular disease (CVD) is the major cause of death in Canada. Both the incidence and prevalence of CVD increase with chronological aging, however, there is a great variability in the susceptibility, age of onset and progression of the disease among individuals. As for aging or other aging-related disease, the notion of biological age (in reference to telomere length) rather than chronological age become determinant in CVD.

The first details on the association between telomere length and cardiovascular disease came from a pilot study in 2001 (Samani et al., 2001). This study compared telomere length of ten patients with severe coronary disease with those of twenty age-matched controls. The telomeres in leucocyte DNA of the ten patients were 303 bp shorter than

controls, and were equivalent in size with control individuals who were 8.6 years older. Similar larger scale studies reported that individuals with cardiovascular disease and hypertension had shorter telomeres (Huda et al., 2007; Serrano and Andres, 2004).

In patients with coronary artery disease, coronary endothelial cells demonstrate shorter telomeres at the atherosclerotic lesion (Ogami et al., 2004). Similarly, an increased rate of telomere loss is found in the endothelial cells of arterial wall areas that are subjected to more hemodynamic stress and thus more prone to form atherosclerotic plaques (Chang and Harley, 1995). It is believed that the higher stress in these regions leads to faster cell turnover. Consequently, endothelial and smooth muscle cells near atherosclerotic lesions are positive for the senescence-associated β -galactosidase activity test (Fenton et al., 2001; Minamino et al., 2002). Telomeres in cardiac muscle cells from patients with end-stage heart failure are 25% shorter than in age-matched controls (Oh et al., 2003).

Whether telomere length in peripheral blood mononuclear cells (PBMC) or human leucocytes reflects telomere length in the diseased tissue remains to be determined. A small-scale study reported that circulating leukocyte telomeric DNA can predict vascular cells telomeric

content (Wilson et al., 2008). Other studies reported that there is a high degree of correlation between telomere lengths in different cell types in one individual. Although the absolute lengths varied between cell types, they varied proportionally in leukocytes, skin and synovial tissues (Friedrich et al., 2000) or leukocytes and fibroblasts (von Zglinicki et al., 2000b).

1.9.5 Frailty : a marker for biological age

In a similar manner that telomere length is used at the molecular level to predict the biological age of individuals, the frailty index (FI) is used at the clinical level. In medicine, frailty is a geriatric syndrome used to define older individuals with impaired resistance to stressors due to a decline in physiologic reserve (Bergman et al., 2007). The impaired resistance to stressors such as acute illness, injury, surgery or hospitalization, ultimately represent a greater risk or morbidity and mortality.

1.9.5.1 Frailty and cardiovascular disease are related

Frailty and CVD are closely related both at the mechanistic level and the epidemiologic level. Frailty has been implicated as a causative and prognostic factor in CVD. Older adults who are frail as determined by the gait speed factor have a threefold higher risk of developing CVD incident and mortality (Dumurgier et al., 2009; Newman et al., 2006). Conversely, the Women's Health Initiative Observational Study showed that older women with CVD and CVD risk factors are associated with the highest risk of developing incident frailty (Woods et al., 2005). Four epidemiological studies also suggested an association between prevalent frailty and prevalent CVD (Chaves et al., 2005; Chin et al., 1999; Klein et al., 2005; Newman et al., 2001).

Frailty and CVD share molecular characteristics. They are both related to chronic low grade inflammation, which can be measured by markers such as circulating neutrophils, monocytes, high-sensitive Creactive protein, and interleukin-6 (IL-6). These inflammation markers are all increased in both frailty and CVD. Inflammation plays a key role in CVD by initiating, propagating and activating lesions in the arterial system (Hansson, 2005). In frailty, inflammation provokes a redistribution of amino acids from skeletal muscles to the other organs, which leads to the loss of muscle mass (Chevalier et al., 2003).

1.10 Objectives of the thesis

The induction of telomere dysfunction is currently investigated as an alternative anticancer strategy that would be independent of telomere length and mechanism of telomere maintenance (telomerase or recombination based) (Cerone et al., 2006a; Goldkorn and Blackburn, 2006; Guiducci et al., 2001; Kim et al., 2001; Li et al., 2004; Marusic et al., 1997; Stohr and Blackburn, 2008). However, it is important to consider the possibility that such approaches could lead to the emergence of alternative mechanisms of telomere maintenance.

Dyskeratosis congenita is a rare and fatal congenital disorder that is estimated to occur in 1 in 1 million people. The disease is a multisymptoms disorder, and to date no effective treatment has been found. Individuals with dyskeratosis congenita share mutations in genes or abnormal levels of proteins involved in telomere maintenance. For this reason, dyskeratosis congenita is now regarded as a disease of telomere maintenance. Future potential approaches could aim to restore telomerase function in patients and the understanding of dyskerin regulation by post-translation might provide new insights into the treatment of patients.

The association between telomere length and aging has led to the suggestion that telomere length could be used as an aging biomarker. Associations between telomere length and aging, mortality and various age-related diseases have indeed been reported however, other studies failed to detect such associations. Peripheral blood mononuclear cells (PBMC) being the most accessible, they are usually used for telomere length measurements and could represent a potential non-invasive tool for diagnostic and prognostic purposes. Again, the conclusions between different studies diverge, and whether circulating leukocyte telomere length can be used to predict the telomere length or state of other tissues remains to be determined.

Chapter 2

Material and methods

2.1 Cell culture

YCC-B2 breast cancer cells(Park et al., 1998) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). VA13 ALT cells were cultured in α-DMEM supplemented with 10% FBS. Human embryonic kidney HEK293 cells were maintained in minimum essential medium (MEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin and streptomycin.

2.2 Plasmids and constructs

The plasmid pCDNA3.1-FLAG-hDKC1 was obtained from François Dragon (Université du Québec à Montréal). DKC1 point mutations or deletions were created in pCDNA3.1-FLAG-hDKC1 using Quickchange site-directed mutagenesis (Stratagene) and specific primers. The constructs pCDNA3.1neo/SUMO1 and pCDNA3.1neo/SUMO2/3 were obtained from Frédérick Mallette (Lady Davis Institute).

2.3 RNA interference and rescue experiment

For DKC1 knockdown, 293T cells were co-transfected with a DKC1 3'UTR-specific short hairpin (shRNA)-pLKO.1 plasmid construct (TRCN0000039738, Open Biosystems) or negative control scrambled shRNA-pLKO.1 plasmid construct (plasmid 1864, Addgene) and the packaging pLKO.1 constructs pMD2.G (plasmids 12259, Addgene) and psPAX2 (plasmid 12260, Addgene) using FuGENE HD (Roche). The supernatant containing lentiviruses was collected, filtered through 0.45µm filter and stored in aliquots at -80°C or immediately used to infect HEK293 cells in the presence of 8µg/ml hexadimethrine bromide (Millipore). 24h after infection, stable cells were selected using 1µg/ml puromycin (Gibco). 7 days later, stable DKC1 knockdown cells were transfected with rescue pCDNA3.1-FLAG-hDKC1 variants using Fugene HD (Roche). Stable rescue cells were selected 24h after transfection with 500µg/ml G418 for 14 days. Total RNA was isolated and positive clones were screened using PCR and primers specific for exogenous *DKC1* (F:5'-TGCCACCATG GACTACAAAGACGA-3' and R:5'-GCAGCCGGACAATCCCCACA-3').

2.4 UBC9 knockdown

HEK293 cells were transfected with two different pSUPERpuro/shUBC9 plasmids (gift from K. Mann, Lady Davis Institute). Cells were analyzed 48h after transfection by TRAP assay.

2.4 In vitro sumoylation assay

³⁵S-Methionine-labeled DKC1 variants were obtained by *in vitro* transcription/translation using the T7-coupled transcription/translation rabbit reticulocyte lysate (RRL) system (Promega) in the presence of L-[³⁵S]methionine. In vitro sumovlation reactions were carried out as previously described using E1 enzyme (120ng SAE1/SAE2, Alexis Biochemicals), E2 enzyme (720ng UBC9, Alexis Biochemicals), SUMO1 or SUMO2 protein (500ng, Enzo Life Sciences) and 1µl 35S-Met-labeled RRL substrate in SUMOylation buffer (50 mM Tris-HCl pH 7.5, 5mM MgCl₂, 5mM dithiothreitol (DTT) and protease inhibitors) in an ATPregenerating system (2mM ATP (Sigma), 10mM creatine phosphate (Sigma), 3.5U/ml creatine kinase (Sigma) and 0.6U/ml inorganic pyrophosphatase (Sigma)) and incubated for 6 hours at 37°C before separation on a NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen). Gels were dried on a gel dryer (Biorad) for 2 hours and exposed to a phosphorimager (Storm 840, GE Healthcare Life Science).

2.5 Real-time quantitative PCR

Total RNA was prepared using the RNeasy kit (QIAGEN) according to the manufacturer's protocol. cDNA was obtained from reverse transcription of 1µg total RNA using SuperScript II (Invitrogen). Real-time quantitative PCR (qPCR) analyses were conducted using Power SYBR Green master mix (ABI) in 7500 fast qPCR system (ABI) for 10 min at 95 °C, followed by 40 cycles of 15s at 95 °C and 1min at 60°C. *hTR* primers (F3B: 5'-TCTAACCCTAACTGAGAAGGGCGTAG-3' R3C: 5'-GTTTGCTCTAGAATGAACGGTGGA-3') were used (Yi et al., 1999). Data were analyzed using the 7500 analysis software v2.0.4 (ABI) and the

comparative $\Delta\Delta$ Ct method.

2.6 CO-FISH

Cells were incubated in fresh medium containing 5'-bromo-2'deoxyuridine (BrdU) /5'bromo-2'deoxycytidine (BrdC) (3:1 ratio; Sigma, St. Louis, MO) for 16 h, and Colcemid (Life Technologies, Rockville, MD) was added to the medium for the last 3 h of incubation to accumulate mitotic cells. Chromosome preparations were then obtained according to standard cytogenetic methods. Slides were treated with 0.5 mg/ml RNase A (Fermentas, Hanover, MD) in phosphate-buffered saline (PBS) for 10 min at 37°C and then stained with Hoechst 33258 (Invitrogen, Carlsbad, CA) in 2× NaCl–sodium citrate for 15 min at room temperature. Cells were exposed to 254 nm UV light (GS Gene linker; Bio-Rad, Hercules, CA) at a dose of 180 mJ, and the BrdU/C-substituted DNA strands were digested with 800 U of Exonuclease III (Promega, Madison, WI) in 50 mM Tris-HCI (pH 8.0), 5 mM MgCl₂, and 5 mM dithiothreitol for 10 min at room temperature. Dehydrated slides were hybridized with an fluorescein isothiocyanate (FITC)-conjugated (TTAGGG)₃ peptide nucleic acid (PNA) probe (Cambridge Research Biochemicals, Cleveland, UK) at a final concentration of 0.5 µg/ml in 70% formamide, 0.5% blocking reagent (Roche), and 10 mM Tris-HCI (pH 7.2) for 2 h at room temperature; rinsed with 70% formamide, 0.1% bovine serum albumin (BSA), and 10 mM Tris-HCI (pH 7.2) for 5 s; and hybridized with a Cy3-conjugated (C3TA2)3 PNA probe (Cambridge Research Biochemicals) at a final concentration of 0.5 µg/ml in 70% formamide, 0.5% blocking reagent (Roche, Indianapolis, IN), and 10 mM Tris-HCI (pH 7.2) for 2 h at room temperature. After hybridization, cells were washed two times in 70% formamide, 0.1% BSA, and 10 mM Tris-HCI (pH 7.2) for 30 min and three times in 0.1 M Tris-HCI (pH 7.2), 0.15 M NaCl, and 0.08% Tween-20 for 5 min. The slides were then dehydrated through an ethanol series (70%, 95%, 100%), air-dried, and mounted in a 4',6-diamino-2-phenylindole (DAPI)/antifade solution (Chemicon, Temecula, CA). Images were captured using a Zeiss M1 fluorescence microscope (63× and 100×). Only T-SCE events observed

with both leading- and lagging-strand probes simultaneously were scored as positive.

2.7 Immunofluorescence

For detection of APBs, cells were grown on coverslips for 24 h, fixed in 4% formaldehyde for 10 min at room temperature, and permeabilized in 0.5% NP-40 (Sigma) for 10 min at room temperature. Slides were incubated with 1:200 goat anti-PML (N-19, Santa Cruz Biotechnology, Santa Cruz, CA) and 1:4000 rabbit anti-TRF1 (kindly provided by Titia de Lange) in phosphate-buffered gelatin (PBG; 0.2% fish gelatin [Sigma], 0.2% BSA in PBS) at 37°C for 2 h. Slides were washed in PBG and incubated with 1:250 Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA) and 1:250 FITC-conjugated donkey anti-goat (Jackson ImmunoResearch) at 37°C for 1 h. Slides were washed in PBG and mounted as described above. Three-dimensional images were captured using a Leica DMI600B confocal laser scanning microscope (63×) equipped with a Hamamatsu EM-CCD camera. At least 50 cells per condition were captured using Volocity 5.4.1 (Improvision, Lexington, MA), and the experiment was repeated three times. Images were obtained at 0.2-µm vertical intervals for each cell, and colocalization

was analyzed using Volocity. For TRF1 foci, a threshold of 15 SD above the mean of the total fluorescence intensity was used, and foci below 0.05 µm³ were excluded. For PML foci, a threshold of 9 SD was used, and foci below 0.05 μ m³ were excluded. Erosion was applied to every PML foci. The protocol calculated the number of PML foci touching the TRF1 foci. TIFs were visualized in interphase cells, fixed, and permeabilized as described above. Slides were incubated with 1:100 mouse anti-53BP1 (BP13; Upstate, Temecula, CA) or 1:500 goat anti-ATM Ser1981 (10H11.E12; Cell Signaling, Danvers, MA) and 1:4000 rabbit anti-TRF1 (#370; kindly provided by Titia de Lange) in PBG (0.2% fish gelatin [Sigma], 0.2% BSA in PBS) at 37°C for 2 h. Slides were washed in PBG and incubated with 1:250 Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch) and 1:250 FITC-conjugated donkey anti-goat (Jackson ImmunoResearch) at 37°C for 1 h. Slides were washed in PBG and mounted as described above. Images were captured using a Zeiss M1 fluorescence microscope (63× and 100×).

2.8 Q-FISH and pq ratios

Metaphase spreads from Colcemid-arrested cells were prepared according to standard cytogenetic methods. Cells were fixed in 4%

formaldehyde in PBS for 2 min, then washed three times in 1× PBS for 5 min. Slides were treated in a preheated solution of 1 mg/ml pepsin in 10 mM glycine (pH 2.0) for 10 min at 37°C, washed twice in 1× PBS for 2 min, and then subjected to a second round of formaldehyde fixation and 1× PBS washes. After dehydration through an ethanol series, cells were hybridized with a Cy3-conjugated (C3TA2)3 PNA probe, washed, and mounted as described above for CO-FISH. Telomere lengths were measured with the TFL-Telo v2.0 software kindly provided by Peter Lansdorp (British Columbia Cancer Center, Vancouver, Canada), and values were used to measure telomere pg ratios. A total of 15–30 metaphases were examined for each clone. Chromosomes with singlefree ends or end-to-end fusions were given a q/p score of 5 (Morrish and Greider, 2009). Two-fold ratios values ($q/p \ge 2$ or $q/p \le 0.5$) and fivefold ratio values $(q/p \ge 5 \text{ or } q/p \le 0.2)$ were plotted and normalized according to the total number of chromosomes examined.

Telomeric repeat amplification protocol (TRAP) and TRF analysis Protein extracts were prepared using 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonate lysis buffer, and TRAP assays were performed as previously described(Marie-Egyptienne et al., 2009). To compare telomerase activity between extracts, serial dilutions of telomerase extension products were assayed in the PCR reaction.

Genomic DNA was digested with *Rsa*l and *Hin*fl, and the fragments were separated by 2D-pulse field gel electrophoresis (PFGE) (Cerone et al., 2005a). After denaturation and neutralization, agarose gels were dried for 2 h at 60°C. In-gel hybridization was performed using γ -³²P-ATP–labeled (C3TA2)4 probe and exposed to x-ray film.

2.9 Affymetrix analyses

Gene expression profiles were measured in triplicate by Affymetrix Human Gene 1.0 ST Array (performed at Genome Quebec facilities) and analyzed with the FlexArray program.

2.10 Frailty project study design

The study sample consisted of 64 elderly patients 70 years of age and older and undergoing cardiac surgery (coronary artery bypass and/or valve replacement or repair). The 64 patients were part of a larger cohort of 131 patients recruited for a frailty and cardiovascular disease study as previously described (Afilalo et al., 2010). The patient characteristics are summarized in Table 5-1.

2.11 Telomere length measurement

2.11.1 Blood collection and genomic DNA purification

Whole blood samples were collected in lavender top tubes and processed immediately or stored at 4°C no longer than 24 hours. Buffy coats were isolated using Ficoll Paque Plus according to standard procedure (GE Healthcare). Genomic DNA was isolated from buffy coat using the Gentra Puregene Blood Kit according to the manufacturer's protocol (QIAGEN).

2.11.2 Telomere length qPCR

min, 40 cycles of 95°C for 15 s and 60°C for 2 min. Fluorescence data collection was performed during the annealing steps. The primers for the single-copy gene (human beta-globin) were HBG-F 5' GCTTCTGA CACAACTGTGTTCACTAGC-3' and HBG-R 5'-CACCAACTTCATCC ACGTTCACC-3, both used at a final concentration of 400 nM . The PCR reaction was carried out as follow : 50°C for 2 min, 95°C for 15 min, 40 cycles of 95°C for 15 s and 60°C for 1 min.

Aortic wall tissues were collected and immediately stored at -80°C. The aortic tissues were homogenized with a rotor–stator homogenizer and total RNA was isolated using the All Prep DNA/RNA Kit (QIAGEN) according to the manufacturer's protocol.

2.12 Predictor variables

2.12.1 Gate speed test

Although frailty can be evaluated using several variables, the slow gate speed alone has been found to be the most predictive of frailty and adverse outcomes (Studenski et al., 2003). We classified patients in two groups according to the 5m gait speed test as described in (Afilalo et al., 2010). A cutoff of \geq 6s was chosen to differentiate normal and slow gate speed.

2.12.2 STS risk score

The Society of Thoracic Surgeons (STS) mortality and morbidity risk score was included in our analysis. The STS risk score is specifically designed to measure the probability of mortality and morbidity of patients following the most common type of open heart surgery. The score is based on several clinical and demographic factors including gender, age, general medical history and cardiac history (Shahian and Edwards, 2009). Importantly, it has been previously demonstrated that the slow gait speed is associated with a threefold increase in postoperative mortality or major morbidity when adjusted for the STS risk score (Afilalo et al., 2010).

2.13 Outcome variables

For the outcomes, mortality or major morbidity after cardiac surgery were determined by the following : postoperative death, stroke, prolonged ventilation, renal failure, deep sternal wound infection, or reoperation. The patients were considered to have major morbidity when they experienced one or more complications mentioned above. Chapter 3

Telomeric recombination induced by

dysfunctional telomeres

3.1 Preface

Previous work in our lab reported that telomere destabilization induced by the expression of a mutant human telomerase RNA template (MuA-hTR) increases the sensitivity of human breast cancer cells such as YCC-B2 cells to chemotherapeutic drugs (Cerone et al., 2006a). The MuA-hTR reconstitutes an active mutant telomerase enzyme that adds TTTGGG instead of wild-type TTAGGG repeats to the ends of chromosomes; thus the sensitization of the MuA-hTR-expressing cells to chemotherapy is most likely due to an improper recruitment of shelterin proteins and telomere uncapping (Cerone et al., 2006a; Guiducci et al., 2001; Kim et al., 2001; Li et al., 2004). In the current chapter, we further analyzed three MuA-hTR-expressing clones derived from the YCC-B2 cancer cell line (telomere length 11 kb) with telomere lengths of approximately 6 kb (clone 17), 15 kb (clone 23), and 8 kb (clone 27) as assessed by telomere terminal restriction fragment (TRF) analysis (Cerone et al., 2006a). Q-FISH analysis at the single-cell level showed an increase in telomere-length heterogeneity in all three clones expressing MuA-hTR compared to the control cells (Cerone et al., 2006a). We demonstrate that telomere dysfunction increases HR at the telomeres of

these human breast cancer cells as shown by increased levels of T-SCEs and variation of pq ratio. We also show that some of the MuA-hTR– expressing cells share some characteristics with ALT cells, including the presence of APBs and t-circles.

3.1 Increased telomere dysfunction–induced foci in MuA-hTR– expressing telomerase-positive breast cancer cells

Various tumor cell lines expressing different mutant telomerase RNAs induce a DNA damage response at telomeres and mild phenotypes such as growth inhibition to more severe phenotypes including apoptosis (Cerone et al., 2006a; Goldkorn and Blackburn, 2006; Guiducci et al., 2001; Kim et al., 2001; Li et al., 2004; Marusic et al., 1997; Stohr and Blackburn, 2008). However, in our earlier study, MuA-hTR expression had only a mild effect on cellular viability, probably because of the presence of endogenous hTR, which can likely partially restore normal telomere function due to the synthesis of wild-type telomeric repeats. We determined whether MuA-hTR expression could engage a primary DNA damage response at telomeres of YCC-B2 cells, which might account for its ability to exacerbate the antiproliferative effects of chemotherapeutic drugs(Cerone et al., 2006a). The cytological manifestation of a DNA damage response at telomeres is called telomere dysfunction-induced

foci (TIF) and can be observed by the colocalization of DNA damage protein foci with telomeric proteins (d'Adda di Fagagna et al., 2003; Takai et al., 2003).

MuA-hTR expression resulted in the formation of foci containing the DNA damage protein 53BP1 and the telomeric protein TRF1 (Figure 3-1A). TIFs containing the phosphorylated (Ser1981) form of ataxia telangiectasia mutated (ATM) and BRCA1 were also observed in cells expressing MuA-hTR, consistent with deprotected telomeres (Figure 3-1B) and data not shown). Quantification of TIF-positive cells is reported in Figure 3-1C. Hence, the incorporation of mutant telomere repeats in YCC-B2 cells can elicit a primary DNA damage response at telomeres, but this response is insufficient to lead to apoptosis or senescence (Cerone et al., 2006a). Instead, MuA-hTR-expressing cells maintained growth rates similar to parental and vector-containing cells despite extensive, continuous passaging for 3 months. Persistence of mutant telomerase activity and mutant telomeric repeats confirmed that the mutants' proliferation was not due to loss of MuA-hTR expression.

Previous studies have shown that hTR levels are limiting in telomerase-positive human cell lines and that increasing hTR levels results in increased telomerase activity and telomere lengthening

(Cristofari and Lingner, 2006; Pickett et al., 2009). To address the possibility that increased telomere length of MuA-hTR-expressing YCC-B2 clone 23 and increased telomere-length heterogeneity of all three clones expressing mutant hTR could result from increased endogenous wild-type telomerase activity, we assessed the telomerase activity of the three YCC-B2 clones expressing the mutant hTR compared with the parental YCC-B2 cells. We found that telomerase activity was not increased (Figure 3-1D) in the cells expressing the mutant hTR, consistent with previous studies reporting the retention of endogenous wild-type telomerase activity levels upon expression of mutant hTR (Kim et al., 2001).





(A)YCC-B2 parental, vector-containing, and MuA-hTR–expressing cells were coimmunostained with an antibody recognizing 53BP1 (BP13, green) and an antibody recognizing TRF1 (#370, red). 53BP1 is colocalized with TRF1, indicating telomere dysfunction–induced foci (yellow). (B) YCC-B2 cells were also coimmunostained with an antibody against the activated and phosphorylated (Ser1981) form of ATM (10H11.E12, green) and anti-TRF1 (#370, red). Telomere dysfunction–induced foci are shown in yellow. (C) Quantification of TIF-positive cells. Cells with three or more 53BP1 or phospho-ATM Ser1981 foci colocalizing with TRF1 were counted as TIF positive. For each cell type, 100 cells were counted. Data are mean ± standard error of the mean (SEM) for three independent experiments. Asterisks indicate statistical significance from vector-containing cells (***P < 0.001). (D) Relative telomerase activity was measured by TRAP using serial fivefold dilutions of protein extracts.

3.2 Telomeric recombination between sister chromatids (T-SCEs) after

MuA-hTR expression

Telomere protection is essential in repressing HR of sister telomeres (T-SCEs). Elevated levels of T-SCEs are observed in cells utilizing the HR-based ALT pathway (Bechter et al., 2004; Londono-Vallejo et al., 2004). We verified whether the potential telomere deprotection in YCC-B2 cells could favor T-SCE. T-SCEs can be detected by the CO-FISH technique using a fluorescein isothiocyanate (FITC)–conjugated probe against the C-rich strand and a Cy3-conjugated probe against the G-rich strand, and exchanges between two sister chromatids can be visualized by a double signal at one telomere. Consistent with recombination occurring at telomeres of MuA-hTR–expressing cells, T-SCE events increased in the MuA-hTR–expressing clones when

compared with vector-containing cells (Figure 3-2, A-E). The increased T-SCE events were statistically significant for MuA-hTR–expressing clones 17 and 23, which showed an average of 0.89% and 0.53% T-SCE events, respectively, compared with 4.1% for the control telomerase-negative ALT cell line VA13 (Figure 3-2, C-F). Quantification of T-SCE events is reported in Figure 3-2G.





Figure 3-2: Telomeric recombination between sister chromatids (T-SCE) after MuA-hTR expression.

(A–F) Representative CO-FISH images of metaphase spreads of YCC-B2 cells. Slides were hybridized with an FITC-conjugated probe (FITC-[TTAGGG]3) against the C-rich strand (green) and a Cy3-conjugated probe(Cy3-[CCCTAA]3) against the G-rich strand (red). Chromosomes were stained with DAPI (blue). Chromosomes from (A) parental cells and (B) vector-containing cells exhibit two telomeric signals of each color per chromosome. No significant increase in T-SCEs was found in these cells. (C–E) The three MuA-hTR–expressing clones contain low levels of T-SCEs represented by chromosomes with double signals at both sister telomeres. (F) The VA13 control cell line also shows T-SCEs. Examples of T-SCEs are indicated by arrows and enlarged in inset. (G) Quantification of the T-SCEs for each cell type. The total number of T-SCE out of the total number of chromosomes analyzed is indicated over bars. Error bars give standard deviations for two independent experiments. Asterisks indicate significant differences from vector-containing cells (***P < 0.001 and *P < 0.05).

3.3 Mutant telomerase RNA expression is associated with changes in telomere pq ratios

To determine whether the observed telomere-length heterogeneity in MuA-hTR–expressing cells could be due to telomeric recombination, we measured the pq ratio of the telomere signals. In telomerase-positive cells, telomere lengths of the p and q arms of a chromosome should conserve a constant ratio. However, telomere recombination results in either

increased or decreased telomere length at one chromosome arm. Thus the pq ratio of the telomeres of a chromosome that has undergone recombination will vary from the pq ratio of the other copies of that chromosome in a cell population (Perrem et al., 2001). Originally, pg ratio analysis was performed on a specific chromosome in a growing population of cells; however, the analysis can also be expanded to all chromosomes of a metaphase spread, as described in Morrish and Greider (Morrish and Greider, 2009). Telomere lengths were assessed by Q-FISH and TFL-Telo analysis, and pq ratios were measured and plotted (Figure 3-3, A-E). Significant increases in pg ratios were observed in the three MuA-hTRexpressing clones when compared with parental and vector-containing cells. The pg ratios for each metaphase were plotted along the x-axis of the graph from left to right. The distribution of the changes along the x-axis of each graph demonstrates that changes in pg ratios were observed in all metaphases examined and cannot be attributed to changes in only a subset of metaphases. Chromosomes with ends lacking detectable telomeric signals (signal-free ends, or SFEs) or telomere fusions were given a pq ratio value of 5 and were not plotted for better clarity. The VA13 cell line was used as a control for elevated pg ratios (Figure 3-3F). To quantify the changes in pq ratios, the percentage of chromosomes

showing a variation greater than twofold or greater than fivefold was measured (Figure 3-3G). All three clones showed a higher proportion of chromosomes with a pq ratio \geq twofold and a pq ratio \geq fivefold compared with parental and vector-containing cells. We also observed telomere fusions with detectable telomere signals as well as SFEs in the MuA-hTR– expressing cells (10.1%, 13.5%, and 15.5% for the MuA-hTR–expressing clones 17, 23, and 27, respectively, versus 1.9% and 1.2% for the parental and vector-containing cells and 30.8% for the control VA13 cells), although we did not detect them previously (Cerone et al., 2006b). We propose that extensive passaging might have allowed the emergence of fusions and SFEs.




represented on a log scale on the *y*-axis. SFEs and telomere fusions were given a pq ratio value of 5 but were excluded from these graphs for better clarity. The total number of chromosome ends analyzed is indicated below graphs. (G) The percentage of chromosomes with a telomere pq ratio greater than twofold (q/p \ge 2 or q/p \le 0.5) or telomere pq ratio greater than fivefold (q/p \ge 5 or q/p \le 0.2) is represented.

3.4 MuA-hTR expression results in the accumulation of circular

extrachromosomal telomeric DNA

ALT cells contain abundant t-circles or extrachromosomal telomeric repeat (ECTR) DNA in a circular form. T-circles are generated by resolution of the t-loop junction, which creates a truncated telomere in addition to a t-circle. We analyzed the MuA-hTR-expressing cells for tcircles by performing two-dimensional pulse-field gel electrophoresis (2D-PFGE) followed by hybridization with a telomeric-specific probe (Cesare and Griffith, 2004). No arcs were detected in the YCC-B2 parental and vector-containing cells or MuA-hTR-expressing clone 17 and clone 27 (Figure 3-4, A-C and E). We detected an arc of double-stranded circular DNA in both the control VA13 cells and the YCC-B2 MuA-hTR clone with the longest telomeres (cl23) (Figure 3-4, D and F) suggesting that the expression of a mutant telomerase RNA can lead to the formation of tcircles. The generation of t-circles in the MuA-hTR clone harboring the

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longest telomeres is in accordance with a recent study suggesting that the generation of t-circles is a consequence of a telomere-length control mechanism, by which telomeric repeats would be trimmed when the telomeres reach a certain threshold length (Pickett et al., 2009).



Figure 3-4: The incorporation of mutant telomeric repeats results in accumulation of t-circles.

2D-PFGE of TRFs from YCC-B2 (A) parental, (B) vector-containing, (C–E) MuA-hTR–expressing, and (F) VA13 cells, digested with *Rsa*l and *Hin*fl. Hybridization was performed in gel with a γ -³²P–labeled C-rich probe under denaturing conditions. The black arrows indicate circular telomeric DNA.

3.5 Increased formation of PML bodies associated with telomeric DNA in telomerase-positive cells

ALT cells contain specific nuclear structures called APBs (Yeager et al., 1999). We analyzed MuA-hTR-expressing cells for the presence of APBs by immunostaining with antibodies against the PML and TRF1 proteins (Figure 3-5A). We found an increased proportion of cells with APBs in the three MuA-hTR-expressing clones compared with the parental and vector-containing cells (Figure 3-5, B and C), although the increase compared with vector-containing cells was statistically significant only in YCC-B2 MuA-hTR clone 23. The VA13 cell line was used as a positive control for APBs.



Figure 3-5: MuA-hTR-expressing cells contain APBs.

(A) Immunostaining with an antibody against the PML protein (N-19, green) and an antibody against the telomeric-binding protein TRF1 (#370, red). APBs were detected by the colocalization of the fluorescent signals in merged images (yellow). Images represent only one slice in the Z-stack. Arrows indicate colocalization. (B) Quantification of APBs in the YCC-B2 MuA-hTR–expressing cells compared with parental, vector-containing, and VA13 control cells. The percentage of cells showing four or more colocalization events per nucleus is shown. A minimum of 50 cells were

scored for each population. Three-dimensional images were obtained using a Leica DMI600B confocal laser scanning microscope (63×), and colocalization was analyzed with Volocity 5.4.1 software. (C) The average number of APBs per cell is represented. Data are mean \pm SEM for three independent experiments. Asterisks indicate significant differences from vector-containing cells (***P < 0.001 and **P < 0.01).

ALT is only observed in abnormal conditions such as in cancer cells or immortalized cell lines. It is thus tempting to speculate that ALT is actively repressed in telomerase-positive cells and that a deregulation in ALT repression is required to activate the alternative pathway. To better understand the molecular mechanisms regulating the decision to activate ALT, we performed gene expression profiling of the parental YCC-B2 cells and the YCC-B2 MuA23 cells showing recombination using Affymetrix Human Gene 1.0 ST Array. Gene expression profiling did not reveal any changes in gene expression greater than 2-fold in the YCC-B2 MuA23 cells when compared to the YCC-B2 cells. The most significant variations are summarized in Table 3-1.

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Table 3-1: Affymetrix gene array analysis of candidate genes implicated in

cancer cell resistance	by reactivation of	f telomeric r	ecombination
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Gene	Gene Name	GeneBank ID	Fold- change	P-value
Upregulated in	n telomeric recombination phenotype			
RBMY2EP	Homo sapiens RNA binding motif protein, Y-linked, family 2, member E pseudogene (RBMY2EP), non-coding RNA (Homo sapiens)	NR001574	1.79	0.007
CFHR1	Homo sapiens complement factor H-related 1 (CFHR1), mRNA (Homo sapiens)	NM002113	1.74	0.041
DDI1	Homo sapiens DNA-damage inducible 1 homolog 1 (S. cerevisiae) (DDI1), mRNA (Homo sapiens)	NM001001711	1.59	0.004
ZNF730	Homo sapiens cDNA FLJ16640 fis, clone TESTI4028938, moderately similar to Zinc finger protein 85 (Homo sapiens)	AK131472	1.51	0.054
Dowregulated in telomeric recombination phenotype				
SNORD116- 22	Homo sapiens small nucleolar RNA, C/D box 116-22 (SNORD116-22), small nucleolar RNA (Homo sapiens)	NR003336	1.91	0.023
SUMO1	Homo sapiens SMT3 suppressor of mif two 3 homolog 1 (S. cerevisiae) (SUMO1), transcript variant 2, mRNA (Homo sapiens)	NM001005781.1	1.64	0.049
SUMO1P3	Homo sapiens SUMO1 pseudogene 3 (SUMO1P3), non-coding RNA (Homo sapiens)	NR002190	1.60	0.078
SNORA62	Homo sapiens small nucleolar RNA, H/ACA box 62 (SNORA62), small nucleolar RNA (Homo sapiens)	NR002324	1.59	0.093
FIGF	Homo sapiens c-fos induced growth factor (vascular endothelial growth factor D) (FIGF), mRNA (Homo sapiens)	NM004469	1.52	0.063

Because it was detected twice in the array, we chose to validate the decrease expression in SUMO1 expression by qPCR. To further explore the role of SUMO1 expression in the ALT mechanism, we further examined the expression of SUMO1 in the YCC-B2 MuA23 cell line showing telomeric recombination, the telomerase-positive cell lines YCC-B2 and HeLa and in three ALT cell lines, VA13, GM847 and U2OS (Figure 3-6). We found a 2-3 fold reduction in SUMO1 expression in the ALT cell lines compared to the telomerase-positive cell lines. Interestingly, the YCC-B2 MuA23 cell lines showed SUMO1 expression levels lower than in ALT cells, but higher than in telomerase-positive cells, consistent with the presence of both telomerase activity and telomeric recombination activity

in this cell line. The significance of SUMO1 decreased expression and regulation of SUMO1 at the transcriptional level in ALT remains to be evaluated.



Figure 3-6: Expression levels of SUMO1 were validated by qPCR in the telomerase-positive cell line HeLa and ALT cell lines VA13, GM847 and U2OS. Each bar represents the mean and standard error of triplicate reactions. Data are normalized to *GAPDH* and relative to YCC-B2 parental.

Among the other interesting candidates, we noted an increase expression of the DNA damage-inducible Ubiquitin-like domain and Ubiquitin-associated domain (UbL-UbA) protein Ddi1, a protein that is specifically required for the degradation of ubiquitylated Ho, an endonuclease, in *S. cerevisiae* (Kaplun et al., 2005). Ddi1 serves as a shuttle to bind polyubiquitylated proteins in the cytoplasm or nucleus via its UbA domain and deliver them to the proteasome via its UbL domain (Bertolaet et al., 2001). The role of DDI1 in ALT will be investigated.

Chapter 4

Sumoylation regulates DKC1 trafficking to maintain human telomerase RNA stability and promote telomere length maintenance

4.1 Preface

Based on the observation in Chapter 3 that SUMO1 expression is reduced in ALT cells, we initiated the screening of telomerase and telomerase-associated protein amino acid sequences to find putative sumoylation sites by using the SUMOsp 2.0. program. Computational analyses revealed that one of the components of the telomerase holoenzyme, dyskerin, possesses the consensus hydrophobic cluster sumoylation motif (HCSM) (Matic et al., 2010) defined by six amino acids with the sequence $\Psi\Psi\Psi KX(D/E)$. Strikingly, the consensus sumoylation sequence corresponds to a mutation "hotspot" in dyskerin, where a mutation for each amino acid in the sequence $\Psi\Psi\Psi KX(D/E)$ has been reported in dyskeratosis congenita patients. We thus decided to focus on the role of dyskerin sumoylation in Dyskeratosis congenita. The role of SUMO1 decreased expression in ALT will be the subject of future studies.

In the current chapter, we identified that mutations in the consensus hydrophobic cluster sumoylation motif ΨΨΨΚX(D/E) and in the NLS of dyskerin impair *in vitro* sumoylation of DKC1 by SUMO2/3. ShRNAmediated knockdown of DKC1 in HEK293 cells followed by the expression of various lysine-to-arginine FLAG-DKC1 mutants failed to rescue the phenotypes observed in DKC1 knockdown cells. Most importantly, rescue with the lysine-to-arginine DKC1 mutants recreated the phenotypes associated with DC: telomerase shortening, impaired telomerase activity and decreased hTR stability. Our results are in favor of a new important role for SUMO2/3 in DC. The results of this study could importantly point to new possibilities in the treatment of dyskeratosis congenita.

4.2 DKC1 is sumoylated *in vitro* and *in vivo*

Amino acid sequence analysis of human DKC1 revealed a consensus sumoylation motif at the N-terminus (Fig.4-1A). The identified FLIKPE motif corresponds to the novel hydrophobic cluster sumoylation motif (HCSM) ΨΨΨKXE where three hydrophobic residues replace the single hydrophobic residue present in conventional consensus sumoylation motifs ΨKXE (Matic et al., 2010). This extended sumoylation motif has been shown to enhance sumoylation efficiency of zinc finger and BTB domain protein 1 (ZBTB1) and Ran GTPase-activating protein 1 (RanGAP1) (Matic et al., 2010). Strikingly, every amino acid within DKC1 HCSM sequence has been found to be mutated in DC. The single amino acid substitutions F36V, I38T, K39E, P40R, E41K and L37 deletion (L37del) are all associated with X-linked recessive DC (Cossu et al., 2002; Heiss et al., 1998; Knight et al., 1999) although their effects on DKC1 function remain largely unknown. Morevover, the FLIKPE motif of DKC1 is highly evolutionarily conserved suggesting that this motif is essential for optimal DKC1 function (Fig. 4-1). We hypothesized that DKC1 is sumoylated and that mutations in the HCSM of DKC1 contribute to DC by impairing sumoylation.

DKC1 has been recently identified as a putative nucleolar SUMO target in a proteomic screen using SILAC (Westman et al., 2010) in conjunction with mass spectrometry analyses of tryptic digests from nitriloacetic acid (NTA)-purified protein extracts of cells transfected with His₆-tagged SUMO paralogs (Galisson et al., 2011). To validate that DKC1 is a substrate for SUMO modification, DKC1 was translated in RRL in the presence of [³⁵S]methionine. ³⁵S-Met-labeled DKC1 was then subjected to an *in vitro* SUMOylation assay using purified recombinant E1 (SAE1/2), E2 (UBC9) and SUMO1 or SUMO2 (Desterro et al., 1997; Tatham et al., 2001). Unmodified DKC1 migrates at ~72KDa. However, higher molecular weight species corresponding to SUMO1- and SUMO2-modified-DKC1 were detected in reactions containing E1, E2 and SUMO (Fig. 4-1B). The formation of DKC1-SUMO conjugates was dependent on the addition of SAE1/2, UBC9, and SUMO since no slow migrating bands were observed

in the absence of any of the components (Fig. 1b). We conclude that

DKC1 is sumoylated by SUMO1 and SUMO2 in vitro.





(A) A schematic of the human DKC1 protein with the sequence alignement of mouse (mDKC1), rat (NAP57), S.cerevisiae (Cbf5p) and D. melanogaster (Nop60B) DKC1 homologues. Amino acid with identified disease causing mutations are indicated in bold. Lysines that have been mutated to arginine in this study are indicated below the alignment. Sumoylation sites predicted with medium and high probability are framed by a grey rectancle and the asterisk denote high probability. The consensus hydrophobic cluster sumoylation motif (HCSM) is indicated above the alignment. Note that a mutation for each amino acid found in the HCSM has been found in X-linked recessive Dyskeratosis congenita. NL, nuclear localization signal; TruBI and TruB II, pseudouridine synthase motifs I and II; PUA, pseudouridine synthase and Archaeosine transglycosylase domain. (B) In vitro expressed ³⁵S-Met-labeled DKC1 was incubated with recombinant SUMO1 or SUMO2, UBC9 and SAE1/2 in the presence of ATP. Where indicated, SUMO, UBC9 or SAE1/2 were omitted from the reaction. Reactions were separated on a 4-12% gradient SDS-PAGE gel. The gel was dried and exposed to a Phosphorimager screen followed by a X-ray film. Unmodified DKC1 is indicated.

4.3 Sumoylation of DKC1 requires intact hydrophobic cluster sumoylation motif and nuclear localization signal

Several putative sumoylation sites were identified using the SUMOsp 2.0 program, a site-specific predictor of sumoylation sites (Ren et al., 2009). Among them, K39 is located at the N-terminus of DKC1 in the HCSM motif and has been found to be mutated to glutamic acid (K39E) in DC. K43 is located four residues downstream of K39 and is also changed to glutamic acid (K43E) in the disease. No disease-associated mutation has been found for K46. K16 and K448 are comprised within two nuclear localization signals (NLS) of DKC1, located at the N-terminus and C-terminus respectively. We analyzed the function of several FLAG-DKC1 variants containing single (K39R, K43R, K46R, K16R, K448R) or double (K16R/K39R, K16R/K46R, K39R/K448R, K46R/K448R) lysine to arginine mutations as well as a FLAG-DKC1 variant containing a leucine deletion (L37del) (Fig.4-1A).The conserved glutamic acid residue in the

consensus WKXE motif is known to be important for the efficiency of SUMO addition to the nearby lysine (Rodriguez et al., 2001; Sampson et al., 2001; Zhang and Sarge, 2008). Similarly, the hydrophobic cluster preceding the lysine in the extended $\Psi\Psi\Psi$ KXE motif also enhances sumoylation (Matic et al., 2010). We thus included an additional variant of FLAG-DKC1 containing the DC-associated mutation del37L to test whether this deletion can act by impairing sumoylation of K39 (Fig. 4-1A). ³⁵S-Met-labeled FLAG-DKC1 variants were analyzed for *in vitro* sumoylation (Fig. 4-2). K39R, K43R, K46R, K16R and K448R were less efficiently modified by SUMO2 than WT as indicated by a decrease in slower migrating products. Modification by SUMO1 remained unchanged. Double mutants K16R/K39R, K16R/K46R, K39R/K448R, K46R/K448R further reduced sumoylation by SUMO2 and also impaired SUMO1 modification, supporting the existence of several sumovlation sites on DKC1. Del37L slightly impaired sumoylation of DKC1 by SUMO2 confirming the importance of an intact consensus sumovlation sequence for sumoylation. We are currently testing the sumoylation of WTDKC1 and the various DKC1 mutants in cells by immunoprecipitation using an anti-FLAG antibody.

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Figure 4-2: Identification of the sumoylation sites of DKC1. In vitro expressed ³⁵S-Met-labeled DKC1 WT, L37del, K39R, K43R, K46R, K16R, K448R mutants and K16R/K39R, K16R/K46R, K39R/K448R, K46R/K448R double-mutants were incubated with SUMO1 or SUMO2, UBC9 and SAE1/2 in the presence of ATP and the products were analyzed as in 4-1B. Unmodified DKC1 is indicated. Sumoylation of L37del, K39R, K43R and K46R by SUMO2 is less efficient than sumoylation of WTDKC1 (lanes 6, 9, 12, 15 compared to lane 3) as shown by a decreased signal intensity for higher molecular weight products. Modification by SUMO1 remains unchanged (lanes 4, 6, 8, 10 compared to lane 2). Double-mutations greatly impair SUMO1 and SUMO2 modification (lanes 17, 18, 20, 21, 23, 24, 26, 27, 29, 30, 32, 33 compared to lanes 2 and 3).

4.4 Impaired DKC1 sumoylation reconstitutes the telomere shortening phenotype observed in Dyskeratosis congenita

Telomere length is the prime determinant of the phenotypes observed in Dyskeratosis congenita (Hao et al., 2005). To determine the role of DKC1 sumoylation on telomere length maintenance, we used DKC1 shRNA lentiviruses to stably knockdown DKC1 in HEK293 cells and

rescued DKC1 expression with stable expression of FLAG-tagged hDKC1 variants WT, del37L, K39R, K43R or empty rescue vector. Telomere length was analyzed using Q-FISH after 18 to 40 population doublings (PDs) (Fig. 4-3 A-F, Fig. 4-4 and 4-5). ShRNA knockdown of DKC1 reduced telomere length of HEK293 cells when compared to control shRNA, as shown by a shift in the telomere length distribution toward shorter telomeres (Fig. 4-3 A and B). Stable expression of FLAG-DKC1 WT rescued the DKC1 knockdown and even slightly increased telomere length when compared to control cells, as shown by a shift in the telomere length distribution toward longer telomeres and increase in the frequency of long telomeres (>300 a.u.f.) revealed by a brighter telomeric signal (Fig. 4-3 A-C and Fig. 4-5). Stable expression of lysine-to-arginine FLAG-DKC1del37L, K39R and K43R variants were not able to rescue the telomere shortening observed in the empty vector-expressing knockdown cells (Fig. 4-3 A, D-F). For each DKC1 variant, two clones were analyzed (Fig. 4-3 and 4-5). Importantly, short telomeres (<100 a.u.f.) were more abundant in the FLAG-DKC1del37L, K39R and K43R mutants when compared to control and the amount of long telomeres (>300 a.u.f.) was reduced. In contrast, expression of WTDKC1 not only rescued the DKC1 knockdown but also extended all telomeres, shifting the telomere length

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distribution toward the right and increasing the average telomere length (Fig. 4-3 A-F). The telomere shortening in the HEK293 DKC1 knockdown cells and FLAG-DKC1del37L, K39R and K43R rescues was also obvious from the higher occurrence of telomere signal-free ends (SFEs), or chromosome ends lacking detectable telomere repeats, in these cells (Fig. 4-3G).





(A-F) Histograms of telomere length distribution in DKC1 control, vector, WT, L37del, K39R and K46R variants. Telomere lengths are expressed as arbitrary units of fluorescence (a.u.f.). A shift to the left in the length distribution of DKC1 mutant cells indicates telomere shortening. The total number of telomeres analyzed, mean telomere length ± standard error of

the mean are indicated. Note the greater abundance of short (<100a.u.f.) telomeres in the vector, L37del, K39R and K46R mutants compared to control and greater abundance of long (>300a.u.f.) telomeres in the WTDKC1 rescue cells. Telomeres were hybridized with a Cy3-conjugated PNA probe (Cy3-[CCCTAA]3). A minimum of 9 metaphases per clone were analyzed. (G) Telomere signal-free ends (SFEs) are increased in the lysine-to-arginine DKC1 mutants. Expression of WTDKC1 rescues the SFEs in DKC1 knockdown cells. (***P<0.001, **<0.01, *P<0.05 and n.s.P>0.05).









Representative Q-FISH images for telomere length in DKC1 control, vector, WT, L37del, K39R and K46R variants. Note the brighter telomeric signal in WTDKC1 cells. Signal-free ends are represented by white arrowheads. Telomeres are labeled with a Cy3-conjugated PNA probe (Cy3-[CCCTAA]₃) (red) and chromosomes are labeled with DAPI.

4.5 Impaired DKC1 sumoylation leads to reduced telomerase activity and reduced telomerase RNA levels

DC patients with point mutations in DKC1 exhibit reduced telomerase activity and decreased hTR levels (Mitchell et al., 1999b). We measured telomerase activity by TRAP and found reduced telomerase activity in the FLAG-DKC1del37L, K39R and K43R cell lines when compared to the control cells (Fig. 4-6 A and B). Stable expression of FLAG-DKC1 WT rescued the DKC1 knockdown and increased telomerase activity, in accordance with the increase telomere length observed previously. Surprisingly, telomerase activity was maintained in the empty rescue vector cells. Downregulation of the SUMO E2 conjugating enzyme UBC9 by shRNA also decreased telomerase activity in HEK293 cells (Fig. 4-6 C and D), confirming the importance of sumoylation for telomerase activity. Interestingly, reconstitution of telomerase activity by the addition of recombinant hTR to *in vitro* translated hTERT and DCK1 WT or mutant in a RRL system determined that sumoylation of DKC1 is not required for telomerase activity *in vitro* (Figure 4-7). Thus, sumoylation of DKC1 might

be involved in the maturation, processing or localization of dyskerin, but not in the formation of a catalytically active telomerase enzyme.



Figure 4-6: Impaired telomerase activity in lysine-to-arginine DKC1 mutant cells.

A. Telomerase activity measured by TRAP in control and two clones for each vector, DKC1 WT, L37del, K39R and K46R variants. IC internal control. Neg. negative control. B. Quantification of telomerase activity calculated as the intensity of the telomerase product relative to the intensity of the PCR amplification of the internal control (IC). C. Telomerase activity measured by TRAP in UBC9 knockdown HEK293 cells with two different shRNAs. Range of concentrations represent fivefold serial dilutions. D. Quantification of telomerase activity as in B.



Figure 4-7: In vitro telomerase activity is not impaired in RRL reconstituted telomerase complexes.

A. IC internal control. Neg. negative control. In vitro translated Sam68 was used as a control. B. Quantification of telomerase activity as in Fig. 4-6.

We also quantified hTR levels in the DKC1 knockdown and rescue cell lines using qPCR (Figure 4-8A). We found a marked decrease in the expression of hTR in FLAG-DKC1del37L, K39R and K43R cells while the FLAG-DKC1 WT cells showed increased levels of hTR. We thus conclude that sumoylation of DKC1 is required for hTR biogenesis and/or stability and subsequent telomerase activity.

Similarly to telomerase activity, hTR levels were unexpectedly but markedly increased in the empty vector rescue cells compared to control cells (Figure 4-8A). The unanticipated elevated telomerase activity and hTR levels observed were suggestive of a loss of shRNA-mediated knockdown. Indeed, immunoblot analysis of DKC1 expression in HEK293 DKC1shRNA knockdown cells at late passage and empty vector rescue cells after clonal selection revealed that RNA interference had been lost with population doublings (data not shown). We speculate that the original shRNA-containing cell population died and was subsequently regenerated with HEK293 cells lacking the DKC1shRNA through cell division, leading to the reestablishment of telomerase activity and hTR transcript levels. Exhaustion of a cell population lacking DKC1 and repopulation with HEK293 cells lacking DKC1shRNA is not surprising; DKC1 knockout in

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mice is embryonic lethal (He et al., 2002) and dyskerin possesses essential functions, including ribosome biogenesis, that are distinct from telomere maintenance. Accordingly, the generation of stable clones expressing the empty vector was accompanied by massive cell death, restrained proliferation, and only few clones were able to resume cell growth (data not shown). We measured the expression of p16INK4A, a senescence-associated molecular marker (Campisi, 2005) and found a two-fold increase in p16INK4A levels in the empty vector recue HEK293 cells compared to control cells (Figure 4-8B). We ignore to which extent RNAi was also lost in DKC1del37L, K39R and K43R population but it is tempting to speculate that it may have contributed to a milder phenotype.





4.6 Sumoylation of DKC1 is not required for its accumulation in the nucleolus

The ability of lysine-to-arginine DKC1 mutants to maintain telomerase activity *in vitro* but not *in vivo* suggests that sumoylation of dyskerin is important for the biogenesis or maturation of the telomerase complex and not telomerase catalytic activity. We compared the localization of the DKC1shRNA-expressing HEK293 cells rescued with WT, L37del, K39R or K43R however we found no significant difference in the localization of dyskerin (data not shown). Chapter 5

The role of telomeres in frailty

5.1 Preface

In the current chapter, we conducted telomere length analyses on a cohort of 64 patients 70 years and older and undergoing invasive cardiac surgery in order to find if telomere length, frailty, and outcomes of cardiac surgery are associated. Most importantly, surgery procedures have provided the material to measure telomere length of aortic wall tissues and subsequent comparison with leukocyte telomere length. Our data suggest that leukocyte telomere length does not predict aortic wall tissues telomere length, at least in the elderly. Moreover, we did not find any correlation between telomere length, frailty and cardiac surgery outcomes in the cohort studied.

5.2 Subject characteristics

The subject characteristics are presented in Table 5-1 and include gender, age, frailty, cardiac and telomere markers. As shown, the average age of participants did not differ between the two groups. The study included 36% women and 64% men and they were equally represented in each group. Group assignment was made in function of gate speed score, which varied significantly between the two groups. The Society of Thoracic Surgeons (STS) risk score was similar between the two groups, however the slow gate speed group experienced more mortality and morbidity after surgery than the normal gait speed group. Twenty-five percent of total patients experienced mortality or major morbidity after cardiac surgery, 10% patients from the normal gait speed group and 41% patients from the slow gate speed group. For 54.7% of patients, both aortic wall tissue and leukocytes were available for telomere length analyses. Leukocyte telomere length and aortic wall tissue telomere length were similar between the two groups.

Table 5-1: Characteristics of the total sample and for the two study groupswith normal gait speed or slow gait speed (Values are mean ± SD or n(%), STS = Society of Thoracic Surgeons, T/S = telomere/standard)

	Overall	Normal Gait Speed (n=32, 50%)	Slow gate speed (n=32, 50%)
Age, yrs	75.6 ± 3.4	75.1 ± 3.6	76.1 ± 3.2
Female sex	21 (36)	9 (30)	12 (41)
Frailty marker Gait speed (mean time to walk 5m, s)	6.4 ± 1.4	4.9 ± 0.5	7.7 ± 1.7
Cardiac surgery risks and outcomes			
High STS (≥15%)	37 (63)	17 (57)	20 (69)
Mortality or major morbidity	15 (25)	3 (10)	12 (41)
Telomere markers			
T/S leukocytes	1.2 ± (0.3)	1.1 ± 0.2	1.3 ± 0.3
T/S aortic wall tissue	1.8 ± (1.1)	1.7 ± 1.1	1.9 ± 1.1

5.3 Telomere length

The average relative telomere length (RTL), as measured by

the telomere repeat copy number-to-single gene copy number (T/S) ratio

was 1.2 ± 0.3 in leukocytes and 1.8 ± 1.1 in aortic wall tissue. Leukocyte RTL in women was significantly longer compared to men, as previously reported (Figure 5-1A) (Benetos et al., 2001). However, we found no difference in aortic wall tissue RLT in women and men (Figure 5-1B). We observed a slight decrease in leukocyte telomere length with age, in both men and women, although the decrease was not significant (Figure 5-1C). No correlation between telomere length and age was found in the aortic wall tissues (Figure 5-1D).



Figure 5-1: Association of leukocytes and aortic wall tissue telomere length with gender and age.

A) Shorter leukocytes telomere length in male (n=31, 1.109 \pm 0.054) than female (n=15, 1.383 \pm 0.117), p= 0.0185 was observed. B) No gender difference is observed in aortic wall tissue telomere length of male (n=27, 1.811 \pm 0.308) and female (n=16, 1.856 \pm 0.291), p= 0.9234 was observed. C) No significant correlation between leukocyte telomere length and age of patients in male (n= 31, r= -0.146, p= 0.433) and female (n= 15, r= -0.242, p= 0.386) was observed D) No significant correlation between aortic wall tissues telomere length and age of patients in male (n= 27, r= 0.020, p= 0.920) and female (n= 16, r= -0.232, p= 0.388) was observed.

We did not find any positive correlation between telomere length of circulating leukocytes and telomere length of aortic wall tissues (Figure 5-2), suggesting that leukocyte telomere length does not predict aortic wall tissues telomere length in our model.





No correlation between leukocytes telomere length and aortic wall tissues telomere length (n= 31, r= 0.234, p= 0.208) was observed.
There was no association between leukocyte or aortic wall tissue telomere length with frailty, as measured by the gate speed (Figure 5-3A-B). Similarly, no association between leukocyte or aortic wall tissue telomere length and the outcomes following cardiac surgery were found.



Figure 5-3: Association between telomere length and frailty and cardiac surgery outcomes.

A) No difference in leukocyte telomere length of non-frail (n= 25, 1.140 \pm 0.064) and frail (n= 24, 1.267 \pm 0.084) patients, p= 0.2319 was observed. No difference in aortic wall tissue telomere length of non-frail (n= 21, 1.712 \pm 0.313) and frail (n= 23, 1.873 \pm 0.307) patients, p= 0.7155 was observed. C) No difference in leukocytes telomere length of patients with normal (n= 36, 1.166 \pm 0.053) and high mortality and major morbidity (n= 10, 1.312 \pm 0.172), p= 0.2828 was observed. D) No difference in aortic wall tissue telomere length of patients with normal (n= 33, 1.764 \pm 0.264) and high mortality and major morbidity (n= 10, 2.036 \pm 0.381), p= 0.6067 was observed.

Although the STS risk score and gait speed are independent, they have been shown to classify patients in more distinct risk categories when used in combination (Afilalo et al., 2010). We thus combined the STS risk score with gait speed to divide patients in four risk categories (Figure 5-4). We found no association between leukocyte telomere length and the dual risk factors of gait speed and STS risk score. We observed an increase in telomere length of aortic wall tissue in patients with both low STS risk and normal gait speed when compared to the other three groups but due to small simple size, this difference was not statistically significant.



Figure 5-4: Association between telomere length and dual risk factors of gate speed and STS risk score.

A) No difference in leukocyte telomere length of patients with low STS risk, normal gait speed (n= 12, 1.166 \pm 0.075), high STS risk, normal gait speed (n= 12, 1.125 \pm 0.112), low STS risk, slow gait speed (n= 7, 1.125 \pm 0.133) and high STS risk, slow gate speed (n= 15, 1.316 \pm 0.117) was observed. Difference between the lower and higher risk factor groups, p= 0.3151. No difference in aortic wall tissues telomere length of patients with low STS risk, normal gait speed (n= 7, 2.388 \pm 0.779), high STS risk, normal gait speed (n= 7, 1.867 \pm 0.661) and high STS risk, slow gate speed (n= 15, 971 \pm 0.359) was observed. Difference between the lower and higher risk factor groups, p= 0.5804.

Chapter 6

Discussion

At first sight, cancer and aging appear to be two totally different processes. Cancer is characterized by indefinite cell proliferation while aging is characterized by the exhaustion of the cell proliferative potential. However, research over the past few decades has provided evidence that cancer and aging are not so different, and that they are intricately related. Aging is associated with a high risk of developing many diseases including neurodegeneration, cardiovascular disease and osteoporosis. Still, one of the most prevalent age-related diseases of our century is cancer. Cellular aging has thus been considered as a potent tumor-supressor mechanism that could have been put in place to halt unlimited cell proliferation and the development of cancer. Accordingly, telomere shortening induces senescence in cells that have undergone several rounds of cellular division. However, neoplastic transformation is a multistep process that requires several growth and division cycles. Cancer cells must thus bypass senescence in order to become immortal.

Cellular senescence and organismal aging can be induced by several factors, including the disruption of chromatin organization, or exposure to several types of stress including oxidative stress, but telomere shortening remains a key factor. In accordance with the central role of telomeres in aging, the disease Dyskeratosis congenita is associated with

telomere shortening, and the phenotype of the disease recapitulates many features of aging. Moreover, short telomeres increase Dyskeratosis congenita patients predisposition to cancer. Telomeres and telomerase are also central to cancer, and cells must reactivate telomerase or a telomerase-independent mechanism to become immortal. The right balance between telomere length maintenance and inhibition of aberrant telomere elongation is required and disruption of this fine equilibrium, toward one side or the other, will ultimately lead to altered function and disease.

6.1 The role of telomeres and telomerase in human cancer

Because telomerase activity and telomere maintenance confer to cancer cells a unique survival advantage compared to normal somatic cells, they represent very attractive targets in the search for a universal and selective anticancer therapy. Several approaches to inhibit telomerase have been investigated including telomerase immunotherapy and telomerase inhibition. However, there is concern that telomerase inhibition might lead to resistance mechanisms. Strategies to validate targeting telomeres integrity have also been examined, including disruption of shelterin, expression of a mutated telomerase RNA

component which results in the synthesis of defective telomeres, or use of G-quadruplex stabilizers (Harley, 2008). G-quadruplexes are fourstranded guanine-rich DNA structure formed notably at telomeres (Paeschke et al., 2005).

The development of anticancer therapies targeting telomerase and telomeres however faces several challenges; one of them is the existence of an unconventional way to maintain telomeres, the ALT pathway (Bryan et al., 1997). ALT has only been observed in abnormal conditions such as in cancer cells, immortalized cell lines or tumors and is thus considered a backup pathway for telomerase that results from a loss of normal function. However, the circumstances and molecular events that lead to the activation of ALT remain largely unknown. Most importantly, it remains to be elucidated whether ALT can be switched-on in telomerase-positive cancer cells treated with anti-telomerase/telomere agents and create resistance to cell death.

Telomeric recombination after telomerase inhibition in conjunction with a MSH6, one of the mismatch-repair genes involved in nonpolyposis colorectal cancer, defect has been reported (Bechter et al., 2004). Additionally, genetic deletion of telomerase can also lead to activation of alternative telomere maintenance mechanisms (Chang et al., 2003; Hande

et al., 1999; Morrish and Greider, 2009; Niida et al., 2000). In Chapter 3, we reported that ALT features can also be reactivated by telomere dysfunction. The telomerase-positive breast cancer cells YCC-B2 mutant telomerase RNA expressing (MuA-hTR) cells were engineered to contain mutant telomere sequences, likely disrupting the binding of shelterin which specifically recognizes wild-type telomere sequences. Telomere uncapping normally elicits a DNA damage response that leads to p53mediated apoptosis or senescence (Smogorzewska and de Lange, 2002). However, we previously showed that the viability and proliferation of YCC-B2 MuA-hTR cells remain unchanged unless they are treated with chemotherapeutic drugs such as doxorubicin, etoposide and paclitaxel (Cerone et al., 2006a). To clarify the molecular mechanisms participating in YCC-B2 MuA-hTR cells resistance to telomere dysfunction-mediated cell death, we examined their telomere length profile and found heterogeneous telomere lengths, suggestive of telomeric recombination characteristic of ALT cells (Cerone et al., 2006a). These cells exhibited additional features of ALT cells, including telomere sister chromatid exchanges, elevated pg-ratio changes, telomeric circles and ALTassociated PML bodies, although not as prominent as in ALT cells. ALT hallmarks developed without an increase in telomerase activity,

suggesting they are a consequence of ALT activity and not telomerase. Although our study does not prove that ALT can act as a resistance mechanism to telomerase inhibition *in vivo*, it provides new evidence that it can maintain telomere length and extend survival in telomerase-positive cells displaying telomere dysfunction.

There is some evidence that alterations in telomere capping may allow the de-repression of recombination at telomeres. Deletion of POT1 in mouse results in aberrant HR at the telomeres as shown by the formation of t-circles and elevated level of T-SCEs (Wu et al., 2006). Mutation in POT1 also increases levels of T-SCEs as well as telomere end-to-end fusions (He et al., 2006; Wu et al., 2006). Simultaneous deletion of TRF2 and Ku in MEFs in a ligase 4-null background also creates levels of T-SCEs similar to levels observed in ALT cells (Celli et al., 2006). Finally, deletion of the basic domain of TRF2 increases formation of t-circles in human cells (Wang et al., 2004). To explain how telomere uncapping can induce telomeric recombination, Cesare and Reddel (Cesare and Reddel, 2008) proposed a model in which uncapped telomeres are unable to fold into the telomere-specific T-loop structure, thereby rendering them accessible to the HR machinery. Because telomeric proteins TRF1, TRF2 and TIN2, and recombination proteins

such as RAD51, RAD52, XRCC3, and NBS1 possess T-loop formation activity *in vitro* and may assist telomere capping via HR, it is also possible that telomere dysfunction deviates their HR activity at the T-loop toward altered HR at the telomeres (Cesare and Reddel, 2008).

Telomere uncapping engages p53-dependent senescence or apoptosis, suggesting that p53 function must be lost to permit ALT activation. Accordingly, the great majority of ALT cell lines and tumors are impaired in the p53 pathway (Reddel and Henson, 2010), including the YCC-B2 cell line used in our study. In a more recent study in glioblastoma multiforme patients, 78% of all ALT-positive tumors were found to be defective for p53 while only 21% of telomerase-positive tumors were defective for p53 (Chen et al., 2006). However, telomere dysfunction and p53 loss do not always correlate with ALT reactivation; growth inhibition and apoptosis were observed in HCT116 p53-null cells harboring mutant telomeres (Li et al., 2004). It also remains to be determined if telomeric recombination can be triggered by dysfunctional telomeres in cells with functional p53. Notably, we could test if p53 is sufficient to inhibit telomeric recombination by overexpressing a transactivation-deficient p53 in the YCC-B2 cells.

Additional factors must be required for the repression of ALT (Figure 6-1). Most repressive factors of telomeric recombination identified so far are involved in the maintenance of a repressive chromatin environment at the telomeres, as reviewed in Schoeftner and Blasco (Schoeftner and Blasco, 2009). Loss of DNA methyltransferase-1 (DNMT1), DNA methyltransferase-3b (DNMT3b), the Suv39h1 or Suv4-20h histone methyltransferases is associated with increased telomeric recombination. Recent work demonstrated that inactivation of αthalassemia/mental retardation syndrome X-linked (ATRX) and deathdomain-associated protein (DAXX) strongly correlates with the ALT phenotype in several types of tumors, as monitored by the presence of large ultrabright telomere FISH signals (Heaphy et al., 2011). It was proposed that ATRX-DAXX loss leads to increased homologous recombination at telomeres by impairing their heterochromatic state via reduced H3.3 histone variant incorporation. Similarly, absence of the linker histone Hho1p facilitates recombination-dependent telomere maintenance in S. cerevisiae (Downs et al., 2003). It will be interesting to explore if telomere dysfunction can influence telomere chromatin state and in turn activate recombination.

Many questions remain about the reactivation of telomeric recombination in cells with dysfunctional telomeres. Could resistance involving ALT also be observed in cells treated with telomere-disturbing agents such as G-quadruplex ligands? Identifying new ALT repressors genes could help to understand when and how ALT is reactivated instead of telomerase, which is extremely significant for cancer prognosis and development of anticancer therapies.



Figure 6-1: Model for the de-repression of telomeric recombination and cancer therapy resistance.

Expression of a mutant telomerase RNA leads to telomere uncapping. Exposed telomeres induce telomeric recombination when accompanied by p53 loss, which creates resistance to cell death. It is not known if telomeric recombination can be de-repressed in the presence of a functional p53. In some cases, telomere dysfunction induces apoptosis or senescence independently of p53 status. Opening of telomeric chromatin allows recombination events at the telomeres. Dysfunctional telomeres could alter the state of telomeric chromatin. Other telomere-disturbing agents might favour ALT de-repression and cancer cell resistance to death.

6.2 The role of telomeres and telomerase in the premature aging

syndrome Dyskeratosis congenita

Cancer and aging also intersect in the disease DC, a premature aging syndrome characterized by an increased incidence of a variety of cancers. Since the identification in 1999 that dyskerin is mutated in the disease Dyskeratosis congenita, six additional DC-associated genes have been identified. The proteins encoded by the seven genes are all components of the enzyme telomerase or telomere-capping shelterin complex, and because the patients are all characterized by telomere shortening DC has been referred to as a syndrome of telomere shortening (Armanios et al., 2005). Dyskerin is essential for hTR stability and telomerase biogenesis. Consequently, patients with DC show less than half the amount of telomerase than normal people. More than 50 different DKC1 single point mutations have been identified so far but strikingly, none of them map to the RNA binding domain of DKC1. In vitro analyses revealed that the mutations do not impair the assembly of the DKC1/NOP10/NHP1 pre-RNP complex nor the association of the RNP complex with the H/ACA domain of hTR, with the exception of the A353V

mutation which has a mild effect on pre-RNP assembly (Trahan et al., 2010). Hence, DKC1 mutations impair hTR stability in a manner that is not related to the assembly of the telomerase complex.

A clue to this conundrum might come from a recent study in which a family with DC but in which no DKC1-causing mutation has been identified (Parry et al., 2011). Genome wide linkage analysis however implicated the DKC1 locus, and affected family members showed reduced levels of DKC1 protein to 50-72% of normal level and reduced levels of hTR, implying that DKC1 protein levels can alone be responsible for DC. We propose that many DKC1-causing mutations identified so far might impair DKC1 protein stability and that even 75% of DKC1 normal protein level is not sufficient to confer hTR stability.

In Chapter 4, we showed that DKC1 sumoylation is important for the stability of hTR , telomerase activity *in vivo*, and telomere maintenance. Importantly, we showed that mutation of the predicted sumoylation site K46 recapitulates the DC phenotype, although this mutation has not been found in patients. Notably, telomerase activity reconstituted *in vitro* was not impaired by mutations in DKC1 sumoylation sites, suggesting that sumoylation of DKC1 is not required for telomerase catalytic activity.

The sumoylation modification pathway has been shown to regulate the localization of many proteins. The first SUMO substrate identified, RanGAP1, is stabilized at the nuclear pore complex when modified by SUMO1 (Mahajan et al., 1997; Matunis et al., 1996). SUMO1 also targets RanGAP1 to mitotic spindles and kinetochores. The binding partner of RanGAP1 at the nuclear pore, RANBP2, has been shown to have E3 ligase activity. Additionally, the protein MDM2, an important negative regulator of the p53 tumor suppressor, is a substrate of both RANBP2 and the E3 ligase PIAS E3, and it has been proposed that MDM2 is modified by RanBP2 at the nuclear pore and re-modified by PIAS once inside the nucleus (Miyauchi et al., 2002). This type of nucleocytoplasmic transport has been suggested for proteins containing a sumoylation consensus site and a NLS (Dejean and Seeler, 2003). Interestingly, DKC1 contains both a sumovlation consensus site and a NLS. The NLS mutations K16R and K448R could potentially impair DKC1 localization to the nuclear pore complex in a similar manner to MDM2. Moreover, we have shown that DKC1 double-mutants further impair sumoylation *in vitro* when compared to single-mutants.

Several proteins have been reported to act as substrates for both sumoylation and ubiquitylation. Consistent with the ubiquitination of DKC1,

we mapped several putative ubiquitination sites. In addition, we repeatedly observed a higher-molecular weight form of DKC1 that is not recognized by SUMO antibodies, and we are currently testing if DKC1 is also ubiguitinated. A crosstalk between sumoylation and ubiguitination has been reported numerous times. Sumoylation of the nuclear factor-kB (NF- κ B) inhibitor α (I κ B α) inhibits its phosphorylation-induced ubiquitination and promotes its degradation (Desterro et al., 1998). Several similar antagonistic effects of SUMO and Ubiquitin have been reported, however sumoylation and ubiquitination can also work cooperatively as SUMO can act as a signal for the recruitment of E3 ubiquitin ligases and lead to the degradation of the protein. The precise role of sumoylation and possible role of ubiquination for DKC1 function needs to be further investigated. Precisely, we will use the proteasome inhibitor MG132 to verify if the lysine-to-arginine mutant phenotypes can be diminished.

A role for sumoylation in RNP biogenesis has been recently reported (Westman et al., 2010). Sumoylation of Nop58, a core component of box C/D RNP, was shown to be important for the accumulation of box C/D snoRNA into the nucleolus. Although not characterized, DKC1 was also identified as a SUMO2 substrate in this study. The biogenesis pathway of snoRNPs is complex and highly regulated and box H/ACA, like box C/D, are most likely transported from the nucleoplasm to the nucleolus through the Cajal bodies. Based on our observations, we speculate that DKC1 L37del, K39R and K43R impair the transport of DKC1 from the Cajal bodies to the nucleolus, and not from the nucleoplasm to the nucleolus.

6.3 The role of telomeres and telomerase in frailty and cardiovascular diseases

The development of novel telomere length assessment techniques has proven to be extremely useful in the clinic. For example, telomere length measurements allow the correct identification of dyskeratosis congenita in patients that do not present the classical mucocutaneous triad of features. The development of high throughput telomere length assays like quantitative qPCR assays has allowed the study of telomere length in many epidemiological or clinical contexts. As a consequence, the number of studies investigating the correlation between telomere length and a particular disease or feature has exploded in recent years.

In chapter 5, we explored the association between telomere length and the outcomes of open heart surgery in elderly patients. The patients had also been evaluated for frailty, based on gate speed and STS risk

score (Afilalo et al., 2010). Our goal was to find a triple risk score that would better predict the outcomes of cardiac surgery in elderly patients, by combining 1. telomere length, 2. STS risk score and 3. gate speed test. However, we failed to find an association between telomere length and markers of aging, CVD, or surgery outcomes.

The conclusions of correlation studies based on telomere length are often controversial, and a recent study suggested that the technique used and measurement error account largely for the different results observed (Aviv et al., 2011). We speculate that the lack of correlation in our study is likely due to the narrow range of the cohort studied. Most telomere length studies have evaluated particular traits in two distinct populations: normal and affected patients. Our cohort included patients chosen in function of very specific criteria: 70 years of age and older and undergoing cardiac surgery. Such narrowing of the cohort studied combined with typical measurement error associated with qPCR makes the observations of significant differences problematic.

The lack of predictive value of telomere length in our 70-year and older cohort is in accordance with recent similar studies. No association between telomere length and a variety of cognitive, physical or social traits was found in a cohort of 1071 relatively healthy 70-years old Scots (Harris

et al., 2010). Similarly, a large cohort study measured the correlation between 74 biomarkers of aging and 4 health status measures (multimorbidity, cognitive impairment, disability and proximity to death) in 85year and older individuals. While 10 biomarkers were significantly associated with 2 or more health measures, telomere length was not associated with health status (Martin-Ruiz et al., 2011). Although it is not known exactly why the predictive value of telomere length decreased in older individuals, it has been shown that telomere length is highly unstable in older patients, as measured by a greater variation between different samples when they are taken at different time points over a 10 day period and compared to younger control patients (Martin-Ruiz et al., 2005). Our data reinforce the notion that telomere length cannot be used as a biomarker in every disease or all cohorts of individuals. It could be interesting to verify if telomere length could predict the outcomes of cardiac surgery in younger patients. In addition, the addition of a group of 70-years old patients without any CVD would have been a good control for our type of analysis.

6.4 Conclusions

The biology of telomeres and telomerase is complex, and can be extended to many other areas of research. Our work explored the wide range of applications that telomeres and telomerase research could have for human health. Understanding how telomerase and the ALT pathway are regulated and related will certainly help to develop safer and more effective anti-cancer therapies, and will allow the targeting of a wider range of human cancers. Understanding how protein function is modulated post-transcriptionally will undoubtedly help to understand and eventually cure cancer or diseases. Finally, the development of new tools to measure telomere length and a better understanding of telomere dynamics in different populations might help to develop more accurate biomarkers, with both diagnostic and prognostic value.

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