Department of Dermatology and Allergic Diseases
Ulm University, Germany

Chair: Prof. Dr. med. Karin Scharffetter-Kochanek



Telomerase stimulates ribosomal DNA transcription under hyperproliferative conditions

Dissertation for gaining the Doctoral Degree of Human Biology (Dr. biol. hum.)
of the Medical Faculty of Ulm University

Omar García González
Havana – Cuba

MMXIV

Dean/Chairman: **Prof. Dr. Thomas Wirth**

Thesis Advisory Committee:

First Supervisor: **PD Dr. Sebastian Iben**
Department of Dermatology and Allergic Diseases
University of Ulm, Germany

Second Supervisor: **PD Dr. Cagatay Günes**
Leibniz Institute for Age Research
Fritz Lipmann Institute - FLI, Germany

Day doctorate awarded:
February 27, 2015

STATUTORY DECLARATION:

I hereby declare that I wrote the present dissertation with the topic: “Telomerase stimulates ribosomal DNA transcription under hyperproliferative conditions”, independently and used no other aids that those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.

I also hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current rules of the University of Ulm for Assuring Good Scientific Practice].

Ulm, 1st December 2014

Omar García González

..."La ciencia trascendental es la verdad única, generadora y matriz de todo género y toda clase de verdades"

José Martí

A mis queridos padres y mi familia
con todo mi cariño y amor

TABLE OF CONTENTS

ABBREVIARIONS vi

1. INTRODUCTION 8

1.1 RNA polymerase I and telomerase 8
1.2 The mechanism of transcription by RNA polymerase I 9
1.2.1 Organization of the rDNA genes 11
1.2.2 RNA polymerase I transcription cycle. 13
1.2.2.1 Pre-initiation complex formation 13
1.2.2.2 Initiation and promoter escape 15
1.2.2.3 Elongation of transcription 15
1.2.2.4 Termination and reinitiation of transcription 16
1.2.3 Regulation of rRNA transcription 17
1.2.3.1 DNA methylation and gene expression 18
1.2.4 The role of rRNA synthesis in cell growth and cell cycle progression. 18
1.3 The telomerase structure and its canonical function 19
1.3.1 TERT and TERC complex 20
1.3.2 Non-canonical functions of telomerase 24
1.3.3 Telomerase and human diseases: ageing and cancer. 28
1.4 Aims of the study 30

2. MATERIALS & METHODS 31

2.1 Cell culture 31
2.1.1 Cell lines 31
2.1.2 Cell culture conditions 31
2.1.3 Transfection 32
2.2 Molecular biology methods 34
2.2.1 Antibody generation 34
2.2.2 Peptide competition assay 35
2.2.3 Chromatin Immunoprecipitation (CHIP) 35
2.2.3.1 Isolation and fragmentation of the chromatin 35
2.2.3.2 Chromatin preclearing and immunoprecipitation 36
2.2.3.3 DNA extraction and purification 37
2.2.4 Polymerase chain reaction (PCR) 38
2.2.5 Gel Electrophoresis 38
2.2.6 Quantitative real-time PCR (qPCR) 39
2.2.7 ChIP – re – ChIP assay 40
2.2.8 Quantification of Telomere-DNA 40
2.2.9 Partial hepatectomy (PH) and proliferation assay 42
2.2.9.1 Animals 42
2.2.10 RNA preparation and cDNA synthesis. 42
2.3 Protein biochemical methods 43
2.3.1 Preparation of nuclear extracts 43
2.3.2 Immunoprecipitation assays 43
2.3.3 Telomerase repeat amplification protocol (TRAP) 44

2.3.4 Western Blot analysis 45
2.3.5 Purification of telomerase recombinant proteins 46
2.3.6 RNA polymerase I *in vitro* transcription experiment. 46
2.3.6.1 Metabolic Labelling 47
2.3.6.2 Nuclear run-on transcription 48
2.3.7 Template immunoprecipitation (TIP) assay 48
2.3.8 Statistics 49

3. RESULTS 50

3.1 Telomerase binds to ribosomal DNA 50
3.1.1 Telomerase binds to promoter and gene internal regions of rDNA. 50
3.1.2 TERT binds to the TBE sequences of the c-Myc promoter. 52
3.1.3 TERT binds un-methylated active ribosomal RNA genes 53
3.1.4 Characterization of a newly generated anti-TERT antibody (Ab-8313) 54
3.2 Telomerase binds rDNA under hyperproliferative conditions. 56
3.2.1 TERT is present at the telomere in proliferating carcinoma cells. 60
3.2.2 rDNA occupancy of telomerase in proliferating hepatocytes 60
3.3 Telomerase stimulates Pol I transcription. 62
3.3.1 Growth signal-dependent stimulation of rDNA reporter transcription by TERT 63
3.3.2 Telomerase regulates cell proliferation by modulating rDNA transcription 63
3.4 Telomerase stimulates initiation complex at the rDNA promoter. 68
3.4.1 Telomerase does not leave the rDNA promoter during transcription 68
3.4.2 rDNA promoter occupancy of TERT is impaired by telomerase inhibition leading to a reduction of Pol I activity in vitro. 69
3.4.3 Binding of telomerase to rDNA gene internal regions might not directly influence the Pol I transcription elongation stage. 71
3.4.4 Telomerase stimulates initiation complex formation at the rDNA promoter. 73
3.5 Inhibition of telomerase or Pol I retards proliferation. 76

4. DISCUSSION 79

4.1 Telomerase beyond telomeres: New roles for an enzyme 79
4.2 Binding capacity of telomerase to ribosomal DNA repeats 80
4.2.1 TERT binds to rDNA in regenerating hepatocytes 82
4.3 Telomerase stimulates ribosomal transcription 83

5. SUMMARY 87

6. REFERENCES 91

ACKNOWLEDGEMENTS 103

APPENDICES 105
· Curriculum vitae
· Publication list

ABBREVIARIONS

ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
cDNA	complementary DNA
ChIP	Chromatin ImmunoPrecipitation
CTP	Cytidine triphosphate
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	DiMethyl SulfOxid
DTT	DiThioThreitol
EDTA	EthylenDiaminTetraacidic Acid
EtBr	Ethidium bromide
ETS	External transcribed spacer
FBS	Fetal Bovine Serum
GAPDH	GlycerAldehyde-3-Phosphate DeHydrogenase
GTP	Guanosine TriPhosphate
HA	Human influenza hemagglutinin protein
HEPES	4-(2-HydroxyEthyl) Piperazine-1-EthaneSulfonic acid
IGS	Inter Genic Spacer
IP	Immunoprecipitation assay
ITS	Internal transcribed spacer
MNase	Micrococcal Nuclease
mRNA	Messenger RNA
NP40	Nonidet 40
NTP	Nucleotides
NTS	Nontranscribed spacer
PAGE	PolyAcrylamid GelElectrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PH	Partial hepatectomy
PIC	Pre-initiation complex
Pol I	RNA polymerase I

Pol II / Pol III	RNA polymerase II / III
RP	Ribosomal protein
RPA135	Subunit of RNA polymerase I
rRNA	Ribosomal RNA
SDS	Sodium Dodecyl Sulphate
shRNA	Small or short hairpin RNA
siRNA	Small interfering RNA
SL1	Selectivity factor 1
TAFs	TATA-box-associated factors
TBE	Tris-Borate-EDTA buffer
TBE2	TCF binding element 2
TBP	TATA-box-binding protein
TEMED	N,N,N',N'-TEtraMEthylethyleneDiamine
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase subunit
TFIIH	Transcription factor II H
TIF-IA	Pol I basal transcription initiation factor
TIP	Template immunoprecipitation assay
TRAP	Telomerase repeat amplification protocol
Tris	TRIS hydroxymethylaminomethane
TTD	Trichothiodystrophy
TTF-I	Transcription termination factor
UBF	Upstream Binding Factor
UCE	Upstream control element
UTP	Uridine TriPhosphate
3´UTR	3´ Untranslated region
WB	Western blot



1. INTRODUCTION

1.1 RNA polymerase I and telomerase

Cellular growth is the accumulation of cell mass and is closely related to cellular proliferation (increase in cell number) and ribosomal biogenesis is a key step regulating both phenomena. Ribosomal ribonucleic acids (rRNA) are the predominant components of the ribosome and are essential for protein synthesis in living organisms. The increased demand for protein synthesis that characterizes proliferating cells is accomplished by changes in the rate of ribosome biogenesis; thereby ribosome biogenesis is a limiting factor for cell growth and proliferation.

A progressive increase in the synthesis of rRNA, which is transcribed in the nucleolus by the RNA polymerase I (Pol I) is a requisite in cells stimulated to proliferate. Thus, tumour cells show an excessive increase in the synthesis of rRNA, which is correlated with an adverse prognosis in cancer *[Drygin, D et al., 2011[1]].* Moreover, enlarged nucleoli, reflecting accelerated rRNA synthesis, have long been recognized as a marker for aggressive tumour cells *[Derenzini, M et al., 2000[2]; Maggi, LB et al., 2005[3]].*

Telomerase is the enzyme responsible for maintenance of the telomere length at the chromosome ends. This catalytic telomere elongation is known as the canonical function of telomerase, and it is essential for the long-term proliferation potential in immortal cells (i.e. cancer cells, germ cells, embryonic stem cells and

in vitro immortalized cells) *[Wright, WE et al., 1996[4], Greenberg, RA et al., 1998[5]]*. With the exception of stem and germ cells and some other highly proliferating cells, the telomerase ribonucleoprotein complex is continuously repressed in most normal somatic tissues, therefore telomeres shorten progressively with each cell division due to the end-replication problem. In contrast, 70 – 90% of cancer cells stably express the telomerase enzyme, which is reactivated during malignant transformation conferring unlimited proliferation capacity for the tumour cells. This process is mainly thought to occur through the stabilisation of telomere length to overcome cellular crisis (genome instability). Telomerase activity has also been shown to strongly correlate with the state of malignancy and metastatic potential of cancer cells, emphasising the role of telomerase in tumorigenesis.

However, telomerase may also contribute to tumorigenesis via other mechanisms beyond its action at the telomeric level. Indeed, human telomerase can function as a transcriptional modulator of the Wnt-β-catenin signalling pathway as a cofactor in a transcriptional complex with β-catenin. In addition, although telomerase mainly localises to Cajal bodies *[Zohng, F et al., 20011[6]],* a nucleolar localisation of TERT have been described *[Etheridge, KT et al., 2002[7]]*, which is unrelated to its telomere-elongating function *[Wu, YI et al., 2006[8]]*. Interestingly, the nucleoli are the sites where ribosomal biogenesis takes place through the rDNA transcription by Pol I.

The catalytic activities of both telomerase and RNA polymerase I enzymes are tightly related with cellular growth and cell cycle progression. The dynamics of telomerase in RNA polymerase I transcription and its contribution to tumorigenesis will be discussed in the forthcoming chapters.

1.2 The mechanism of transcription by RNA polymerase I

Synthesis of rRNA is the first step in ribosome biogenesis and a focal point for the regulation of cell growth *[Grummt, I et al., 2003[9]; Moss, T et al., 2007[10]]*. The RNA polymerase I transcription activity accounts for up to 60% of total nuclear transcription in a growing cell and the product rRNA accounts for up to 80% of all cellular RNA *[Warner, JR 1999[11]]*. Ribosome biogenesis is a highly coordinated, multistep process that takes place in the nucleolus, where ribosomal RNAs

(rRNAs) are synthesized, processed and assembled into ribosomal subunits.

At the core of the protein biosynthesis process is the ribosome, a large molecular machine responsible for translating genetic material into functional proteins *[Frank, J 2000[12]]*. Ribosomes are ribonucleoprotein complexes consisting of ribosomal RNA (rRNA) and over 80 ribosomal proteins. Ribosomes of eukaryotic organisms are comprised of ~50% RNA and 50% protein where the large subunit (LSU) contains a 5S rRNA, 25-28S rRNA, 5.8S rRNA *[Sommerville, J 1986 [13]]* and about 42 proteins *[Verschoor, A et al., 1998 [14]]* and the small subunit (SSU) contains an 18S rRNA and about 33 proteins.

The translation of genetic information into proteins is a major task performed by living cells. The cell must govern both the amounts of specific proteins synthesized as well as the total protein synthesized in response to environmental signals and internal programming. The protein synthetic capacity of a cell is primarily regulated by the steady state number of the ribosomes, which in turn is dictated by the relative rates of ribosome synthesis and degradation *[Moss, T et al., 1995[15]]*. Regulation of cell growth ultimately depends on the control of synthesis of new ribosomes *[Warner, JR 1999[11], 2001[16]]*, which is relatively stable in the majority of cells.

A striking feature of ribosome synthesis is the requirement for the coordinated activity of the three forms of RNA polymerase (Pol I, Pol II and Pol III) to produce the building blocks for ribosome construction. Recently it was shown that the accumulation of large rRNAs, as a result of deregulated Pol I transcription, led to concomitant accumulation of 5S rRNA, mRNAs encoding ribosomal proteins and of fully assembled ribosomes *[Laferte, A et al., 2006[17]]*. This observation suggested a central role for Pol I activity in ribosome synthesis *[Chédin, S et al., 2007[18]]*. In addition to rDNA transcription, ribosome biosynthesis requires pre-rRNA processing and ribosome assembly, which takes place in nucleoli within the nucleus.

1.2.1 Organization of the rDNA genes

The rRNA gene is the most conserved DNA in all cells. The rDNA array of a eukaryotic nuclear genome typically consists of several hundred tandem repeated copies of the transcription unit and non-transcribed spacer. The mammalian rDNA transcription units are large, comprising ~43 kb in humans and ~45 kb in mice *[Grozdanov, P et al., 2003[19]; Sylvester, JE et al., 2004[20]]*. The sequences that encode pre-rRNA (13 – 14 kb) are separated by long intergenic spacers (IGS) of approximately 30 kb. Regulatory elements, including gene promoters, spacer promoters, repetitive enhancer elements, and transcription terminators, are located in the IGS (Figure 1). The rDNA promoter consists of a core promoter element adjacent to the transcription start site and an upstream control element (UCE) approximately 100 nucleotides further upstream *[Haltiner, M et al., 1986[21]; Learned, RM et al., 1986[22]]*. The rDNA transcription units are flanked at their 5´ and 3´ ends by one or more terminator elements that are recognized by the transcription termination factor (TTF-I), a specific DNA binding protein that stops elongating Pol I and serves an important role in epigenetic regulation of rRNA genes *[Grummt, I et al., 1985[23], 1986[24]; Henderson, S & Sollner-Webb, B 1986[25]; McStay, B & Reeder, RH 1986[26]]*.

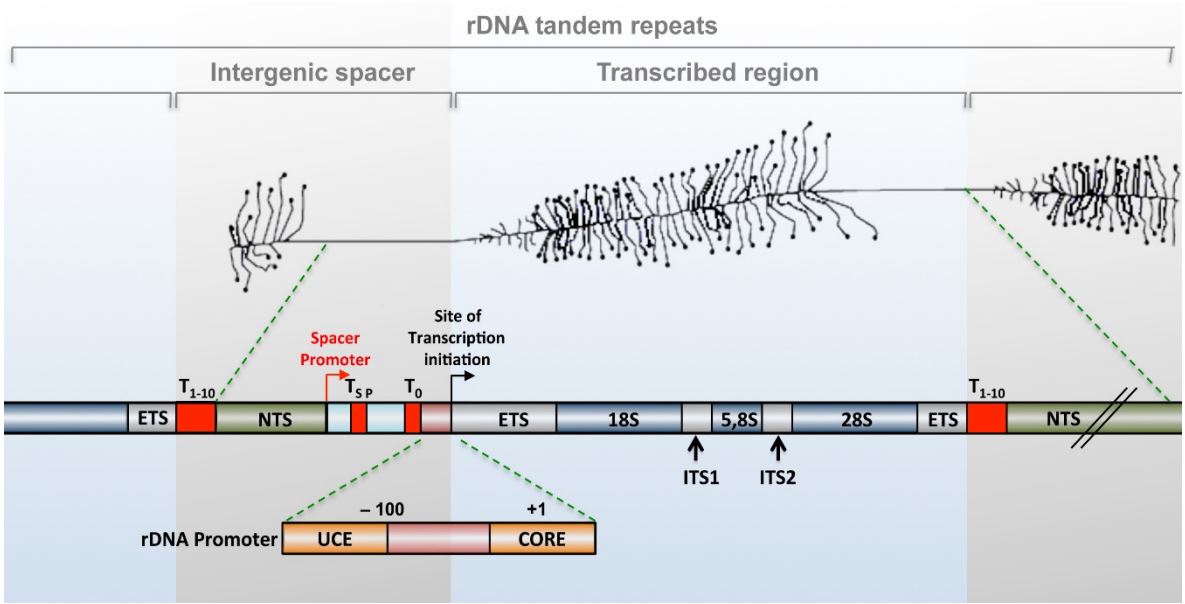


Figure 1: *The diagram shows the structural organization of mammalian rDNA transcription unit. The site of transcription initiation of 47S pre-rRNA (black arrow) and intergenic transcripts from the spacer promoter (red arrow) are indicated. Terminator elements are located downstream of the transcription unit (T_{1-10}), downstream of the spacer promoter (T_{SP}), and upstream of the gene promoter (T_0) (red boxes).*

Ribosomal RNA (rRNA) genes in eukaryotic genomes are arranged in tandemly repeated clusters in the nucleolus organizers of one or more chromosomes, separated by several spacers, namely the NTS (nontranscribed spacer), ETS (external transcribed spacer), and ITS1 and ITS2 (internal transcribed spacers). The NTS separates neighbouring repeat units, ETS is located between the promoter and 18S gene, ITS1 is located between the 18S- and 5.8S-like coding regions, and ITS2 lies between the 5.8S- and 28S-like genes (Figure 1) *[Gerbi, SA 1985[27]]*.

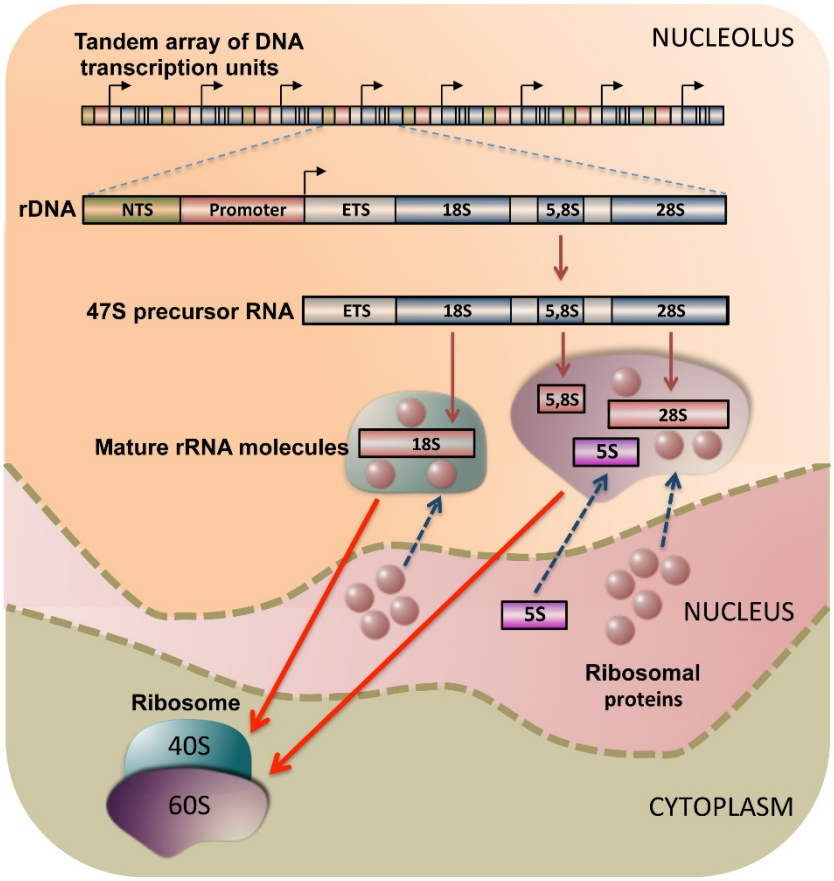


Figure 2: *Schematic representation of the main processes involved in ribosome biogenesis. One ribosomal gene unit is repeated hundreds of times in the genome and includes the promoter and the transcribed region. After transcription, the 47S pre-rRNA is processed. The mature rRNAs are assembled with ribosomal proteins (RP) to generate mature ribosomal subunits. In particular, 18S is assembled with small subunit RPs to generate the 40S small ribosomal subunit, whereas 5.8S and 28S rRNA are assembled with 5S rRNA (which is transcribed by Pol III in the nucleus), and with large subunit RPs to form the 60S large ribosomal subunit. Mature 40S and 60S subunits are then exported to the cytoplasm to constitute the ribosomes.*

As shown in Figure 2, each active rRNA gene is transcribed by RNA polymerase I to generate a 47S precursor rRNA. After synthesis, this primary transcript is

subsequently processed and modified to generate one molecule each of mature 18S, 5.8S, and 28S rRNA, which together with 5S rRNA (which is transcribed by Pol III) forms the RNA backbone of the ribosome *[Drygin, D et al., 2010[1]]*.

1.2.2 RNA polymerase I transcription cycle.

The synthesis of rRNA is confined to the nucleolus and is catalysed by RNA polymerase I, using the rDNA repeats as template. In order to efficiently transcribe the ribosomal gene, RNA polymerase I requires a number of accessory factors, which play essential roles during every stage of the transcriptional process (e.g. recruitment of Pol I to promoter and formation of pre-initiation complex, transcription initiation, elongation, termination and re-initiation).

1.2.2.1 Pre-initiation complex formation

The first stage of transcription by RNA polymerase I is the formation of a pre-initiation complex (PIC) at the gene promoter (Figure 3). The rDNA promoter region contains two important domains, the core promoter element and the upstream control promoter element (UCE) that enable the formation of a transcriptionally competent complex *[Xie, L & Rothblum, WQ 1992[28]; Hannan, LI et al., 1995[29]]*. In humans, the core promoter element is located from approximately +6 to –31, with respect to the transcription initiation site and the upstream control element (UCE) extends from the core to ~ –167.

Pol I is the most complex component of the PIC and has a molecular weight of ~600 kDa, comprising 14 polypeptide subunits in yeast, homologues for 13 of which have been identified in mammals. A catalytic core is formed by ten of these subunits, which are shared with or homologous to subunits found in Pols II and III *[Kuhn, C et al., 2007[30]; Werner, M et al., 2009[31]]*. Transcription by Pol I in mammalian cells is dependent upon the upstream binding factor (UBF) and the promoter selectivity factor 1 (SL1), which is a complex of TATA-box-binding protein (TBP) and at least four Pol I-specific TBP associated factors (TAFs) *[Goodfellow, S & Zomerdijk, J 2013[32]]*. UBF binds as a dimer to the UCE and core regions of the *promoter [O'Sullivan, A et al., 2002[33]]*, playing critical roles as a nucleolar scaffold protein and in promoting decondensation of rDNA chromatin *[Chen, D et al., 2004[34];*

Mais C et al., 2005[35]; Sanij, E et al., 2008[36]. Crucially, UBF can also activate promoter-specific transcription by Pol I. UBF interacts cooperatively with SL1 at the rDNA promoter *[Beckmann, H et al., 1995[37]]*. This stabilises the association of UBF with the PIC *[Friedrich, JK et al., 2005[38]]*, hence facilitating promoter-specific transcriptional activation. In addition, SL1 confers promoter specificity by recognising and binding the core promoter element in the rDNA repeat, it is essential for Pol I recruitment to the transcription start site, and it promotes a stable interaction between UBF and the rDNA promoter.

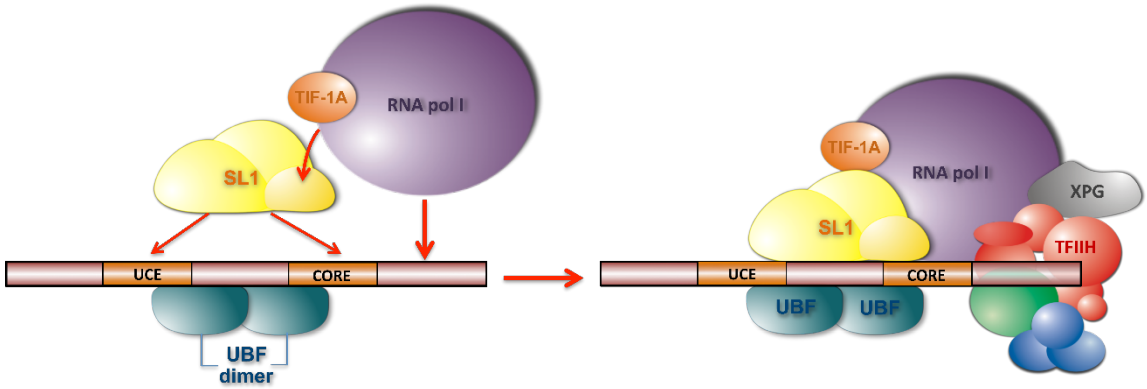


Figure 3: *Assembly of the RNA polymerase (Pol) I pre-initiation complex (PIC) at ribosomal RNA gene (rDNA) promoters begins with the binding of upstream binding factor (UBF) to the upstream control elements (UCEs) and core element of the rDNA promoter, leading to the recruitment of the SL-1 initiation factor, which contains TATA-box-binding protein (TBP) and at least five TATA-box-associated factors (TAFs). The resultant stable UBF/SL-1 complex recruits an initiation-competent form of RNA Pol I, which contains TIF-IA that mediates interactions between Pol I and SL-1*

For the assembly of the PIC, SL1 recruits Pol I to the promoter via interaction of its TAFI63 and TAFI110 subunits with the Pol I basal transcription initiation factor (TIF-IA), which in turn, interacts with the Pol I core subunit A43 (RPA43) and the associated factor PAF67, thereby serving as a bridge between Pol I and the pre-initiation complex at the rDNA promoter *[Moss, T et al., 2007[10]]*. TIF-IA is an essential transcription factor that regulates Pol I activity in response to growth status and the cellular need for ribosomes and protein synthesis *[Peyroche, G et al., 2000[39]]*. Fluctuations in rRNA synthesis in response to external signals correlate with phosphorylation of TIF-IA at specific serine residues by ERK and RSK kinases. Mutations of these phosphorylation sites abolish TIF-IA activity, impair Pol I transcription, and retard cell growth. Moreover, signals that affect cell proliferation and metabolism alter the phosphorylation pattern of TIF-IA; for

example, in growth-arrested cells TIF-IA is hypophosphorylated and incapable of binding to Pol I. In summary, a series of cooperative protein-protein and protein-DNA interactions involving SL1, UBF and specific promoter elements are required for the recruitment of polymerase poised for the activated transcription of rRNA genes.

1.2.2.2 Initiation and promoter escape

Following the assembly of a productive initiation complex formation at the rDNA promoter, promoter opening and transcription initiation by Pol I can start, defined by the incorporation of the first ribonucleotides of the RNA chain. However, for productive RNA synthesis to ensue, Pol I must dissociate from the promoter-bound initiation factors in a process known as promoter escape *[Panov, KI et al., 2006a, b[40,41]; Russell, J and Zomerdijk, J 2005[42]]*. Productive initiation complex formation leads to promoter opening, whereupon the first ribonucleotide becomes incorporated and transcription initiates. The RNA polymerase then stutters over the synthesis of the first few nucleotides until the inhibitory interactions between the enzyme and the transcription factors at the promoter are surmounted, and Pol I escapes the promoter to engage in elongation of the transcript *[Russell, J & Zomerdijk, J 2005[42]]*. Interestingly, promoter escape or clearance is the rate limiting step in a reconstituted Pol I transcription system. The mammalian activator of Pol I transcription, UBF, also plays an important role in stimulating promoter escape *[Panov, KI et al., 2006a[40]]*.

1.2.2.3 Elongation of transcription

The transcription elongation by Pol I is a highly efficient process. This impressive transcriptional output is achieved through the intrinsic processivity of Pol I and its cooperation with a multitude of other proteins. Pol I-specific transcription factors are also thought to play a role in elongation. As Pol I escapes the promoter, UBF and SL1 remain promoter-bound, ready to recruit another Pol I complex and reinitiate transcription from the same promoter, and so support multiple rounds of transcription. UBF is distributed throughout the rDNA repeats and has been reported to regulate Pol I elongation by phosphorylation-dependent remodelling of the rDNA chromatin *[O'Sullivan, A et al., 2002[33]; Stefanovsky, V et al.,*

2006[43]]. More recently, SL1 has been proposed to assist elongating Pol I via its role in anchoring the core promoter, upstream region and terminator, which provides a spatial arrangement favourable for productive rRNA synthesis *[Denissov, S et al., 2011[44]]*.

Elongation is likely to be interrupted at sites of DNA damage in the rDNA template. Transcription-coupled repair occurs in Pol I-transcribed genes *[Conconi, A et al., 2002[45]]* and this might explain the presence of DNA-repair proteins TFIIH, Cockayne syndrome B (CSB) and Xeroderma pigmentosum group G (XPG) in a complex with Pol I that participates in rDNA transcription in the nucleolus of mammalian cells. Mutations in CSB, and the XPB and XPD helicase subunits of TFIIH (all of which confer Cockayne's syndrome), disrupt the interaction of TFIIH with Pol I in this complex *[Bradsher, J et al., 2002 [46]]*. TFIIH is also found associated with SL1 and is required for productive rDNA transcription. Moreover, TFIIH has been shown for stimulate elongation of RNA polymerase I transcription *[Iben, S et al., 2002[47]; Lebedev, A et al., 2008[48]; Assfalg, R et al., 2012[49]]*.

1.2.2.4 Termination and reinitiation of transcription

Transcription termination by Pol I is a multistep process involving specific DNA sequence elements and regulatory proteins. In mammals, transcription termination factor TTF-I binds terminator elements downstream of the rRNA gene (T_1-T_{10}), causing polymerase pausing. Dissociation of the paused transcription complex is then mediated by Pol I and the transcript release factor PTRF.

The termination step might be rate limiting in cases of high rRNA production. Following termination of transcription, the components of the released polymerase are likely to be recycled and then indirectly stimulate the reinitiation of transcription by Pol I at the same rDNA gene. Correct termination of transcription and release of the nascent rRNA is required for reinitiation by Pol I. Once a gene is activated, the rate of re-initiation will contribute to the overall level of transcripts produced. In addition to terminator elements located downstream of the rRNA coding region, TTF-I binding sites are also found immediately upstream of the rDNA promoter

(termed T_0) and downstream of the spacer promoter (T_{sp}). In cells, efficient recycling and re-initiation by Pol I might be facilitated by TTF-1.

1.2.3 Regulation of rRNA transcription

The rate of cell growth is directly dependent on the rate of protein synthesis, which, itself, is intricately linked to ribosome synthesis and rRNA transcription. Thus, intracellular signals must coordinate the synthesis of rRNA with that of other components of protein translation. Given the repetitive nature of rRNA genes, two specific mechanisms ensure proper control of rRNA synthesis and Pol I-mediated transcription. Pol I transcription may be controlled either by changing the rate of transcription from each active gene or by adjusting the number of genes that are involved in transcription. The majority of short-term regulation affects the rDNA transcription cycle, e.g., preinitiation complex assembly, initiation, promoter escape, and transcription elongation or termination *[Russell, J & Zomerdijk, J 2005 [42]]*. Moreover, posttranslational modifications influence the activity and functional interplay of transcription factors, adding further complexity and fine-tuning to transcriptional regulation in response to external signals that affect cell growth and proliferation *[Grummt, I 2003[9], Russell, J & Zomerdijk, J 2005[42]]*.

In mammalian cells, the number of active rDNA genes varies between different cell types and level of differentiation *[Haaf, T et al., 1991[50]]*. In general, as a cell becomes more differentiated, it requires less growth and, therefore, will have a decrease in rRNA synthesis and a decrease in rDNA genes being transcribed. Thus, long-term changes in rDNA transcription can be achieved by regulating the number of rRNA genes that are transcriptionally active. The Christmas tree view of active ribosomal RNA (rRNA) genes suggests a gene topology in which a large number of nascent rRNA transcripts are prevented from intertwining. The balance between active and inactive rDNA repeats is regulated by epigenetic mechanisms *[Grummt, I & Pikaard, CS 2003[51]; Lawrence, RJ & Pikaard, CS 2004[52] ; McStay, B 2006[53]]*.

1.2.3.1 DNA methylation and gene expression

DNA methylation in vertebrates has been viewed as a mechanism for silencing transcription. The level of cellular rRNA is regulated by changing the rate of transcription initiation at active rDNA genes rather than by activating silent transcription units. While DNA methylation of CpG islands downstream of promoters is an epigenetic mark associated with gene silencing, DNA sequences that are transcriptionally active require being unmethylated, at least at the promoter regions (Table 1).

The epigenetic regulation is stably propagated through the cell cycle to ensure that an appropriate proportion of active and inactive rDNA repeats is maintained *[Conconi, A et al., 1989[54]]*. Only half of the rDNA genes are transcriptionally active and maintained in an "open" chromatin conformation. The other half that corresponds to inactive gene copies resides in a compact, nucleosomal structure.

Table 1: *Features associated with transcriptionally active and inactive chromatin*

Feature	Chromatin conformation	
	Transcriptionally active	Transcriptionally inactive
Chromatin conformation	Open, extended	Highly condensed
DNA methylation	Relatively unmethylated, especially at promoter regions	Methylated, including at promoter regions

1.2.4 The role of rRNA synthesis in cell growth and cell cycle progression.

Certainly there is a crucial role for rRNA synthesis in normal cell growth and in the adjustment of cell growth in response to growth factors and nutrient availability. Cell growth is essential for cell-cycle progression and sustained cell proliferation, and the attainment of a particular cell mass is a prerequisite for cell division *[Saucedo, LJ & Edgar, BA 2002[55]]*. The level of rDNA transcription is increased in transformed cells and, indeed, the corresponding increase in nucleolar size is often used as a prognostic marker for the rapidity of cancer-cell proliferation *[Derenzini, M et al., 1998[56]]*. The increased rRNA levels support the rise in protein synthesis required to accommodate the escalated cell growth and proliferation in transformed cells. The rDNA transcription might be upregulated in

transformed cells by overexpression or increased activity of components of the Pol I transcription machinery or proteins that directly affect rDNA transcription. Constitutively active growth-factor-independent signalling pathways can activate Pol I transcription and drive ribosome biogenesis in cancer cells.

1.3 The telomerase structure and its canonical function

To understand the canonical function of telomerase (telomere elongation) it is important to explain the biology of the telomeres. Telomeres form specialized structures at the end of linear chromosomes that ensure their integrity and stability. Its name is derived from the greek words telos (τέλος) –„end“ and meros (μέρος) – „part“; meaning „the ending part“). Telomeres are long tracts of DNA at the linear chromosomes ends composed of tandem repeats of a guanine rich sequence motif that vary in length according to species. The basic telomere DNA repeat unit in vertebrates is the hexamer TTAGGG, and is bound by a multi protein complex known as shelterin, which serves to stabilize the T-loop and D-loop structures, protecting them from being recognized by the DNA damage machinery as dsDNA breaks.

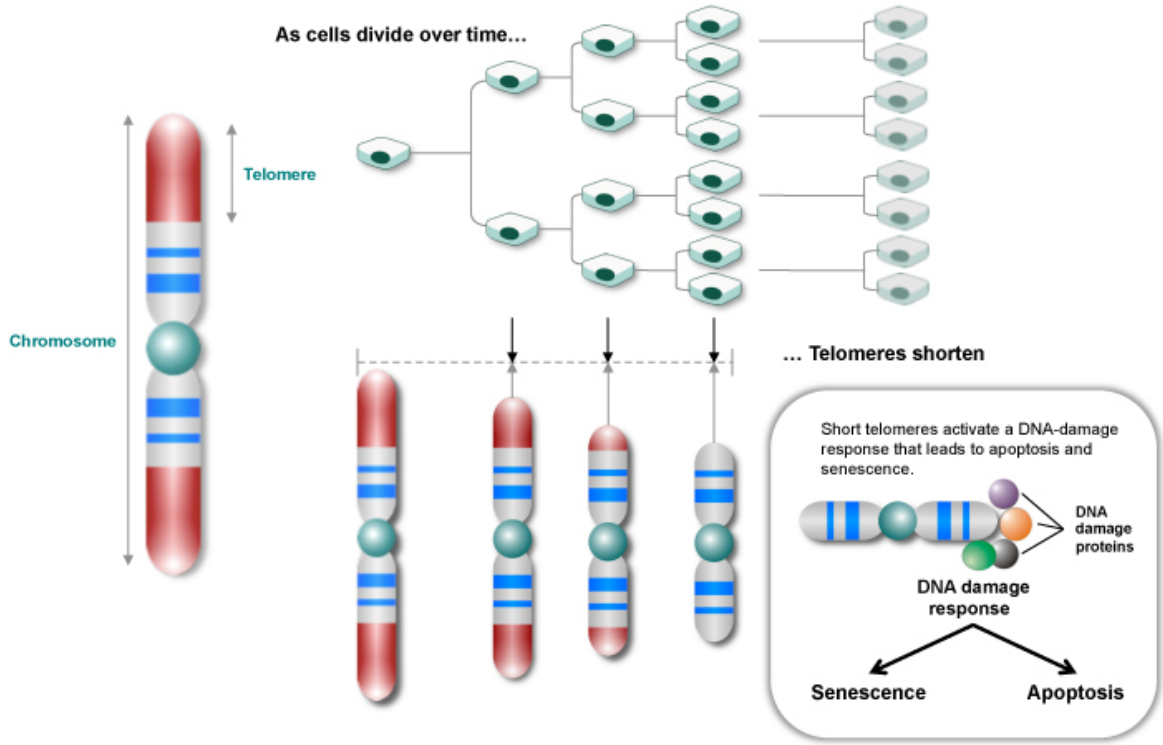


Figure 4: *Telomeres become progressively shorter each time the cell divides. When they get too short, cells reach replicative senescence and can no longer divide. The result can be the various conditions associated with old age.*

With each cell division telomeres become shorter due to the "end-replication problem", which occurs when during replication the DNA of the lagging 3' strand cannot be elongated by DNA polymerase continuously and gets shorter than the leading 5' strand. The DNA polymerase requires an RNA primer to initiate synthesis in the 5´- 3´ direction. At the end of a linear chromosome the DNA polymerase can synthesize the leading strand until the end of chromosome. However, in the lagging strand the synthesis is based on a series of Okazaki fragments requiring RNA primers. Without DNA to serve as template for a new primer, the replication machinery is unable to synthesize the sequence complementary to the final primer event. The result is an end-replication problem in which a short fragment of the telomere (~200 bp) is lost during each round of replication. Once the length of the telomeric DNA reaches a critical level the cell is facing replicative senescence and cell cycle arrest or apoptosis *[Blasco, MA 2005[57]; Galati, A et al., 2013[58]]* (Figure 4). This loss of cell viability associated with telomere shortening is thought to contribute not only to the onset of degenerative diseases that occur during aging, but also to several age associate diseases such as cancer *[Donate, LE & Blasco, MA 2011[59]]*.

1.3.1 TERT and TERC complex

The progressive telomere loss eventually leads to critically short telomeres which leads a DNA damage response (DDR) resulting in chromosomal end-to-end fusions or a permanent cell cycle arrest (termed cellular senescence) or apoptosis (programmed cell death) *[Blasco, MA et al., 2005[57]; Galati, A et al., 2013[58]]*. This loss of cell viability associated with telomere shortening is thought to contribute not only to the onset of degenerative diseases that occur during human aging, but also to several age associated diseases such as cancer *[Donate, LE & Blasco, MA 2011[59]]*. The majority of cells with critical short telomeres and programmed to enter senescence (crisis stage) die due to massive genomic instability, but in rare circumstances cells can escape the crisis stage by stabilizing their telomeres, commonly accomplished by activating the enzyme telomerase *[Counter, CM et al., 1992[60]]*.

The telomerase complex is a ribonucleoprotein composed by a catalytic subunit with reverse transcriptase activity (TERT), a RNA component (TERC) which acts as template for the addition of the telomere sequence, as well as other species-specific accessory proteins (Figure 5).

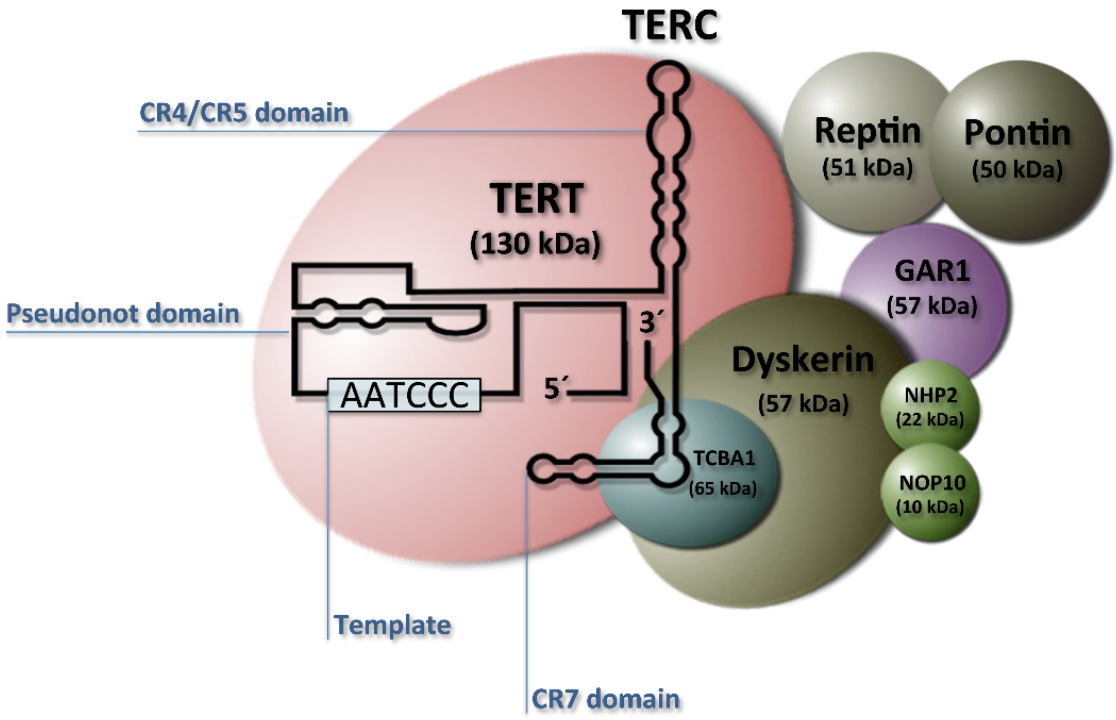


***Figure 5**: Schematic representation of human telomerase and its associated proteins Adapted from Gomez et al. 2012*

The accessory proteins regulate telomerase biogenesis, subcellular localization and its function *in vivo [Wyatt, HD et al., 2010*[61]*]*. In human telomerase, seven associated proteins have been identified: Dyskerin, NHP2 (H/ACA ribonucleoprotein complex subunit 2), NOP10 (nucleolar protein 10), Pontin, Reptin, GAR1 (H/ACA ribonucleoprotein complex subunit 1) and TCAB1 (telomerase Cajal body protein 1) *[Fu, D & Collin, K 2007*[62]*]*. Dyskerin, NHP2 and NOP10 are required for the stability and accumulation of human telomerase RNA (hTR) *in vivo [Fu, D & Collin, K 2007* [62]*]*. Pontin and reptin are two closed ATPases necessary for the stability of Dyskerin and hTERC *in vivo [Veinteicher, A et al., 2008*[63]*]*. The current model is that Dyskerin, Pontin and Reptin form a scaffold that recruits and stabilizes hTERC, and assembles the telomerase ribonucleoprotein particle. Once this complex is formed, Pontin and Reptin are believed to dissociate

from the complex and release the catalytically active enzyme *[Veinteicher, A et al., 2008[63]]*. The subcellular location of telomerase complex assembly is the Cajal body, and it appears to be regulated by the TCAB1 protein *[Zhong, F et al., 2011[6]]*.

The telomerase reverse transcriptase subunit is the rate-limiting component needed for the restoration of the telomerase activity. TERT is a relatively large protein (127 kDa), containing three main structural elements:

i) The N-terminal extension that contains conserved DNA and RNA-binding domains.

ii) The central catalytic domain containing seven evolutionarily conserved reverse transcriptase motifs, essential for the enzymatic activity.

iii) The short C-terminal extension that shows weak sequence conservation suggesting that it may have species-specific functions or that different amino acid sequences have evolved to fold into similar structural domains.

The non-coding telomerase RNA component (TERC) is also essential for telomerase activity *[Blasco, MA et al., 1996[64]]*, but is not a limiting factor for telomerase *[Cairney, CJ & Keith, WN 2008[65]]*. The template part contains a sequence of 11 bp (5-CUAACCCUAAC-3') complementary to the telomeric sequence. TERC consists in three conserved domains:

i) The pseudoknot / template core domain contains a template region for telomeric DNA synthesis, and a pseudonot is essential for telomerase activity either *in vitro* or *in vivo*.

ii) The CR4/CR5 domain that is required for telomerase activity but is not essential for TERC stability in the cell.

iii) The box H/ACA domain, essential for TERC stability, processing, nuclear localization and telomerase activity *in vivo*.

Once TERC precursor is transcribed by the RNA polymerase II, it assembles with the H/ACA core protein of Dykerin and is processed to generate mature TERC, which is then transported to Cajal bodies that are also the sites of telomerase complex assembly and remodelling. Finally TERC binds to TERT in the active telomerase holoenzyme. Both TERT and TERC elements are required and sufficient for the telomerase canonical function.

The maintenance and elongation of telomeres is a process that happens in different stages (Figure 6). First, the nucleotides of the 3' extreme of the telomeric DNA are hybridized to the end of the RNA template, inside the RNA domain of the telomerase complex. The template sequence of 11 nucleotides is complementary to almost two telomeric repeats. Next, the gap in the extreme of the template is completed by synthesis, using triphosphate nucleotides in the catalytic site of the enzyme (hTERT). In this way, a complete hexanucleotidic repeat is assembled in the template. Then telomerase relocates and the cycle is repeated, extending the telomere in the 3´ direction. Finally, the DNA polymerase can synthesize the lagging strand and thus, the end of the chromosome is faithfully replicated.

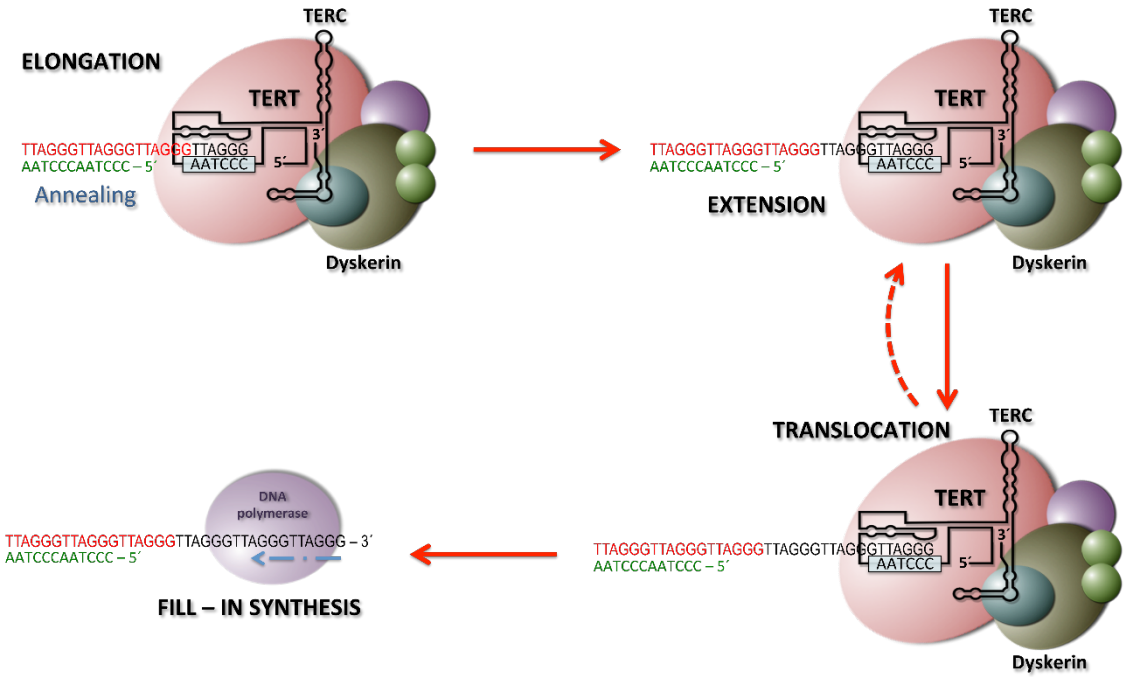


Figure 6: *Telomerase binds to the 3´ flanking end and bases are added using TERC as template. Then, telomerase relocates and telomere is extended in the 3´ direction. Finally, the DNA polymerase can synthesize the lagging strand. This process can maintain telomere length or lead to telomere lengthening.*

Telomerase through its canonical function protects against erosion of telomeres, instability of chromosomes, and against chromosome end fusion. Hence, an active enzyme is necessary to keep an indefinite potential of proliferation in immortal cells (i.e. cancer cells, germ cells, embryonic stem cells and *in vitro* immortalized cells) *[Wright, WE et al., 1996[66]; Martin-Rivera L et al., 1998[67]; Greenberg, RA & Rudolph, KL 2005[68]]*. In the absence of telomerase (i.e. in human somatic cells) telomeres become shorter and activate, at a critical point, a p53-dependent DNA damage response (DDR) in the telomeric region and may lead to cell cycle arrest and senescence or apoptosis *[Saretzki, G et al., 1999[69]; Maser, RS & DePinho, RA 2002[70]]*. Thus, telomerase through its telomere dependent function is involved in a number of processes in cells; most importantly in development, carcinogenesis and senescence.

Mutations or changes in expression of telomerase genes are linked to several diseases. For example, telomerase was found active in over 90% of human malignancies and is responsible for indefinite proliferative capacity of cancer cells. Therefore, a plethora of *in vitro* and *in vivo* studies suggests that telomerase might be a major pharmacological target for new cancer therapies *[Park, YP et al., 2008[71]; Ruden, M & Puri, N 2012[72]]*. In addition to cancer, a number of diseases are related to dysfunctional telomerase genes. For example, Dyskeratosis congenita is a progressive bone-marrow failure syndrome that is characterized by some features of premature ageing, such as abnormal skin pigmentation, leukoplakia and nail dystrophy. This disease is associated to mutations in hTERC gene that are responsible for an autosomal dominant inheritance. The affected patients have an 821-base-pair deletion on chromosome 3q that leads to accelerated telomeres shortening *[Vulliamy, TJ et al., 2001[73]]*. Other examples of diseases where mutations in telomerase genes are risk factors include: Aplastic anaemia, pulmonary fibrosis and hepatic cirrhosis, which are all characterised by abnormally short telomeres *[Young, NS 2012 [74]]*.

1.3.2 Non-canonical functions of telomerase

The canonical function of telomerase is essential for cellular immortality and the long-term proliferation potential of stem cells and cancer cells. However,

there is growing evidence that telomerase has other functions beyond its action at the telomeric level. Furthermore, although telomerase mainly localizes to Cajal bodies, distinct telomerase non-telomeric functions have been described in the cytoplasm, nucleolus, nucleus and mitochondria.

The non-canonical functions can further be divided into those that require telomerase activity but not telomere lengthening (non-canonical I or NC I) and those that require neither telomerase activity nor telomere lengthening (non-canonical II or NC II) (Figure 7). The overexpression of either TERT or TERC appears to be capable of providing NC I functions but NC II functions require neither TERC nor the integrity of the TERT catalytic site *[Parkinson, EK et al., 2008[75]]*.

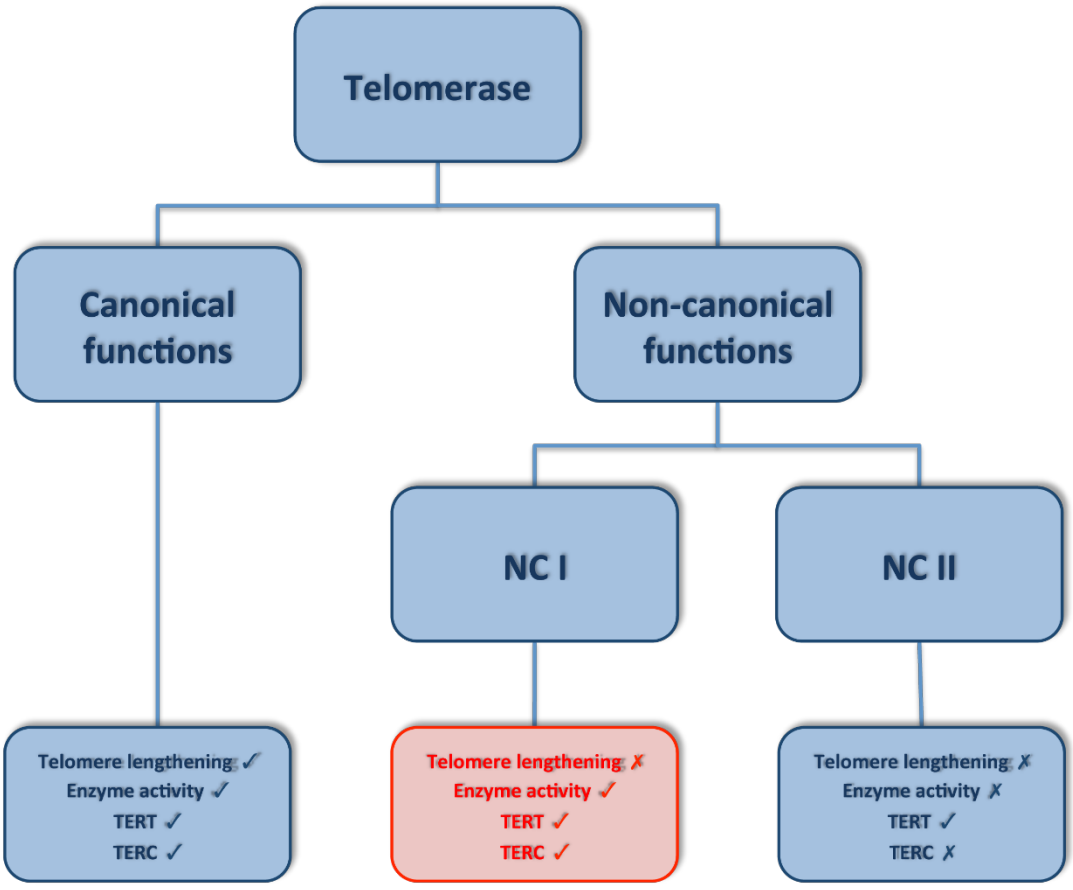


Figure 7: *The diagram summarizes the canonical and non-canonical functions of telomerase and the properties of the enzyme required to fulfill each function. None of the non-canonical functions require telomere lengthening to take place but these functions can be divided into those which require telomerase enzyme activity, the catalytic site and TERC (NC I) and those which do not (NC II). Adapted from Parkinson et al. 2008 (With permission from: S. Karger AG, Basel)*

The first indication that telomerase might have functions other than its established ability to lengthen telomeres came from the observation that telomerase activity is increased in mouse models of epidermal and mammary carcinogenesis *[Bednarek, A et al., 1995[76]; Chadeneau, C et al., 1995[77]; Broccoli, D et al., 1996[78]]*. As mouse cells have a massive telomere reserve compared to human cells, these observations raised the suspicion that telomerase might have telomere lengthening-independent functions. In contrast, subsequent experiments have shown TERC deficient mice with a considerable telomere reserve are resistant to epidermal tumor formation, suggesting that non-canonical functions of telomerase requiring TERC (NC I) are also required for epidermal tumorigenesis *[Gonzalez-Suarez, E et al., 2000[79]]*.

In addition, a variant of TERT was created accidentally by tagging the carboxy-terminus of TERT with haemagglutinin (TERT-HA). TERT-HA retains telomerase activity *in vitro* but cannot lengthen telomeres or immortalize human fibroblasts *[Counter, CM et al., 1998[80]]*. Ectopic TERT expression can augment the tumorigenicity of both normal human fibroblasts *[Hahn, WC et al., 1999[81]]* and fibroblasts which possess a telomerase-independent telomere maintenance mechanism *[Stewart, SA et al., 2002[82]]*. Apart from its role in suppressing genomic instability, TERT overexpression has been associated with the autocrine production of fibroblast growth factor, and epidermal growth factor *[Smith, LL et al., 2003[83]]*.

Many telomere-independent activities of telomerase are possibly involved in the process of tumorigenesis. TERT expression can promote cell growth and proliferation independently of telomere elongation by inducing or inhibiting the expression of pro-proliferative and anti-proliferative genes, respectively *[Geserick, C et al., 2006[84]]*. TERT can increase resistance to chemotherapeutic agents and pro-apoptotic stimuli, possibly blocking the mitochondrial death pathway *[Dudognon, C et al., 2004[85]; Del Bufalo, D et al., 2005[86]; Mondello, C et al., 2006[87]]*. TERT is able to modulate chromatin structure and response to DNA damage *[Masutomi, K et al., 2005[88]]*. TERT also increase cancer cell fitness improving mitochondrial activity and resistance to apoptosis. TERT can stabilize telomeres in

a telomere-capping dependent manner increasing cell's life span without telomere lengthening *[Zhu, Y et al., 1999[89]; Kim, HR et al., 2003[90]; Mukherjee, S et al., 2011[91]]*.

Other experiments have suggested the existence of telomere length-independent activities for telomerase in cellular transformation, proliferation, stem-cell biology, cell survival and chromatin regulation. The ability of TERT to activate tissue stem cells requires neither the telomerase RNA component (TERC) nor the reverse transcriptase activity of TERT and is therefore independent of its catalytic role in adding telomere repeats *[Sarin, KY et al., 2005[92]; Choi, J et al., 2008[93]]*.

TERT has been described as a transcriptional modulator of the Wnt/β-catenin signaling pathway playing a role as a co-factor at the promoters of Wnt downstream genes *[Park, JI et al., 2009[94]]*. In fact, TERT interacts with BRG1, a chromatin re-modeller binding to β-catenin and involved in the Wnt signalling *[Barker, N et al., 2001[95]]*, and promotes the expression of several β-catenin target genes in a BRG1- dependent way. Moreover, TERT was found not only to bind to promoters responsive to Wnt signaling, but also to interact with the same promoter elements recognized by BRG1 and β-catenin *[Park, JI et al., 2009[94]]*.

Furthermore, the ectopic expression of TERT in the hair follicle keratinocyte stem cells activates cell division, migration, and self-renewal in the absence of detectable telomere elongation, a requirement for TERC *[Cayuela, ML, 2005[96]; Sarin, KY et al., 2005[92]]* or the catalytic activity of TERT *[Choi, J. et al., 2008[93]]*. Moreover, TERC is required for the development of epidermal tumours *[Cayuela, ML et al., 2005[96]]*, suggesting that some of the non-canonical functions of TERT can promote stem cell self-renewal and functional maintenance without inducing neoplasia. It has long been known that telomerase can protect neuronal cells from a variety of apoptotic stimuli *[Zhu, H et al., 2000[97]]*. As most of these experiments were conducted with rodent cells harbouring long telomeres, it is assumed that these results are unconnected to telomere length. A subsequent study has shown that human telomerase forms a complex wit the mitochondrial RNA processing endoribonuclease (RMRP) and acts as a RNA-dependent RNA polymerase *[Maida, Y et al., 2009[98]]*. Authors of this publication provided an additional function of telomerase in the nucleus, independent of telomere elongation.

1.3.3 Telomerase and human diseases: ageing and cancer.

In contrast to germ cells, most normal human somatic tissues and adult stem cells do not express sufficient telomerase to maintain telomere length indefinitely, and therefore undergo telomere attrition with age. This telomere shortening can eventually lead to critically short or unprotected telomeres and loss of organismal viability, indicating an important role for telomerase activity and telomere length in the pathobiology of human disease. Indeed, many human diseases of different origins that are associated with ageing, as well as late stages of cancer, are characterized by the presence of short telomeres. Furthermore, telomerase mutations have been detected in the context of several premature aging syndromes:

- Dyskeratosis congenita (DC) also known as Zinsser-Engman-Cole syndrome is a rare multi-system bone marrow failure syndrome characterised by mucocutaneous abnormalities and an increased predisposition to cancer *[Walne, AJ & Dokal, I 2008[99]]*

- Aplastic anemia, a hematological disorder characterized by reduced red blood cell counts, bone marrow failure and liver and lung disease *[Vulliamy, T et al., 2002[100]]*

- Hoyeraal-Hreidarsson syndrome (HH), a clinically severe variant of DC, also includes cerebellar hypoplasia, immunodeficiency, and intrauterine growth retardation *[Kocak, H et al., 2014[101]]*

- Idiopathic pulmorary fibrosis (IPF) is a chronic, and fatal disease that is characterized by a progressive decline in lung function defined as irreversible lung fibrosis *[Armanios, M 2012[102]]*.

The fact that diseases associated with human ageing and premature ageing syndromes are characterized by short telomeres and telomerase dysfunctions, to establish the role of telomerase in these diseases might be important for the design of appropriate therapeutic strategies, based on temporary telomerase

reactivation. Therapeutic agents that could be designed to do this, would preferentially target those cell types that normally divide to maintain organ homeostasis, such as stem cells, which, although telomerase proficient, do not have sufficient telomerase activity to maintain telomere length over time and extend the lifespan of the cells.

Apart from ageing and specific syndromes directly related to telomere dysfunction, mutations or changes in expression of telomerase genes are associated with tumorigenesis *[Prescott, J et al., 2012*[103]*]*. Telomerase has been found active in over 90% of human malignancies and is responsible for its unlimited proliferative capacity, one of the hallmarks of cancer cells. Therefore, telomerase inhibition could be an effective way to develope new cancer therapies. Telomerase can be inhibited through a variety of methods including: oligonucleotides, dominant negative TERT mutants or alternative splice forms of TERT, small molecule inhibitors and RNA interference (siRNA). Telomerase inhibition leads to loss of telomerase activity, telomeres shortening and apoptosis *[Shammas, MA et al., 2005*[104]*]*.

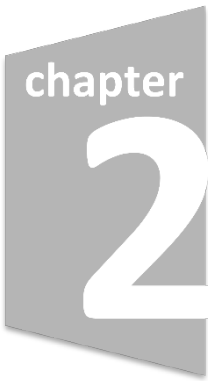
The fact that telomerase deficiency only results in loss of organismal viability when telomeres reach a critically short length is an important point considering the possible therapeutical effects. In particular, anticancer therapies that are based on temporary telomerase inhibition will only trigger loss of viability in those cells with short telomeres that depend on telomerase activity. Presumably, these include tumour cells, but not healthy tissues, which generally lack telomerase activity and have sufficiently long telomeres to maintain viability during the human lifetime, therefore providing a window of opportunity for intervention. The strong association of telomerase and cancer makes telomerase an attractive therapeutic target for the treatment of cancer.

1.4 Aims of the study

In addition to the classical, canonical role of telomerase in telomere maintenance a multitude of different non-telomeric functions have been described so far but not all cellular consequences or molecular mechanisms of these functions are well understood yet. The aim of this work was to gain more insights into the regulatory mechanisms of telomerase/TERT in gene expression, particularly on the rRNA gene transcription. Specific aims of this thesis are:

- To analyse whether telomerase might be involved in the transcription process on the rDNA locus in the nucleoli. While telomerase mainly localizes to Cajal bodies, distinct extratelomeric functions of telomerase have been described in cytoplasm, mitochondria and nucleolus. Notably the nucleolus is the site of rDNA transcription and ribosomal biogenesis. The nucleolar localization of telomerase raises the possibility of an association with the rDNA and leads to hypothesise that the enzyme may cooperate with the Pol I transcription machinery.

- To investigate the role of telomerase in Pol I transcription and its contribution to tumorigenesis. It is known that rDNA transcription is the major determinant of cell proliferation and cell growth. In addition, telomerase expression is associated with enhanced cellular survival detected in most tumour tissues. A question addressed in our study is the potential connection between functional mechanisms of telomerase and ribosome biogenesis under conditions of cellular proliferation and tumour progress.



2. MATERIALS & METHODS

2.1 Cell culture

2.1.1 Cell lines

The eukaryotic cell lines used are listed in table 2.1.

Table 2.1.

Cell line	Specifications
AL-1921	Mouse liver TERC -/- cells *(Kindly gift of Dr. Lechel, Institute of Molecular Medicine)*
AL-2103	Mouse hepatocarcinoma cells *(Kindly gift of Dr. Lechel, Institute of Molecular Medicine)*
HCT116	Human colorectal carcinoma cells
HEK293	Human embryonic kidney cells containing SV40 Large T-antigen
HeLa	Human cervical adenocarcinoma cell line
hHCC	Human hepatocellular carcinoma cells
IMR90	Primary human fetal lung fibroblasts

2.1.2 Cell culture conditions

Reagents and chemicals	Supplier
Dulbecco's modified Eagle's medium (DMEM)	Gibco, Germany
Fetal bovine serum (FBS)	Seromed, Germany
Penicillin/Streptomycin	Biochrom AG, Germany
Phosphate buffered saline modified - w/o Ca^{2+}, Mg^{2+} (PBS)	Gibco, Germany
Trypsin-EDTA solution	Sigma Aldrich, Germany
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, Germany

All cells were cultured under standard conditions maintaining the viability and stability of the cells (37°C, 5% CO_2, 90% humidity). Cell culture works were performed under sterile conditions using a sterile laminar flow box in order to prevent contamination from bacteria, fungi and cross contamination with other cell lines.

Before the cells were put into culture, frizzed cells were thawed and diluted in culture medium, to minimize the toxic effects of DMSO. All cell lines were then cultured in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin and maintained at 37°C in a fully humidified incubator containing 5% CO_2.

Every 3-4 days, according to the growth characteristics of each cell line the confluence degree was examined by microscopy and passages were made when cells upon reached 70 - 80% confluence. For this end the cell culture media was aspirated and the flask washed twice with 20ml PBS, cells were then detached using 1ml Trypsin-EDTA solution. By re-suspending the cells in a small volume of fresh supplemented culture medium the trypsinization reaction was neutralized. Once the majority of the cells (>90%) have been detached and floating a cell count was performed and the required number of cells was transferred into a new-labelled flask containing pre-warmed medium. Subculture of cell lines were performed twice a week at a ratio of 1:10 dependent on the confluence and maintained in DMEM supplemented with 10% FBS and the appropriated antibiotic in a humidified atmosphere at 37°C and 5% CO_2.

2.1.3 Transfection

Reagents and chemicals	Supplier
2,5M $CaCl_2$	In House
2XHBS	10mM KCl / 280mM $NaCl_2$ / 1,5mM Na_2HPO_4 / 50mM Hepes pH 7,05 /12mM Dextrose
Lipofectamine® transfection reagents	Invitrogen
Attractene transfection reagent	QIAGEN
HiPerFect transfection reagent	QIAGEN

To proteins expression and reporter vector experiments several DNA plasmid vectors were used. All constructs are listed in table 2.2.

Table 2.2: List of plasmids (Bact. R=Bacterial resistance / Select. M**=Selectable markers)*

Plasmid	***Bact. R* / Select. M****	***Source / Addgene (#plasmid)***
pBABE-blast-RasV12	*Ampicillin / Blasticidin*	*Generated by PCR*
pBABE-puro-hTERT	*Ampicillin / Puromycin*	Kindly gift of R. Weinberg (#1771)
pBABE-puro-DN-hTERT	*Ampicillin / Puromycin*	Provided by R. Weinberg (#1775)
pBABE-neo large T	*Ampicillin /Neomycin*	Provided by R. Weinberg (#1780)
pBABE-puro-hTERT-HA	*Ampicillin / Puromycin*	Provided by R. Weinberg (#1772)
pWZL-blast-Flag-HA-hTERT	*Ampicillin / Blasticidin*	Provided by R. Weinberg (#22396)
pHrP2BH	*Ampicillin*	Kindly gift of Prof. Grummt
scramble shRNA	*Ampicillin / Puromycin*	Provided by R. Weinberg
pWZL-hygro-H-Ras V12	*Ampicillin/ Hygromycin*	Kindly gift of Scott Lowe (#18749)
pMKO.1-puro-hTERT-shRNA	*Ampicillin / Puromycin*	Kindly gift of William Hahn (*#10688)*

Calcium phosphate has been widely used in the chemical transfection of animal cells. The basic protocol for this method is that DNA in a buffered phosphate solution and calcium chloride are mixed together to generate a DNA-calcium phosphate co-precipitate, which forms on the cells and enter the cells through endocytosis. Transient transfections of HA-tagged hTERT in HEK293 and mouse cells were performed using this standard procedure. One day prior transfections, $1x10^5$ cells were seeded in the appropriate medium supplemented with serum. At the time of transfections the cultures were 60%–80% confluent. The protocol involved mixing of 1,5mg plasmid DNA with 2,5M $CaCl_2$, adding this in a controlled manner to a buffered saline phosphate solution (2XHBS), and allowing the mixture to incubate for 20 minutes at room temperature. The generated precipitate was dispersed onto the cultured cells. The transfected cells were incubated in DMEM for 48 hours.

Lipofectamine reagent was used for stable expression of retroviral vectors in IMR90 cells. Transfections were carried out strictly according to the manufacturers protocol. Lipofectamine was simply mixed with the DNA vectors (pBABE-puro-hTERT, pBABE-neo-large-T, and pBABE-blast-RasV12). The DNA-containing liposomes with positive charge on their surfaces fused the negatively charged cell membrane fused the negatively charged cell membrane allowing the transfected

material cross the cytoplasm to be replicated or expressed by the cells. Three days after transfections the respective antibiotic (see table 2.2) were added and selection was performed over approximately 3 weeks. For knockdown of telomerase activity, HCT116 cells were stably transfected with scrambled, shTERT or DN-TERT constructs by using Attractene (QIAGEN) and selected after 48h by addition of Puromycin. The telomerase RNA component (TERC) was repressed in the same cell line by transfection with siRNAs using HiPerFect reagent (QIAGEN).

The rDNA reporter pHrP2-BH bearing the rDNA promoter and a piece of rDNA flanked by two termination sites was transient cotransfected with pBABE-blast-RasV12 and increasing amounts of the expression plasmid pBABE-puro-hTERT. The transfections were performed in IM90 ST/LT cells with a nucleofector device from Lonza according to the manufacturers protocol. During the electroporation, cells were exposed to a high-intensity electric field that temporally destabilized the cell membrane, becoming highly permeable to exogenous molecules present in the surrounding media. DNA then moved into the cells through the pores. When the electric pulse was turned off, the pores in the membrane were sealed, enclosing the DNA inside the cells.

2.2 Molecular biology methods

2.2.1 Antibody generation

A mouse/human conserved peptide sequence (amino acids 568-581 for human TERT and amino acids 558-571 for mouse TERT) was chosen for the production of the anti-TERT antibody Ab-8313, with an amino acid composition: FQKNRLFFYRKSVWC. The peptide was synthesized at the Heinrich-Pette-Institute on an ABI peptide synthesizer (ABI 433A) and purity was detected by HPLC. The last amino acid, the underlined cysteine, is not part of the TERT sequence but was added for efficient conjugation to KLH (Keyhole Limpet Hemacyanin) and the first conjugate (500µg conjugated peptide) was injected to two rabbits with Complete Freund's Adjuvant (F5881, Sigma) followed by two boost injections with Incomplete Freund's Adjuvant (F5506, Sigma) in the animal facility of University Hospital of Hamburg (UKE, Hamburg, Germany). The third

and final bleedings of both animals were pooled (~100 ml) and were affinity purified according to standard procedures.

2.2.2 Peptide competition assay

The peptide competition assay (PCA) is a recommended procedure for validating antibody specificity. In our PCA, an excess of 500-fold more peptide was added on a molar basis relative to antibody. The antibody was pre-incubated with the peptide (Amino acid composition: PELYFVKDLTEVI) for 1h at room temperature prior to use in ChIP or immunoprecipitation assays. The experiments were run in duplicate; once with antibody pre-incubated with peptide and once with a control antibody not pre-incubated with peptide.

2.2.3 Chromatin Immunoprecipitation (CHIP)

Chromatin immunoprecipitation (ChIP) is a type of immunoprecipitation experimental technique used to identify DNA sequences associated with specific proteins. The association of DNA fragments with a binding protein, such as a transcription factor, can be assessed by PCR after precipitation with an antibody that recognizes the protein of interest.

2.2.3.1 Isolation and fragmentation of the chromatin

Buffers and solutions	Descriptions
Formaldehyde (37%)	Sigma Aldrich, Germany
1M Glycine	In house
Cell lysis buffer	5mM HEPES / 85mM KCl / 0.5% NP-40
NP40 solution	Sigma Aldrich, Germany
Micrococcal Nuclease (MNase)	NEB # M0247S
0,5M EDTA	In house
Nuclei lysis buffer	50mM Tris-Cl pH 8,1 / 10mM EDTA pH 8,1 / 1% SDS

To fix the proteins of interest to its chromatin-binding site, in vivo crosslinking were traditionally achieved by addition of 37% formaldehyde per ml of growth medium, to a final concentration of 1% on 15cm culture plates. After 10

minutes slowly shaking at room temperature, cross-linking reaction is stopped by addition of 1M Glycine to final concentration of 0,125M per ml of culture medium. Cells were subsequently washed with cold 1X PBS, scraped and centrifuged for 5 min at 4°C at 2,500 *rpm*. The cell pellet was suspended in 300µL Cell lysis buffer for ChIP and incubated for 10 min on ice. Cell membranes are dissolved at this stage, by adding a detergent based solution (NP40) and the protein-DNA complexes as well as cellular components are liberated. Cell nuclei were pellet at 1000 rpm for 10 min at 4°C and supernatants were carefully aspirated. The extracted genomic DNA must be sheared into smaller workable pieces. For this end, chromatin fragmentation was achieved by enzymatic digestion with MNase during 25 minutes at 37°C. Adding EDTA to a final concentration of 50mM the digestion is stopped. The digested nuclei are pelleted by centrifugation at 13,000 rpm for 10 minutes at 4°C. Nuclear pellet was suspended in Nuclei lysis buffer, supplemented with protease inhibitors. After incubation for 10 min on ice proceed with sonication using a Bandelin Sonopuls sonicator (5 times 45 sec pulses at 20% energy followed by 60 sec rest on ice). The material was cleared by centrifugation at 14,000 rpm for 20 min at 4°C, to pellet the nuclear membrane. Supernatant containing released chromatin was then analysed on an agarose gel (DNA fragments should ideally be an average length between 300 – 500 base pairs in size). Chromatin was transferred to a new tube and frozen at -80°C for storage.

2.2.3.2 Chromatin preclearing and immunoprecipitation

The sonicated chromatin was pre-cleared in IP dilution buffer with Protein A/ ssDNA and protease inhibitor for 30 minutes on a rotating plate at 4°C. After pre-clearing, agarose beads were pelleted using brief centrifugation and the supernatants were transferred to new tubes. The appropriated antibodies were added in varying amounts and incubated overnight at 4°C on a rotating plate. The next day, Protein A or G agarose beads 50% slurry (depending on antibody) was added and incubated for 1h at 4°C with rotation. The agarose beads were pelleted at 10,000 rpm for 1 min at 4°C and washed once with Low salt wash buffer, high salt wash buffer, LiCl wash buffer and twice with TE buffer. For elution beads and

input samples were suspended in µChIP elution buffer supplemented with proteinase K and incubated for 2h at 65°C to reverse cross-links.

Antibody	Supplier
Rabbit anti RPA135	Kind gift from Prof. Grummt (DKFZ, Germany)
Mouse monoclonal anti HA-Tag	Cell signalling (#2367S)
Normal mouse IgG	Santa Cruz (sc-2025)
Normal rabbit IgG	Santa Cruz (sc-2027)
Rabbit polyclonal anti TERT (Ab-8313)	House generated
Rabbit polyclonal anti TERT	Rockland (600-401-252)

Reagents and solutions	Descriptions
Protein A-agarose beads	Sigma Aldrich, Germany
Complete Protease Inhibitor Cocktail	Roche
IP dilution buffer	0,01% SDS / 1,1% Triton X 100 / 1,2mM EDTA pH 8,1 / 16,7mM Tris-Cl pH 8,1 / 167mM $NaCl_2$
Low salt wash buffer	0,1% SDS / 1% Triton X 100 / 150mM $NaCl_2$ / 500mM EDTA pH 8,1 / 20mM Tris-Cl pH 8,1
High salt wash buffer	0,1% SDS / 1% Triton X 100 / 500mM $NaCl_2$ / 20mM Tris-Cl pH 8,1 / 500mM EDTA pH 8,1
LiCl wash buffer	250mM LiCl / 10mM Tris-Cl pH 8,1 / 1% NP-40 / 1% Sodium deoxycholate / 1mM EDTA pH 8,1
TE wash buffer	1mM BSA / 10mM Tris-Cl pH 8,1
µChIP elution buffer	1% SDS in TE
Proteinase K	Promega

2.2.3.3 DNA extraction and purification

The ChIP-enriched DNA and input chromatin DNA were purified using either the DNA purification kit (QIAGEN, according to the manufacturer's protocol) or standard Phenol/Chloroform extraction *[Sambrook, J and Russell, DW 2006* [105]*]*. The samples were stored at -20°C for further studies.

2.2.4 Polymerase chain reaction (PCR)

The immunoprecipitated DNA fractions were amplified by PCR in a thermal cycler using a standard program comprised a first cycle of 5 minutes at 95°C followed by 30 amplifications cycles at 95°C for 30 seconds, 30 seconds with the appropriate annealing temperature, 40 seconds at 72 °C; and a final extension step of 7 min at 72ºC. Reactions were carried out in a reaction volume of 25µl containing the DNA template, GoTaq® Green Master Mix (Promega) in the presence of 10µM forward and reverse specific primers.

Human primer pairs	Sequence
Hr-ChIP-F (rDNA promoter)	5′–CTGCGATGGTGGCGTTTTTG–3′
Hr-ChIP-R (rDNA promoter)	5′–ACAGCGTGTCAGCAATAACC–3′
H4-F (18S rRNA)	5′–GACGACCCATTCGAACGTCT–3′
H4-R(18S rRNA)	5′–CTCTCCGGAATCGAACCCTG–3′
H8-F(28S rRNA)	5′–AGTCGGGTTGCTTGGGAATGC–3′
H8-R (28S rRNA)	5′–CCCTTACGGTACTTGTTGACT–3′
H13-F (28S rRNA)	5′–ACCTGGCGCTAAACCATTCGT–3′
H13-R (28S rRNA)	5′–GGACAAACCCTTGTGTCGAGG–3′
H23/27-F (IGS)	5′–CCTTCCACGAGAGTGAGAAGC–3′
H23/27-R (IGS)	5′–TCGACCTCCCGAAATCGTACA–3′

Mouse primer pairs	Sequence
M0-F (rDNA promoter)	5′–GTTGTCAGGGTCGACCAGTTGT–3′
M0-R (rDNA promoter)	5′–GTGTCCTTTAGTGTTAATAGGG–3′
M5-F	5′–GACGACCCATTCGAACGTCTG–3′
M5-R	5′–CCTCTCCGGAATCGAACCCTG–3′
M13-F	5′–ACCTGGCGCTAAACCATTCGT–3′
M13-R	5′–GACAAACCCTTGTGTCGAGGG–3′
M16-F (IGS)	5′–AGCATGTAGCAGTTGTAGGAC–3′
M16-R (IGS)	5′–TGGAGAGATGGCTCATCGGTT–3′

2.2.5 Gel Electrophoresis

Amplified products were resolved on 1% agarose gels and visualized by Ethidium bromide staining. To confirm the expected molecular weight of the PCR products, a DNA ladder (a molecular weight marker) was run on gel alongside the PCR products.

2.2.6 Quantitative real-time PCR (qPCR)

To determine the amount of a target DNA sequence relative to its abundance in the input chromatin, qPCR were performed using 5% of each ChIP sample (2μL), 12.5μL of SYBR green PCR master mix (Roche), and 10μM primers. The mixtures were placed in a 96-well plate (Applied Biosystems), and the reaction was carried out in a Real-Time PCR system (Applied Biosystems) using. A cycle threshold (C_T) value was recorded for each sample using software (Applied Biosystems).

Human primer pairs	Sequence
c-Myc-TBE2-F	5′–CGTTTTCCTCCTTATGCCTCTATC–3′
c-Myc-TBE2-R	5′–GTACCAGGCTGCAGGGCGCCTCGCT–3′
c-Myc-3′UTR-F	5′–CTAATGTATCACAAAGTCCTTTA–3′
c-Myc-3′UTR-R	5′–GTGATCTGCTCTGTTAGCTTTTGA–3′
Cyclin-D1-TBE-F	5′–CGCTCCCATTCTCTGCCGGG–3′
Cyclin-D1-TBE-R	5′–CCGCGCTCCCTCGCGCTCTT–3′
Cyclin-D1-3′UTR-F	5′–CAAGAGAAGATTACCGCCCGAG–3′
Cyclin-D1-3′UTR-R	5′–TCCCCAGCCTTTTTGACACC–3′
rPL13a-F	5′–CGGACCGTGCGAGGTAT–3′
rPL13a-R	5′–CACCATCCGCTTTTTCTTGTC–3′
h47S-F	5′–TGTCAGGCGTTCTCGTCTC–3′
h47S-R	5′–AGCACGACGTCACCACATC–3′
18S-F	5′–CGATGCGGCGGCGTTATTCC–3′
18S-R	5′–GAACGGCCATGCACCACCAC–3′
28S/ETS-F	5′–GGTTTCGTACGTAGCAGAGCAGC–3′
28S/ETS-R	5′–AGGGAGGAAGACGAACGGAAGGAC–3′
TERT-F	5′–AGTTTCCGTCAGAGAAGGAACCGC–3′
TERT-R	5′–TTTGATGATGCTGGCGATGACCTC–3′
TERC-F	5′–CCCCAGTTTCAAGGGCGAAT–3′
TERC-R	5′–CTCATGTTACCGCCCGCTAT–3′

Mouse primer pairs	Sequence
m45S-F	5′–CGTGTAAGACATTCCTATCTCG–3′
m45S-R	5′–GCCCGCTGGCAGAACGACAAG–3′

ChIP qPCR data were normalized Standard curve generated from the cycle threshold (Ct) value of the serially diluted chromatin input was used to calculate

the percentage input value of each sample. Average values are from triplicate measurements done per sample.

For analyse the expression levels of genes qRT-PCR, the expression of each gene in each sample relative to the control was calculated using the $2^{-\Delta\Delta CT}$ method to relate the C_T values of gene expression to the C_T values for the housekeeping gene. A mixture of *GAPDH* F-primer and *GAPDH* R primer was used as the primer mix (Table S-1). The PCR conditions are as follows: 98°C for 5 min; 40 cycles at 98°C for 10 s, 64°C for 30s, and 72°C for 30s.

2.2.7 ChIP – re – ChIP assay

ChIP–re–ChIP experiments were performed on the basis of double and independent rounds of immunoprecipitation to examine the co-localization of two proteins at a specific genomic region of interest.

The sheared chromatin was subjected to a first ChIP round with specific antibodies. Subsequently, DNA/protein complexes were washed (as describe above in ChIP assays), and eluted from agarose beads using 10mM DTT in TE buffer to prevent interferences of the first antibody in the second ChIP reaction. The eluted material was then incubated with secondary antibodies that recognize the protein of interest, which is suspected to be located at the same DNA region, either by forming direct or indirect complexes, with the first immunoprecitated antigen. The washing of samples were conducted in a similar manner as for the first ChIP round and after elution the cross-linked DNA/protein complexes were reversed and the released DNA was purified and analysed by PCR using specific primers for the genomic region of interest.

2.2.8 Quantification of Telomere-DNA

The telomere quantification was performed by quantitative real-time PCR using Bio-Rad SYBR green master mix, 25mM DTT, DNA samples and primers in the adequate concentration. Amplification plot of telomeres or HBG was checked for the linear range according to the telomere or HBG standard. Signal in ChIP samples were subtracted by telomere signal of IgG control and corrected for DNA

amount by HBG and then finally relative to the scrambled telomere amount was calculated and plotted.

Telomere primers	Sequence
Tel1b	5´–CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT–3´
Tel2b	5´–GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT–3´

HBG primers	Sequence
HBG 3	5´–TGTGCTGGCCCATCACTTTG–3´
HBG 4	5´–ACCAGCCACCACTTTCTGATAGG–3´

Telomere standard:
TTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTT AGGGTTAGGGTTAGGGTTAGGG

HBG standard:
CCACACCAGCCACCACTTTCTGATAGGCAGCCTGCACTGGTGGGGTGAATTCTTTGCCAAAGT GATGGGCCAGCACACAGA

Telomere-DNA was also quantified by DOT-Blot. 50ng ChIP-ed DNA was denatured incubating for 10 min at 99°C with 0,4N NaOH, 10mM EDTA. The denatured DNA samples were then applied to hybond N+ (Amersham; RPN119B) membrane using DOT-BLOT apparatus connected to a vacuum source and washed with 2X SSC once. DNA was denatured on membrane using (1,5M NaCl / 0,5N NaOH) for 10 min and then neutralized using (1,5N NaCl / 0,5M Tris pH 7). DNA was UV cross-linked using a UV stratagene crosslinker. The membrane was prehybridized by Rapid hybridization buffer (Amersham; RPN1635) for 1 h and incubated overnight with radiolabeled Alu or telomere probes.

Alu probe: 5'-CGGGAAGCAGAGGTTGTAGTGAGCC-3´
Telomere probe: TTAGGG

Reaction mixture: (1µl telomere restriction fragment, 13,5µl H2O, 2µl T4 PNK buffer, 2,5µl 32p-ɣATP, 1µl T4-polynucleotide kinase, New England Biolabs) at 37°C. Membrane was washed next day once with 0,1% SDS/2X SSC buffer and twice 0,1% SDS/1X SSC. Radiolabeled membrane was exposed to phosphorimager (Fuji) and signal intensities were quantified after 3 days exposure.

2.2.9 Partial hepatectomy (PH) and proliferation assay

The liver is one of the few organs that demonstrate a physiological growth response. Within minutes following a two-thirds partial hepatectomy (PH), the majority of cells in the remnant liver, which are normally quiescent, rapidly re-enter the cell cycle accompanied by the induced expression of a large number of growth-response genes. To begin to understand the pathways that regulate telomerase in normal tissues, we studied the telomerase activity of hepatocytes during mouse liver regeneration.

Mice were subjected to 70% hepatectomy by surgically removing the left lateral, left median and right median lobes without disrupting the portal vein, biliary tract and gallbladder. The mice were killed at different time points after PH. For each time point (12h, 48 h, 72 h, and 120 h) we used three different mice. The liver lobes were snap frozen in liquid nitrogen and stored at -80°C until required for further analysis. The tissues were used for chromatin and RNA extraction and ChIP assays or RT-PCR were preformed. The binding dynamic of TERT to the rDNA was determined by qPCR.

2.2.9.1 Animals

For partial hepatectomy and proliferation assays we used 8 weeks old male wild type mice on a C57BL/6J background that were maintained in a pathogen-free environment and fed with a standard diet. Animal experiments habe been approved by the state government of Baden-Württemberg (Protocol Number 35/9185.81-3/919).

2.2.10 RNA preparation and cDNA synthesis.

Total RNA was prepared from mammalian cell pellets using the RNeasy RNA extraction kit (Qiagen) in accordance with the manufacturer's protocol. RNA was eluted with 50µl of RNase-free water. Following elution, the concentration of RNA was determined by spectroscopy and RNA samples were immediately frozen in liquid nitrogen and stored at -80°C.

First-strand cDNA was synthesized using the cDNA synthesis kit (Invitrogen) with 1µg of total RNA according to the manufacturer's protocol. The resulting cDNA was stored at -20ºC.

2.3 Protein biochemical methods

2.3.1 Preparation of nuclear extracts

Reagents and solutions	Descriptions
Dignam A Buffer	10mM Tris pH 7,9 / 1,5mM $MgCl_2$ / 10mM KCl
Dignam B Buffer	20mM Tris (pH 7.9), 25% Glycerol, 420mM KCl, 1.5mM $MgCl_2$, 0.5mM EDTA

To prepare cell lysates, cells were trypsinized, washed with PBS followed by a centrifugation step at 1200 rpm for 5 minutes. Cells were resuspended in 1ml PBS and transferred into 1.5ml microcentrifuge tubes. After centrifugation at 2000 rpm for 10 minutes at 4°C the supernatant was discarded and the undisturbed cell pellet was immediately used for protein isolation. Cell pellets were lysed in Dignam A buffer, supplemented with complete protease inhibitor. After 10 minutes incubation on ice samples were clarified by centrifugation at 13000 rpm for 15 min at 4°C, pelleted containing the nuclear fraction were lysed for 20 minutes in Dignam B buffer. Nuclear lysates were then centrifuged at full speed and pellets were discarded. The supernatants proteins were collected and protein concentrations were determined with the Bradford method in triplicates according to manufacturer protocol. A standard curve was constructed using BSA (Sigma) between the concentrations 0 to 1mg/ml. Absorbance readings were taken at 595 nm, and all samples were measured in duplicate. The nuclear extracts were either stored at -80°C or used immediately for analysis.

2.3.2 Immunoprecipitation assays

Immunoprecipitation (IP) is a method used to enrich or purify a specific protein or group of proteins from a complex mixture using an antibody immobilized on a solid support. In our study we used immunoprecipitation (IP) and co-immunoprecipitation (Co-IP) experiments to investigate proteins interactions and to analyse the specificity of a newly generated antibody.

The common sequential method using Dynal® magnetic beads was to incubate antibody and magnetic beads for 1 hour at 25°C by rocking, in order to bind the antibody to the beads. After addition of Insulin, BSA and Phosphatidylcholine (100µg/µl each one component), in order to block the beads and avoiding unspecific protein binding the beads were then washed and incubate with the sample (e.g., cell lysate or nuclear extracts) for 2h at 4 °C. The immunoprecipitated complexes were then analysed by different techniques, such as Western blotting or TRAP assays. The basic co-immunoprecipitation (Co-IP) protocol was the same as that described for normal immunoprecipitation, and was designed to identify interactions between telomerase and Pol I.

2.3.3 Telomerase repeat amplification protocol (TRAP)

Reagents and solutions	Descriptions
1X CHAPS Lysis Buffer	0,5% CHAPS / 10mM Tris-HCl, pH 7.5 / 1mM $MgCl_2$ / 1mM EGTA / 0,1mM Benzamidine / 5mM β-mercaptoethanol / 10% Glycerol
10X TRAP Reaction Buffer	200mM Tris-HCl pH 8,3 / 15mM $MgCl_2$ / 630mM KCl / 0,5% Tween 20 / 10mM EGTA
50X dNTP Mix	2,5mM each dATP, dTTP, dGTP and dCTP

Telomerase activity was assessed by the TRAPEZE® gel-based telomerase detection Kit (CHEMICON International) which measures the potential enzymatic activity of telomerase in sample of tissue or in cell lysate. In principle, the TRAP assay quantifies the extension of a telomere - like primer (TS – primer) by telomerase from the particular lysate. The assay is a one buffer, two enzymes system utilizing the polymerase chain reaction (PCR). Cell lysates were homogenised in CHAPS buffer for 30 minutes on ice. A total of 100 - 250ng protein per sample was used in the reaction. In the first step of the reaction, telomerase from samples adds a number of telomeric repeats (GGTTAG) onto the 3' end of a substrate oligonucleotide (TS) at 30°C for 30min. In the second step, after telomerase inactivation at 94°C for 5 minutes the extended products were amplified by PCR using the TS and RP (reverse) primers, generating a ladder of products with 6 base increments starting at 50 nucleotides. The product was amplified in the following steps: denaturation at 94°C for 30s, annealing at 50°C

for 30s and polymerization at 72°C for 30s (30 cycles). Reaction products were mixed with DNA loading dye and resolved by a 12,5% w/v (19:1) acrylamide: bisacrylamide non-denaturing gel in 0,5X Tris-Borate-EDTA pH 8,0 (TBE) buffer. The gel was stained with 1:10 000 dilution of SYBR green in 0,5X TBE pH 8,0 for 20 minutes with gentle agitation and protected from light and fluorescent signal were captured.

2.3.4 Western Blot analysis

Reagents and solutions	Descriptions
5X Laemmli Loading Buffer	250mM Tris pH 6,8 / 500mM DTT / 5% SDS / 0,05% Bromphenol Blue / 50 % glycerol.
30% acryl-bisacrylamide mix	Bio-Rad
1.5M Tris pH 8,8	In house
1,5M Tris pH 6,8	In house
10% SDS	In house
10% ammonium persulfate (APS)	In house
Tetramethylethylenediamine (TEMED)	Sigma Aldrich, Germany
10X SDS Running Buffer	In house
Molecular Weight Standards	Bio-Rad, Germany
Nitrocellulose membrane	Schleicher and Schuell, Germany
Whatman Paper	Bio-Rad, Germany
Electro transfer Buffer (Anode 1)	300mM Tris (pH 10,4)
Electro transfer Buffer (Anode 2)	25mM Tris (pH 10,4)
Electro transfer Buffer (Cathode 3)	25mM Tris pH 9,4 / 40mM ε-aminocaproic acid / 0,1% SDS
5% Blocking buffer	5g dry milk powder, 50µl Tween-20 in 100ml 1X PBS
20X Lumiglo Reagent A	Cell Signalling Technologies, Germany
20X Lumiglo Reagent B	Cell Signalling Technologies, Germany

A total of 40µg protein per sample was loaded. After preparing samples by mixing them with 15 µl Laemmli buffer the mixtures were denaturised on a heating block at 95°C for 5 min. and shocked on ice afterwards. Proteins from samples

were separated on a 10% SDS – polyacrylamide gel at 35mA. After separation proteins were transferred onto a nitrocellulose membrane. The membranes were blocked for 1h with 5% non fat dry milk in PBS, washed with PBS and incubated with the primary antibodies overnight at 4°C. After subsequently washing in PBS. Membranes were incubated for 1h at RT with the horseradish peroxidase (HPR)-coupled secondary antibodies dissolved in 5% PBS-milk, followed by three washing steps in PBS. Antibody binding to membranes was visualized using chemiluminescent reagents solution incubated with the membranes for a maximum of 2 min in the dark.

2.3.5 Purification of telomerase recombinant proteins

HA-tagged telomerase fusion proteins were expressed after transient transfections of HEK293 or mouse cells with pBABE-Puro-hTERT-HA or pWZL-blast-Flag-HA-hTERT plasmid. Cells were harvested in buffer AM600 and sonicated 3X 15s. NP40 was added to 0,5%, and lysates were clarified by centrifugation. Monoclonal anti-HA agarose beads (Sigma) were used to isolate HA-tagged hTERT by incubation overnight on an orbital shaker at 4°C. The resin was sequentially washed with buffers AM300 0.1% NP-40AM and HA-hTERT was eluted in buffer AM100 0.1% NP40 with 0.5 µg/µl HA peptide (Sigma). The purified telomerase recombinant proteins were analysed by Western blotting and assessed in reconstitution RNA polymerase I in vitro transcription experiments.

2.3.6 RNA polymerase I *in vitro* transcription experiment.

Reagents and solutions	Descriptions
Proteinase K mix	2% SDS / 20mM EDTA / 0,5mg/ml tRNS / 0,4mg/ml proteinase K
Sample loading buffer	80% formamid / Bromphenol blue and Xylencyanol FF in water
Reaction conditions	16mM Tris-HCl pH 8,0 / 5mM $MgCl_2$ / 0.2mM EDTA / 80mM KCl / 10% (v/v) glycerol / 0.66mM each ATP, CTP, and GTP / 12.5µM UTP / 1µCi of [α-^{32}P] UTP (800 Ci/mmol)

In vitro RNA Pol I transcription reactions are generally used for replicate the process of RNA synthesis outside of the cellular environment. The *in vitro*

transcription reactions were prepared essentially under standard transcription conditions in a total reaction volume of 25µl. After a 1h incubation at 30°C, the reaction mixtures were terminated by digestion with 25µl of Proteinase K mix for 30 minutes at 40°C. The synthesized RNA was precipitated on ice for 30 minutes by adding 200 µl of ice-cold 100% ethanol and 25µl of 7,5M Ammonium acetate. Precipitates were then centrifuged for 20 minutes (13000 rpm) at room temperature, washed with 100µl 80% ethanol and centrifuged for 2 minutes at room temperature. Dry pellets were re-suspended in 10µl of sample loading buffer and resolved on 4% polyacrylamide gels. The gels were dried, exposed overnight in Phosphorimager screen and analysed by autoradiography detecting the incorporation of radioactively labelled nuclide in the transcripts.

DNA template	Transcription type	Characteristics
$pHrP_2$	RNA polymerase I transcription	Linearized with EcoRI and containing human rDNA promoter sequence from – 411 to + 389.
pUC18	Non-specific transcription	Constructed by excising the 267bp EcoRI/NdeI fragment

For non-specific in vitro transcription assays, the transcription reactions additionally contained 100µg/ml α-amanitin to block RNA polymerases II and III activities. Transcription was performed as described above using a promoterless template (pUC18 - EcoRI/NdeI).

Two purified recombinant constructs (HA-TERT and TERT-HA) were assessed in reconstitution of RNA polymerase I *in vitro* transcription experiments. Reactions mixtures contained nuclear extracts from in HCT116 telomerase knockdown cells, the purified recombinant proteins and rDNA template were pre-incubated prior the transcription reactions were performed.

2.3.6.1 Metabolic Labelling

One of the most widely used approaches for measuring general protein synthesis rate is metabolic labelling, typically in the form of radioactive amino acid incorporation into nascent polypeptides. For this experiment, $3x10^5$ cells/vial were starved in methionine/cysteine free medium with dialysed 10% FBS for 20 minutes

at 37°C. After 3h incubation at 37°C with 10% normal medium containing 2-5µCi/vial 35S-labeled methionine/cysteine, the proteins were precipitated by addition of cold 10% TCA, incubated 90 minutes on ice, spotted on glass filters, washed with 10% TCA and 70% ethanol, air dried and counted in a scintillation counter.

2.3.6.2 Nuclear run-on transcription

Newly transcribed rRNA could be identified using the nuclear run-on transcription assays. HCT116 telomerase knockdown cells were counted and lysed with NP-40 lysis buffer by incubation on ice for 5 minutes. The first step of the nuclear run-on assay was the isolation of nuclei by centrifugation. The nuclei were suspended in nuclear freezing buffer and incubated with nucleotides in the presence of α-amanitin for 30 minutes at 30°C allowing the transcription reaction. After DNase digestion the RNA was isolated, reverse transcribed and analysed by qPCR.

2.3.7 Template immunoprecipitation (TIP) assay

To investigate the kinetics of protein–rDNA interactions throughout the transcription process, we developed an assay combining in vitro transcription with formaldehyde crosslinking and immunoprecipitation followed by semi-quantitative PCR analysis. This new developed assay allows to study a time course of the association of a particular protein with the rDNA template after transcription initiation e.g. promoter clearance of RNA polymerase I in vitro.

In this assay transcription was started after synchronization of the reaction through formation of initiated complexes by pre-incubating during 40 minutes at 30°C an amount of 40ng rDNA template (pHrP2 – EcoRI linearized) with 40mg of the indicated nuclear extracts in the presence of the first nucleotides (0,825mmol guanine). Addition of sarcosyl (final concentration: 0.025%) and salmon sperm DNA (final concentration: 400ng) was used to restrict transcription to a single-round. Transcription was started by addition of the lacking ribonucleotides and stopped after the indicated time by crosslinking proteins to the template with

formaldehyde (final concentration: 1%) for 10 minutes at 30°C. Crosslinking was stopped with glycine (final concentration 0.125 M) for 5 min at 30°C. Each sample was diluted with IP buffer and incubated overnight at 4°C with antibodies against the proteins of interest. Isolation of precipitated complexes, washing steps, digestion of proteins and DNA extraction were done as described for ChIP analysis. Template-proteins associations were detected in semi-quantitative PCR with primers directed against the human rDNA promoter.

2.3.8 Statistics

Every experiment has been repeated at least three times and the statistical analyses were performed using Graph Pad Prism (GraphPad Software, Inc., La Jolla, CA 92037 USA). Significant differences were calculated using t-test (unpaired or paired two tailed).



3. RESULTS

3.1 Telomerase binds to ribosomal DNA

Though it has been well established since many years that the canonical function of telomerase is the maintenance of telomere length, recent studies have demonstrated telomere-independent activities of telomerase including transcriptional modulation of the Wnt – pathway by acting as a transcriptional co-regulator *[Park, JI et al., 2009[94]]* or directly regulating the NF-kB – dependent transcription *[Ghosh, A et al., 2012[106]]*. Furthermore, although the telomerase catalytic subunit (TERT) mainly localizes to Cajal bodies *[Zhong, F et al., 2011[6]]*, TERT also harbours nucleolar localization signals and can be traced in the nucleolus *[Chiodi, I & Mondello, C 2012[107]; Lee, JH et al., 2014[108]]*, the site where ribosomal biogenesis takes place. These findings prompted us to investigate a possible role of telomerase in regulating RNA polymerase I transcription.

3.1.1 Telomerase binds to promoter and gene internal regions of rDNA.

The ribosomal DNA (rDNA) consists of a tandem repeat of a unit segment that codes for ribosomal RNA. Each repeating unit of rDNA is comprised of a non-transcribed intergenic spacer (IGS), the promoter region, and an operon or gene internal region that codes for a transcript, which includes the 18S, 5.8S, and 28S rRNA sequences. The possible association between telomerase and specific DNA sequences of ribosomal gene was investigated by chromatin immunoprecipitation (ChIP) assays in human embryonic kidney (HEK) cells overexpressing

haemagglutinin-tagged (HA-tagged) TERT. According to the procedure the crosslinked proteins were selectively immunoprecipitated by antibodies against HA-tag and as a positive control one of the Pol I subunits (RPA135). Bound DNA to the proteins of interest was analysed by quantitative polymerase chain reaction (qPCR) using primers sets flanking different regions of the rDNA (Figure 8).

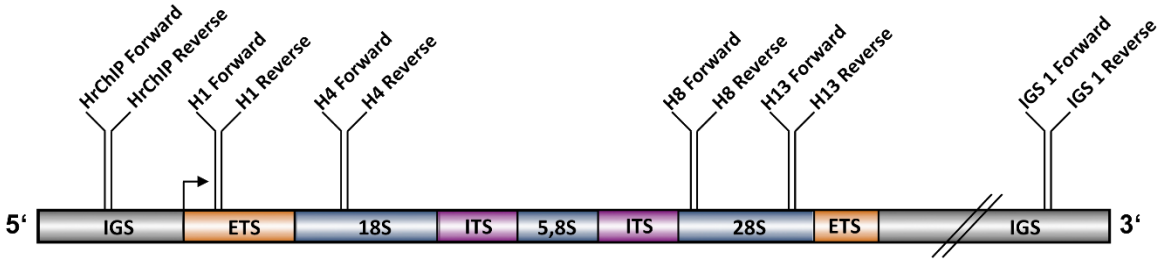


Figure 8: *Maps of primer location in human rDNA*

The ChIP analysis demonstrated TERT occupancy at the rDNA promoter and gene internal regions, excluding the intergenic spacers (IGS). In contrast with the previous finding, anti-HA antibody did not yield rDNA sequences when a control experiment was carried out using chromatin from non-transfected HEK293 cells (Figures 9).

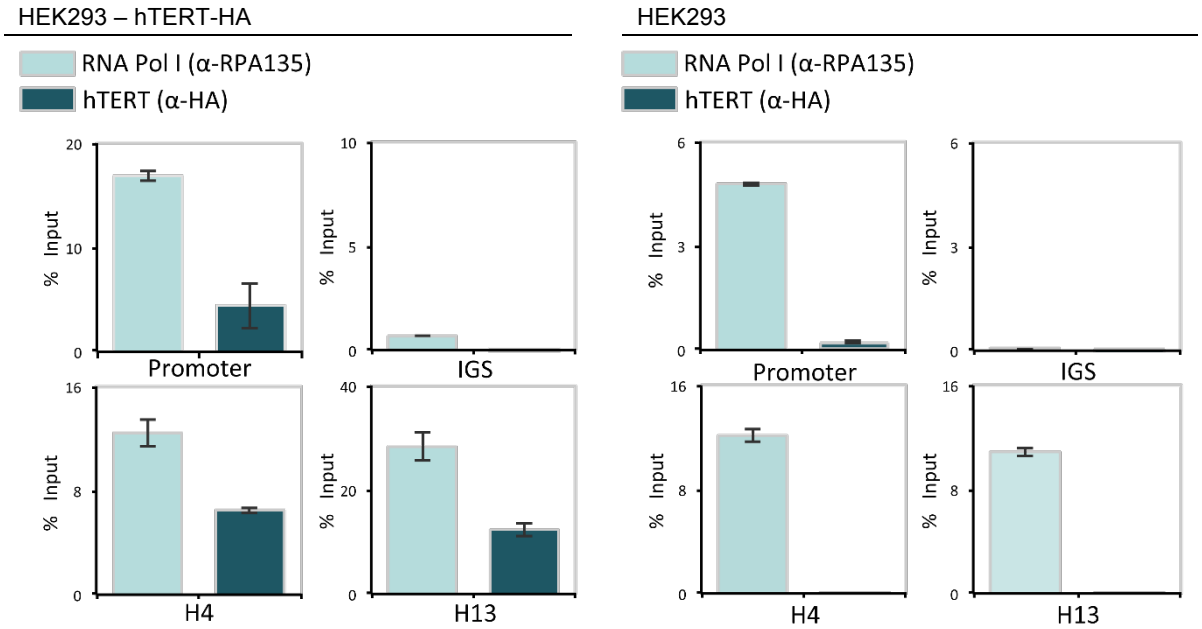


Fig. 9: *Telomerase binds to promoter and gene-internal regions of rDNA. HEK293 cells were transfected with HA-tagged hTERT and subject to ChIP experiments with the indicated antibodies and qPCR with primers flanking different rDNA regions. As a control, the same experiment was performed with chromatin from un-transfected HEK cells.*

To elucidate the question whether TERT association with the rDNA locus is a consequence of the over-expressed telomerase, we used two different specific anti-TERT antibodies in ChIP experiments with chromatin from un-transfected HEK293 cells. Either, the widely accepted commercially available anti-TERT antibody from Rockland or the newly generated 8313 antibody precipitated endogenous TERT associated with rDNA sequences (Figure 10).

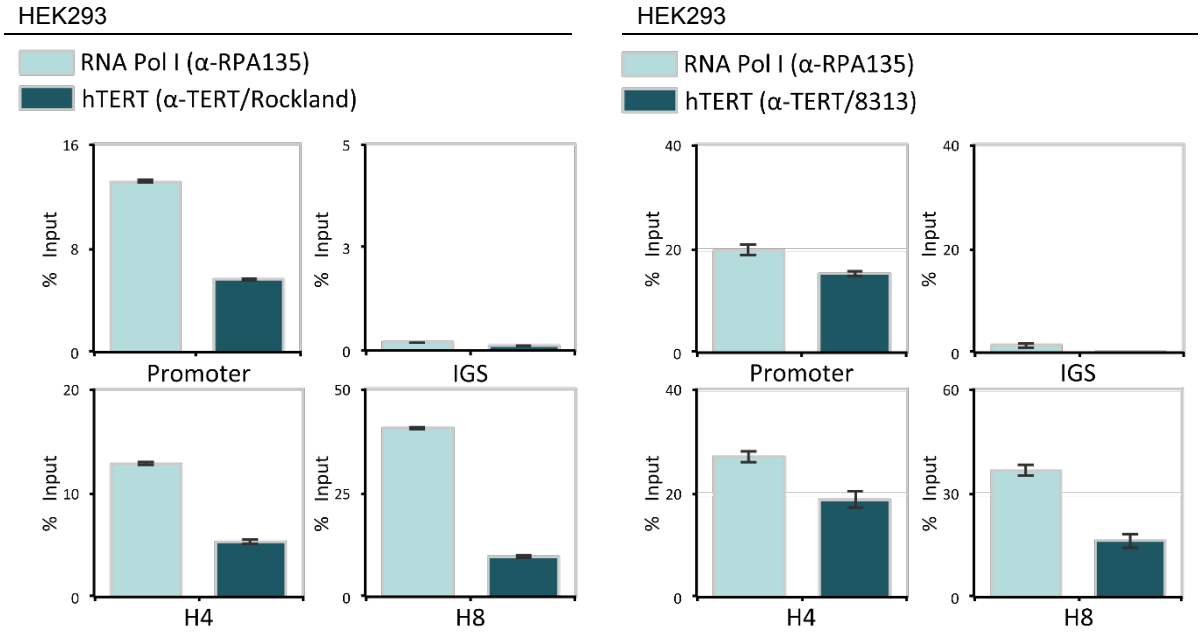


Fig.10: *Chromatin from HEK293 un-transfected cells were subject to ChIP experiments with two different anti-hTERT antibodies, confirming the association of endogenous hTERT with rDNA promoter and gene internal regions.*

3.1.2 TERT binds to the TBE sequences of the c-Myc promoter.

As reported by Park in 2009, TERT directly modulates the Wnt/β-catenin signalling by association with the TCF binding elements (TBE) located near the transcription start site of the Myc and Cyclin D1 genes; which play important roles in embryonic development and the process of tumorigenesis.

Consistent with these findings, our ChIP experiments revealed that over-expressed HA–TERT also binds to TBE sequences of the Myc promoter, whereas the 3' untranslated regions (3´UTR) were not precipitated. Furthermore, as demonstrated by ChIP experiments performed in parallel using different established anti-TERT (Ab8313 and Rockland) antibodies and chromatin from non-transfected HEK293 cells, endogenous TERT similarly occupied the TCF binding elements of c-Myc

promoter. All these data demonstrate the specificity of the TERT-DNA interactions (Figure 11).

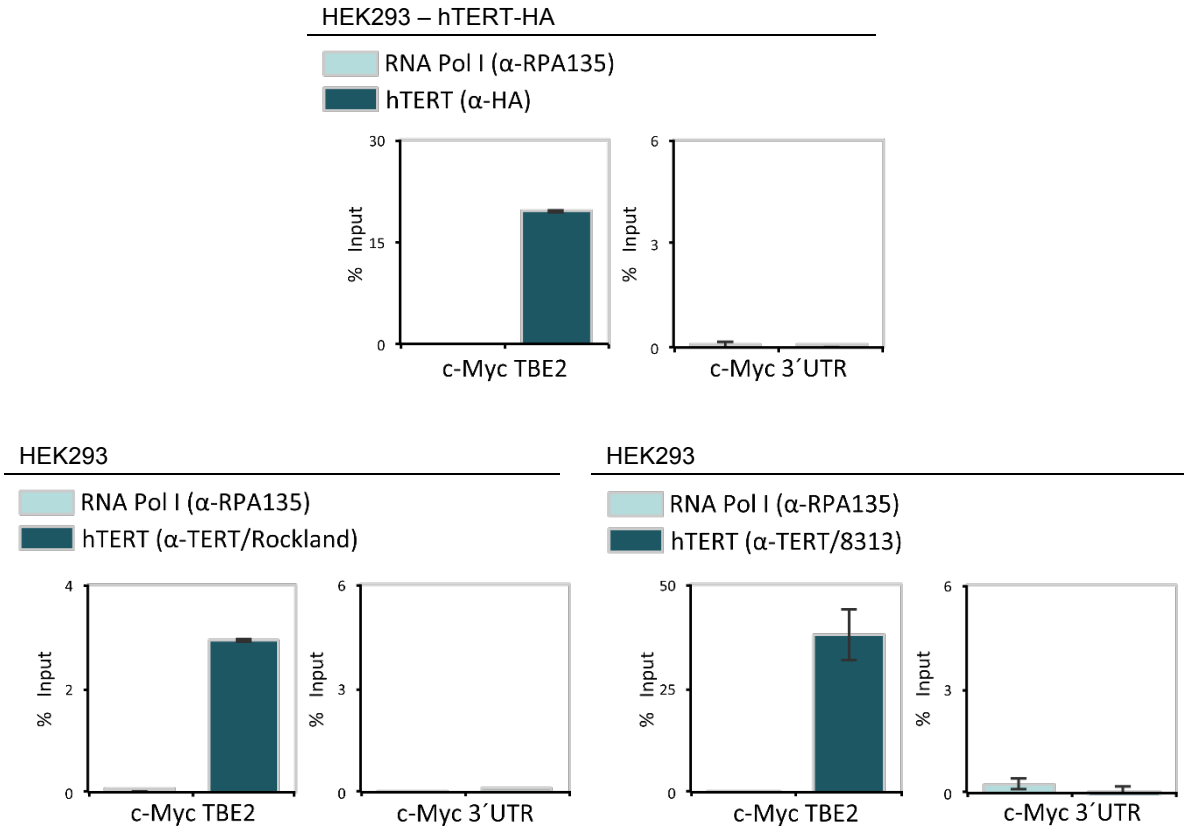


Fig.11: *The c-myc TBE sequence but not the 3`UTR is enriched in telomerase ChIP experiments with different antibodies*

3.1.3 TERT binds un-methylated active ribosomal RNA genes

rDNA sequences which are transcriptionally active are un-methylated, while methylation of CpG islands at promoter regions are correlated with transcriptional silencing. To investigate whether hTERT binds to active or inactive rDNA copies, we used ChIP assays and the immunoprecipitated DNA was subsequently treated with the isoschizomeric restriction nucleases HpaII and MspI, which exhibit different methylation sensitivities. Whereas HpaII cleave only on un-methylated DNA copies, MspI recognizes and cuts DNA sequences either are methylated or un-methylated.

Methylation-sensitive digestion was followed by PCR amplification using a primer pair that covers the promoter sequences. As result, we found that both restriction enzymes cleave completely the rDNA promoter region associated with hTERT as well as with the RNA polymerase I, compared to the 50% effective digestion

observed with HpaII and a complete cleavage with the MspI enzyme of the input-chromatin. These results demonstrated that TERT binds the active fraction of the rDNA (figure 12).

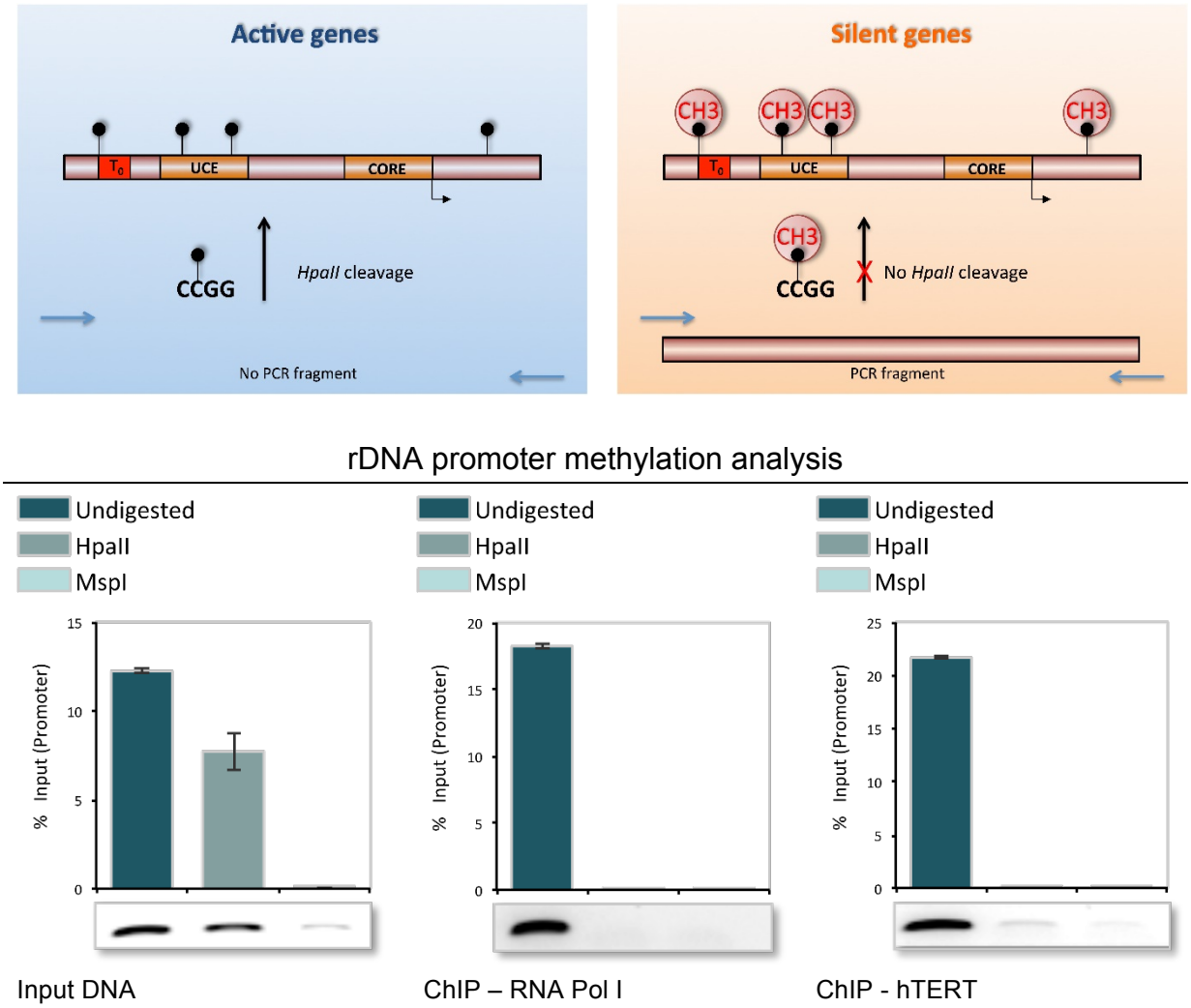


Figure 12. *The scheme (upper panel) illustrates the organization of the rDNA promoter from active (left) and silent (right) rRNA genes that can be distinguished by cleavage with methylation-sensitive restriction enzymes. If CpG residues are un-methylated they are sensitive to HpaII digestion and cannot be amplified by PCR using a primer pair that covers the promoter sequences. On silent genes, the CpG residues are methylated and resistant to HpaII cleavage. The immunoprecipitated chromatin was digested with the indicated enzymes, and the rDNA promoter was detected by semiquantitative PCR and qPCR (lower panel).*

3.1.4 Characterization of a newly generated anti-TERT antibody (Ab-8313)

Given the limited number of commercially available specific antibodies directed against the telomerase reverse transcriptase subunit (TERT), in the present study we used a newly generated antibody (Ab-8313), raised against a

sequence motif conserved between mouse and human TERT. The specificity validation and characterization of this homemade antibody was addressed in multiple ways. First, to validate the specificity of the Ab-8313 antibody we analysed the TERT-rDNA binding capacity in mouse and human tissues and cells by ChIP experiments coupled with a peptide competition assay (PCA). After pre-incubation with an excess of peptide the Ab-8313 antibody was not able to precipitate TERT associated with rDNA promoter sequences, as compared with the no-peptide control (Figure 13 – upper panel). Furthermore, Western Blot analysis showed the anti-TERT house generated Ab-8313 antibody recognizing purified human and mouse TERT (Figure 13 – lower panel left). The Ab-8313 antibody was also assayed in subsequent immunoprecipitation reactions of ChIP-re-ChIP experiments demonstrating simultaneously association of RNA Pol I and TERT with the same chromatin fragments harbouring the rDNA promoter region (Figure 13 – lower panel right).

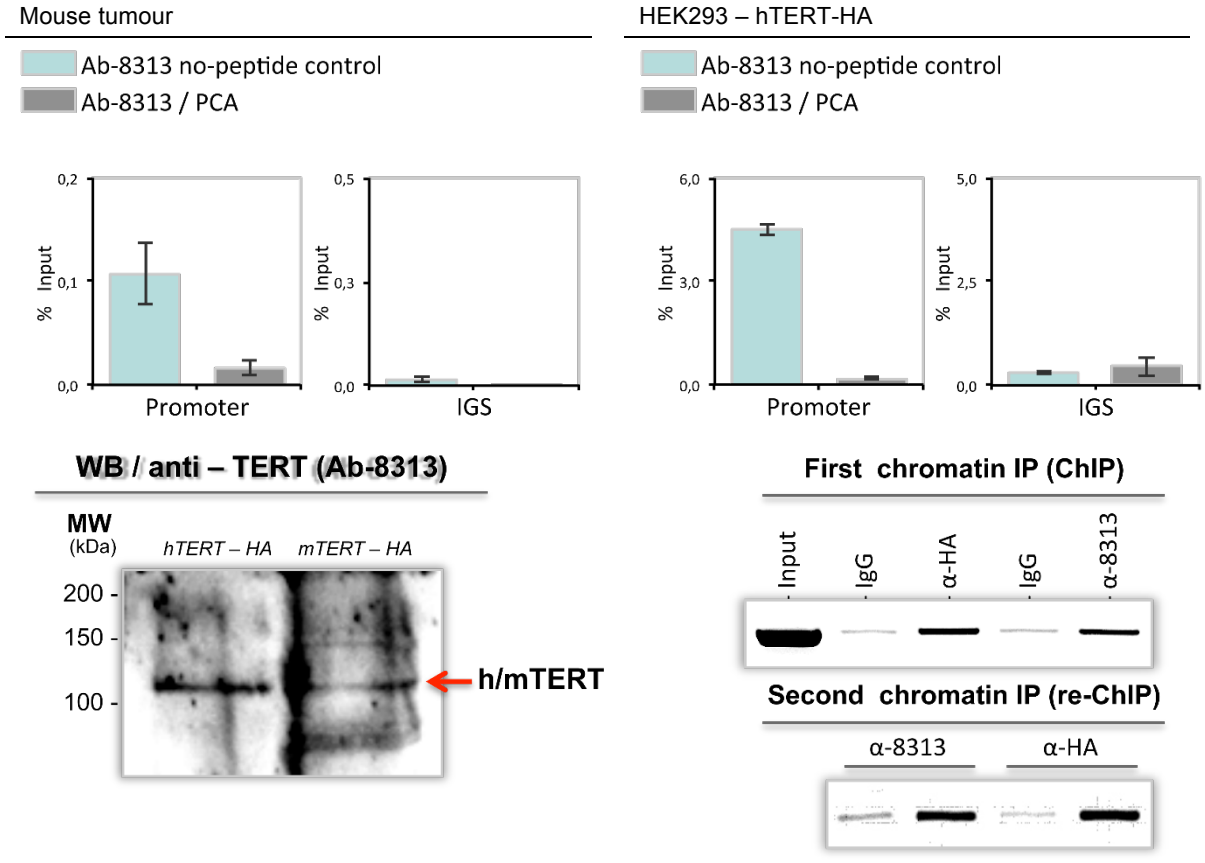


Figure 13: *The upper lane shows peptide competition assay (PCA) in ChIP analysis of TERT-rDNA binding in mouse and human tissue and cells. The newly generated anti-TERT (AB8313) recognizes purified TERT from human and murine cells overexpressing h/mTERT – HA (lower panel - left). ChIP-re-ChIP experiment showing that TERT occupies the same molecules of rDNA as RNA polymerase I (lower panel - right)*

Although the results from ChIP–re–ChIP experiment can suggest the probable physical interaction between TERT and Pol I associated with the same genomic region, a direct protein-protein contact cannot be addressed by this method. For such aims, we performed standard co-immunoprecipitation assay followed by Western blotting (Co-IP/WB).

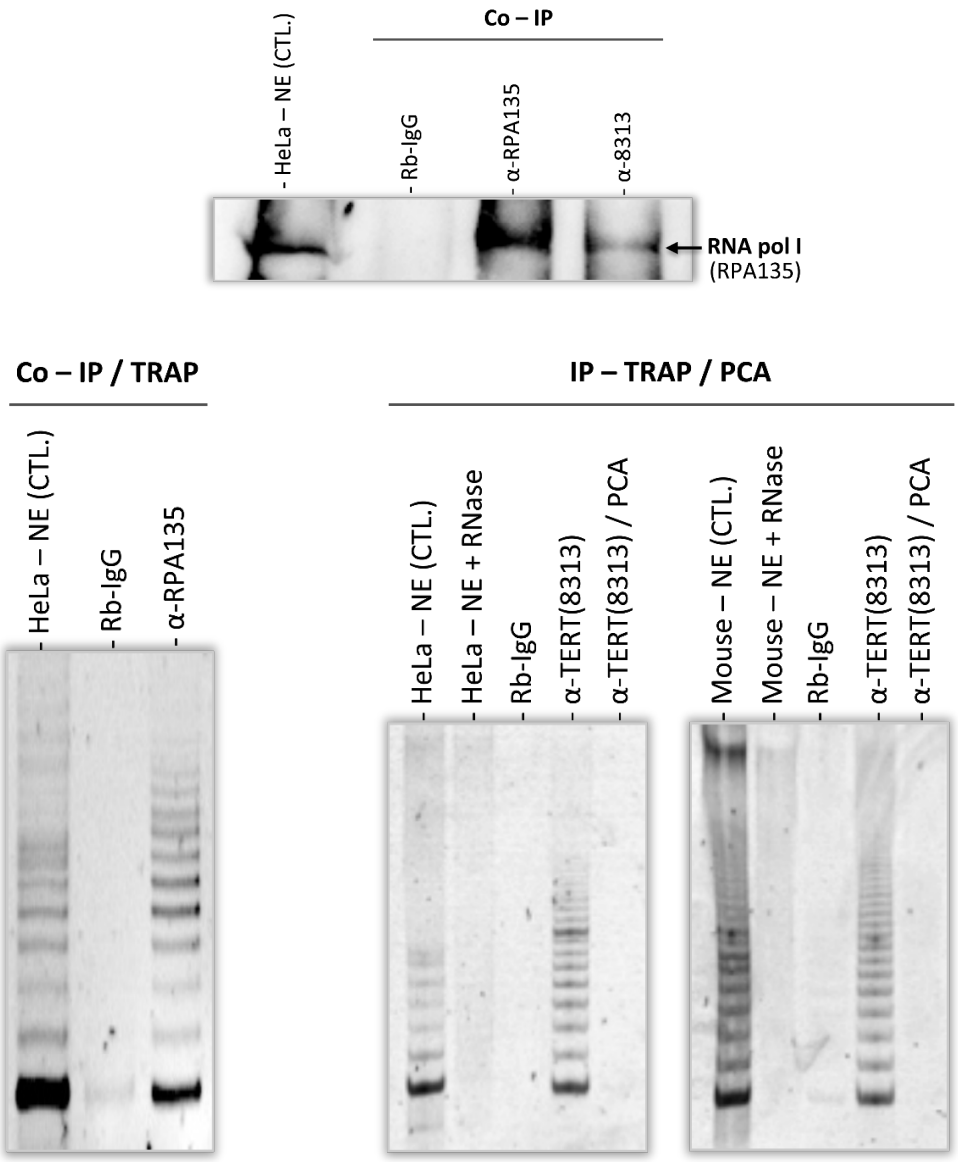


Figure 14: *Telomerase associate with Pol I. Coimmunoprecipitation of Pol I with anti-TERT Ab8313 (upper panel). IP-TRAP assay with an anti-Pol I antibody showed telomerase associated activity (lower panel – left). IP-TRAP experiments with human and mouse nuclear extracts and Ab8313 antibody (lower panel – right). Controls include RNase digestion and peptide competition assay (PCA).*

Western blot analysis demonstrated that the home-generated telomerase antibody precipitated substantial amounts of Pol I from HeLa nuclear extracts (Figure 14 –

upper panel). Conversely, telomerase activity could be detected associated to the RNA polymerase I fraction precipitated with the indicated antibody (anti-RPA135), as shown in TRAP assay performed after Co-IP (Figure 14 – lower panel left). The binding specificity of anti-TERT (Ab-8313) antibody was further characterized by IP-TRAP experiments controlled with RNase digestion and peptide competition (Figure 14 – lower panel right).

3.2 Telomerase binds rDNA under hyperproliferative conditions.

As described above, telomerase-rDNA association was observed in immortalized HEK293 cells under TERT overexpression conditions. It is well known that the telomerase ribonucleoprotein is continuously repressed in most normal somatic tissues with the exception of stem, germ cells and some other highly proliferating cells. Otherwise, most human tumours display an enhanced telomerase activity. All theses evidences lead us to address the question whether rDNA binding of TERT is a feature of all telomerase positive cells, if it is only a specific property acquired under cellular transformation and hyperproliferation conditions. For this end, the telomerase association to the rDNA was further assessed under different physiological conditions.

A first line of investigation was to elucidate the binding activity of telomerase during cellular transformation. ChIP assays using two experimental systems helped us to approach this question:

i) An experimental *in vivo* system comparing normal liver tissue versus hepatocellular carcinoma from mice.

ii) The human biological environment of normal telomerase positive cells was replicated in vitro with telomerase immortalized human fibroblasts (IMR90), which contain the SV40 small- and large-T antigens; on the other hand the same cell line transformed with oncogenic Ras (RasV12) resembles the tumour cellular conditions.

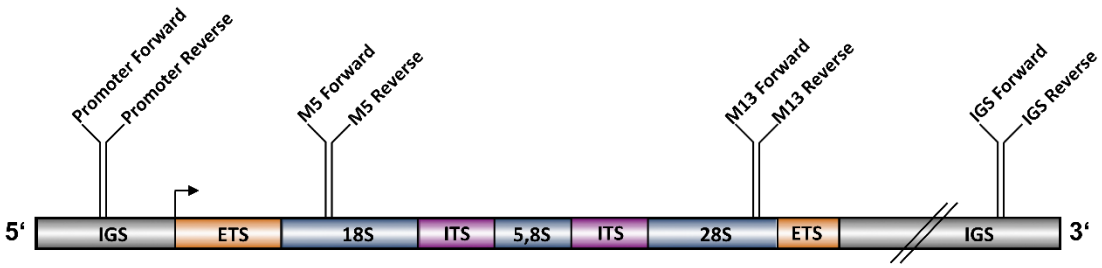


Figure 16: *Maps of primer location in mouse rDNA.*

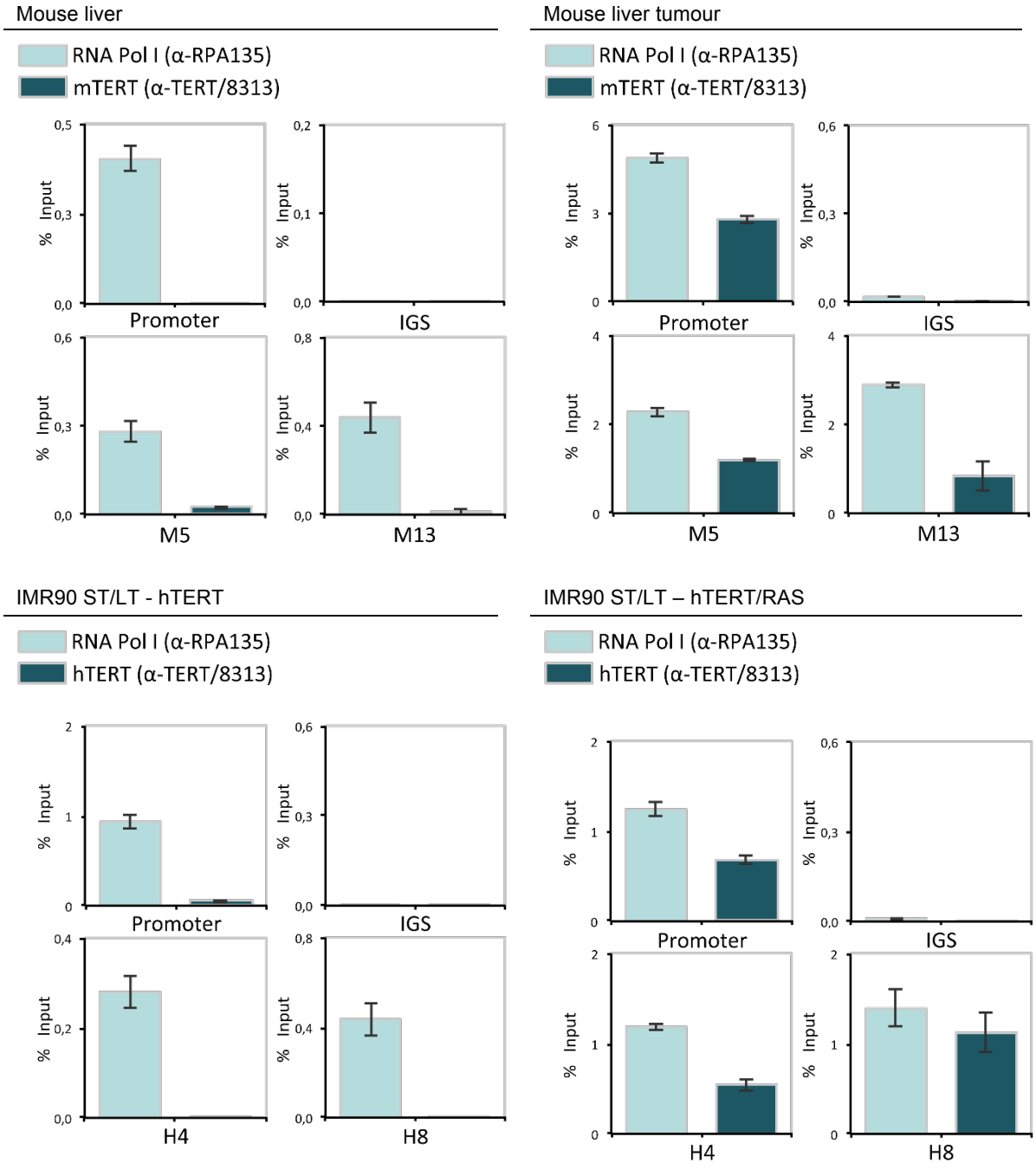


Figure 17: *Quantitative PCR following ChIP experiments on the indicated tissues and cell lines using antibodies against Pol I and telomerase (Ab8313), demonstrating the growth signal dependence of the binding of TERT to rDNA.*

In contrast with the similar high telomerase activity observed in both tissues and cell lines (Figure 18), binding of TERT to rDNA was exclusively detected in tumour and Ras-transformed cells (Figure 17). In addition, telomerase ChIP experiments carried out in human hepatocellular carcinoma cells also exhibited strong binding of telomerase to the rDNA (Figure 19).

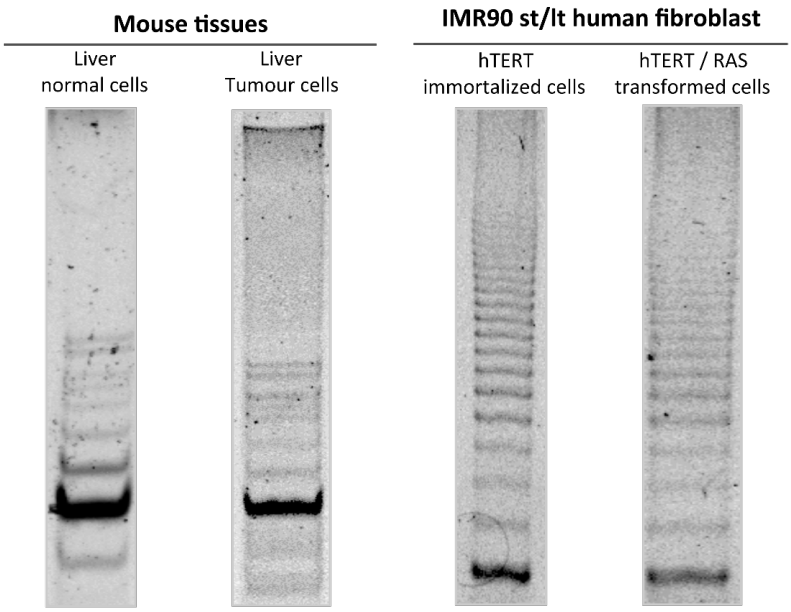


Figure 18: *Similar telomerase activity observed in normal liver tissue vs. tumour (left), and telomerase immortalized IMR90 human fibroblast vs. the same cell line transformed with oncogenic Ras (right).*

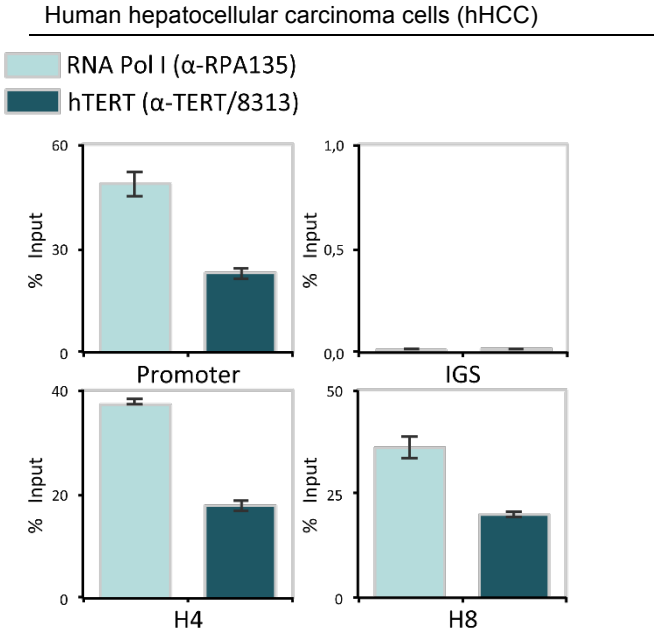


Figure 19: *ChIP analysis of TERT binding to the rDNA in human hepatocellular carcinoma*

3.2.1 TERT is present at the telomere in proliferating carcinoma cells.

Despite the comparable levels of telomerase activity observed in both mouse samples, telomere binding of telomerase can only be detected in liver carcinoma containing highly proliferative cells and not in normal non-proliferating hepatocytes, as revealed telomeric ChIP assay in which protein-associated telomeric DNA was detected by dot blots and quantitative PCR analyses (Figure 20). Thus, our data are also consistent with the solid evidence that active telomerase is specific recruited to telomere ends in the S-phase of the cell cycle *[Taggart, AK et al. 2002[109]; Tomlinson, RL et al., 2006[110]; Xin, H et al., 2007[111]]*.

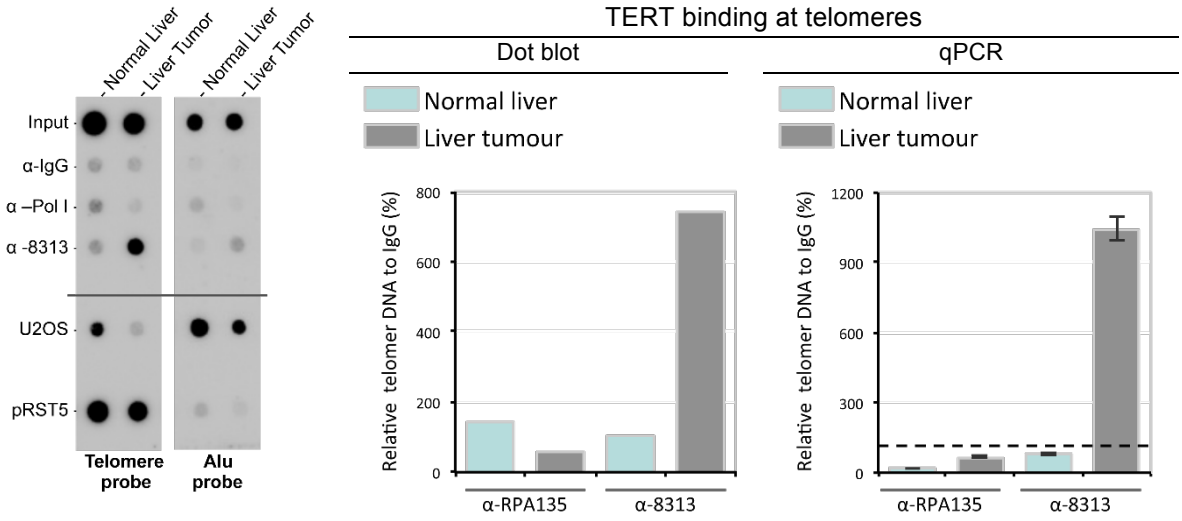


Figure 20: *Telomerase binds telomeric regions in tumour cells. Dot blot (left) and qPCR analysis (right) following ChIP experiments using the indicated antibodies and mouse liver /mouse liver tumour chromatin. Two amounts of U2OS genomic DNA or a plasmid harbouring telomeric sequences (pRST5) served as hybridization controls.*

3.2.2 rDNA occupancy of telomerase in proliferating hepatocytes

To better understand the molecular mechanism underlying telomerase activity at the rDNA locus in vivo, and to elucidate whether TERT binding to rDNA is restricted to transformed cells we performed partial hepatectomy (PH) in mice. This surgical procedure typically involves removal of two-thirds of the liver inducing quiescent hepatocytes to enter the cell cycle and actively proliferate. After PH mice were killed at different time points, rDNA transcription and the binding kinetics of telomerase to rDNA were monitored (Figure 21).

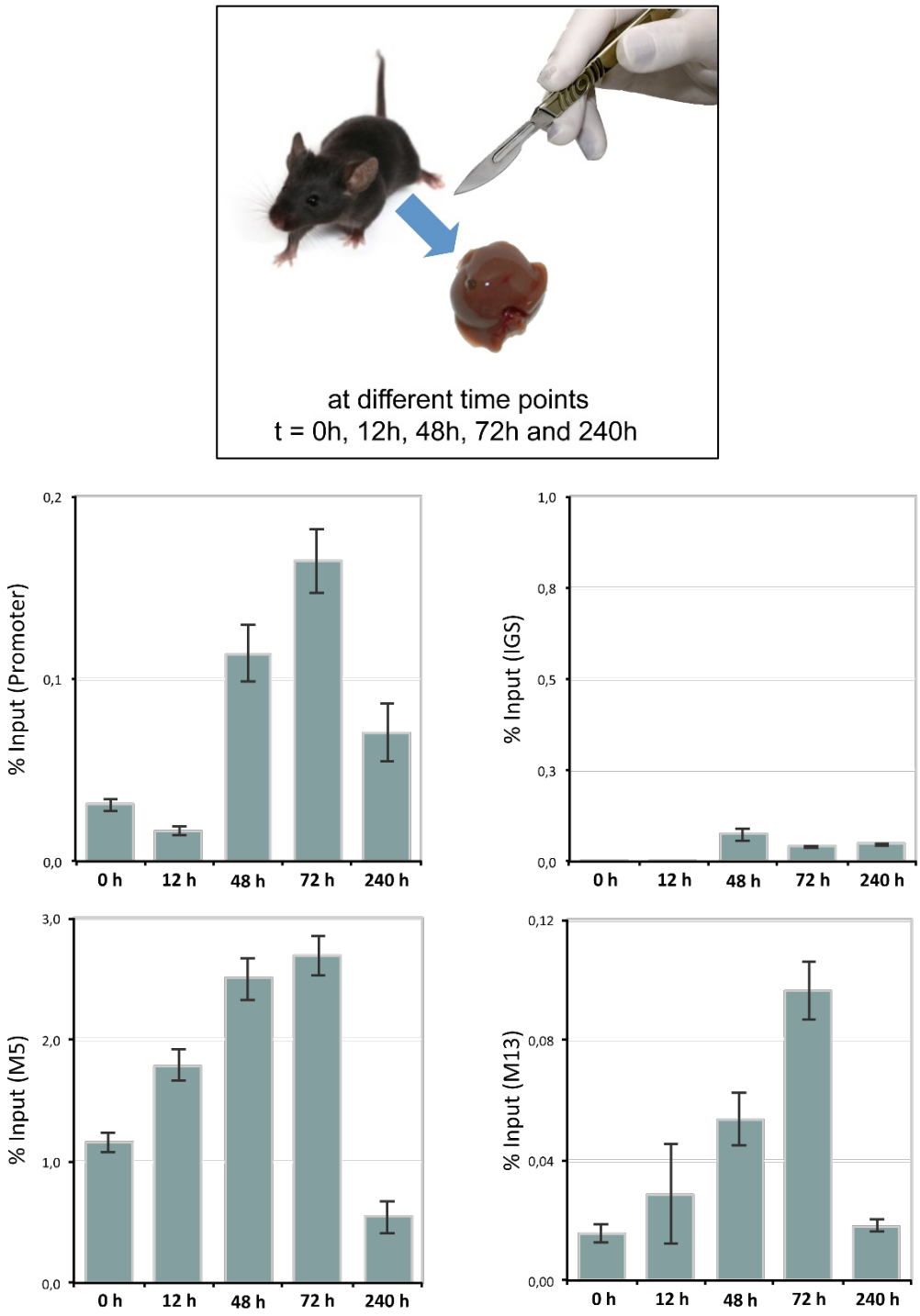


Figure 21: *Binding dynamics of telomerase to the rDNA after partial hepatectomy (HP). qPCR on chromatin harvested at the indicated time points after surgery, immunoprecipitated using Ab-8313 antibody.*

The association of telomerase with rDNA was detected as soon as 12 hours following partial hepatectomy, reaching a maximum at 3 days. 10 days post PH, no significant rDNA could be precipitated with the telomerase antibody Ab-8313. Interestingly, Pol I transcription is still stimulated 10 days after hepatectomy while

the binding of TERT is low at this time. One possible explanation for this phenomenon is that telomerase is activated and elongates telomeres and stimulates rDNA transcription only during the hyper-proliferation phase of liver regeneration (peak of S-phase between 48-72 h post hepatectomy). 7-14 days after partial hepatectomy 93% of liver mass has already regenerated *[Martins et al., 2007*[112]*]* and it might be that after a proliferative phase cell division stops and residual liver mass is restored by growth. It is tempting to conclude, that the rDNA occupancy of telomerase is a feature of proliferating hepatocytes. On the other hand, the expression of 45S precursor rRNA, analyzed by qPCR showed that Pol I activity was increased threefold by HP and remained elevated until 10 days after partial hepatectomy, indicating that Pol I activity might not depend on telomerase binding to the rDNA (Figure 22).

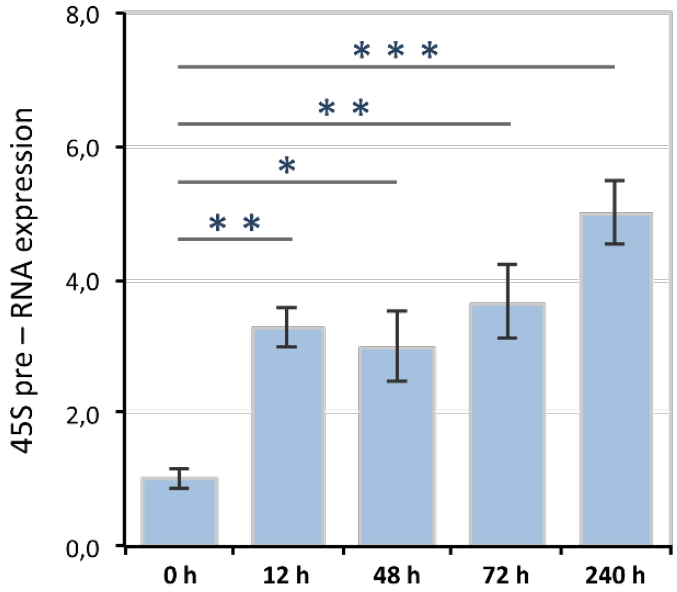


Figure 22: Pol I transcription stimulation after PH. Quantitative PCR analysis of the 45S pre-RNA synthesis at the indicated time points

3.3 Telomerase stimulates Pol I transcription.

TERT has been reported as a direct modulator of the Wnt/β-catenin pathway by occupying TBE containing promoter fragments of Wnt target genes (cyclin D1 and c-myc) and serving as cofactor in a β-catenin transcriptional complex *[Park, JI et al., 2009* [94]*]*. Our previous results showed TERT binding to promoter and internal regions of ribosomal gene, suggesting a novel telomerase molecular mechanism at the rDNA locus linked with RNA polymerase I transcription.

3.3.1 Growth signal-dependent stimulation of rDNA reporter transcription by TERT

To determine the implication of TERT in Pol I transcription, IMR90-ST/LT cells were co-transfected with constant levels of rDNA reporter plasmid pHrP2-BH and increasing amounts of hTERT vector. The reporter transcription was quantified by qPCR and indicated rDNA promoter activity. Remarkably, increasing amounts of TERT in the absence of Ras repressed Pol I transcription in a dose-dependent manner (Figure. 23 - left), whereas transfecting cells with increasing amounts of TERT in the presence of Ras resulted in a fourfold stimulation of rDNA reporter transcription (Figure 23 - middle). Thus, TERT stimulates the key step of ribosomal biogenesis, transcription of RNA polymerase I, in a growth signal-dependent manner.

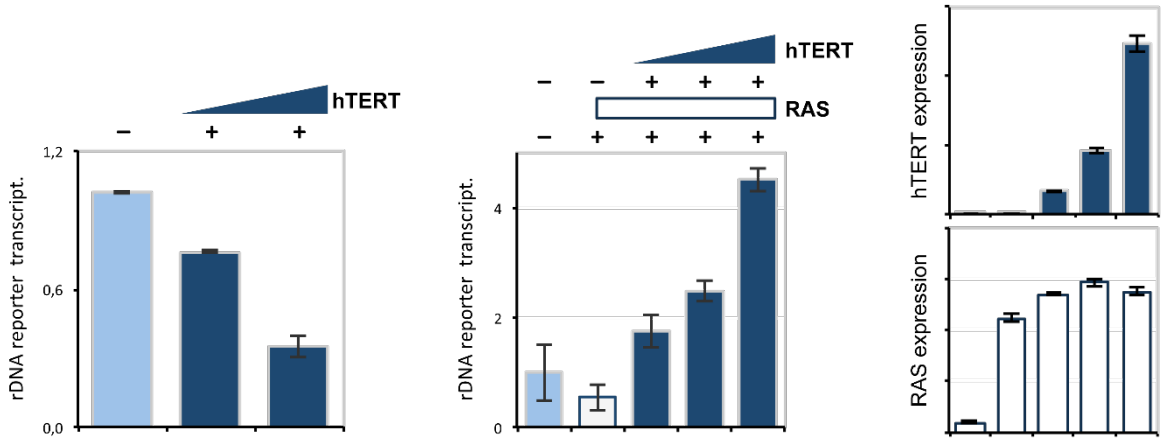


Figure 23: *qPCR analysis of rDNA reporter transcription after co-transfection with increasing amounts of TERT in IMR90 ST/LT cells (left). Analysis of transcription of the rDNA reporter plasmid after co-transfection of TERT and Ras in IMR90 ST/LT cells (middle). TERT and RAS expression levels were monitored by qPCR (right)*

3.3.2 Telomerase regulates cell proliferation by modulating rDNA transcription

The biogenesis of ribosomes is tightly linked to cell growth and proliferation, therefor upregulation of rRNA synthesis is mandatory for all tumors. Moreover, high levels of telomerase activity have been reported in cancer cells. These evidences suggest a connection between telomerase and Pol I transcription, involving the mechanism of cell proliferation, and growth-factor signaling pathways. For a

comprehensive analysis of this relationship, we examined the effect of telomerase inhibition on Pol I transcription.

First, IMR90-ST/LT cells co-transfected with RAS and TERT were treated with the telomerase inhibitor imetelstat (GRN163L) or a control oligo (GRN140833L) and the Pol I activity was monitored. As depicted in Figure 24 (graphic - left), Imetelstat, but not the control oligo, severely affected Pol I transcription, indicating that half of Pol I activity in transformed cells is due to stimulation by telomerase. Furthermore, the inhibition of endogenous telomerase activity by transfecting in human colorectal carcinoma cells (HCT116) with a TERT-specific shRNA or with a DN-TERT mutant (DN-TERT), reduced significantly Pol I transcription as measured by endogenous 47S transcription to 56% or 42%, respectively, of the control level.

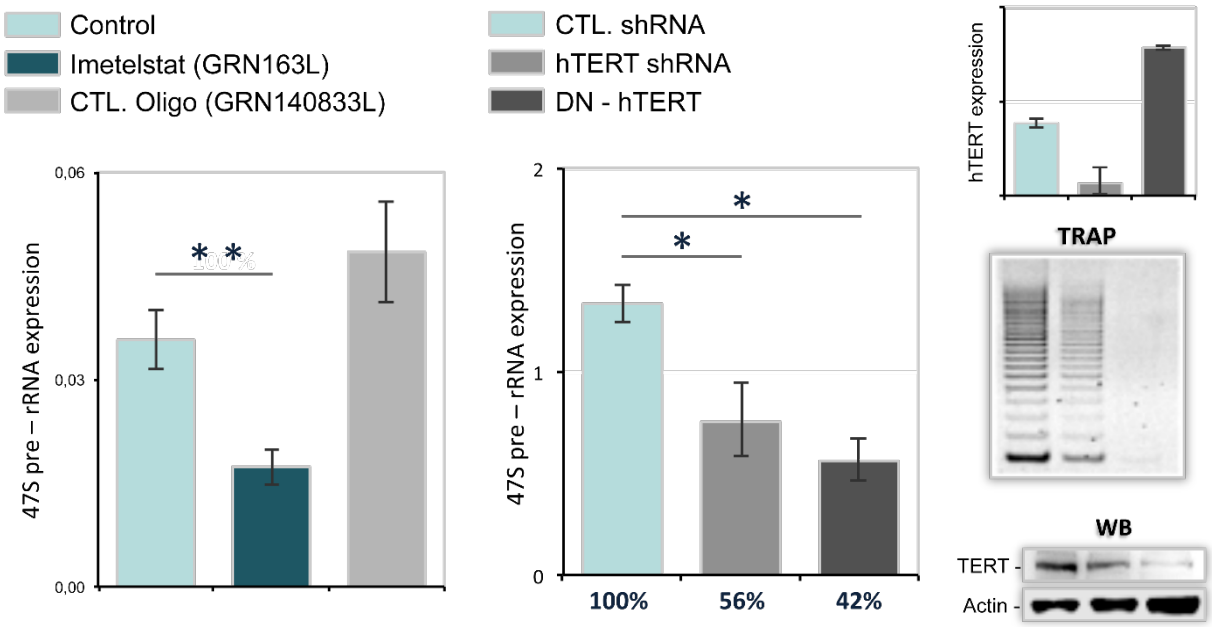


Figure 24: *Telomerase inhibitor Imetelstat inhibits Pol I transcription. IMR90 ST/LT – TERT/RAS cells were treated for 96h with 5μM of Imetelstat or control oligo. RNA was harvested, and the synthesis of the 47S rRNA precursor was analysed by qPCR (left). Knockdown of telomerase reduces Pol I transcription. HCT116 cells were transfected with control shRNA, TERT shRNA or a DN-TERT construct. After antibiotic selection, 47S pre-rRNA (middle) and telomerase expression levels (right – upper graphic) were monitored by qPCR. Telomerase activity was analysed by a TRAP assay, and TERT protein expression was analysed by western blotting with Rockland antibody (right – lower lanes).*

The expression levels of endogenous TERT and DN-TERT were monitored by qPCR and western blotting, demonstrating that DN-TERT expression is followed by the degradation of endogenous TERT protein *[Nguyen, BN et al., 2009* [113]*]*. TERT

shRNA, and to a greater extent DN-TERT, significantly reduced telomerase activity, as demonstrated in the TRAP assay (Figure 24, right panel on the middle). Thus, repression of telomerase activity in tumour cells directly reduced the rDNA transcription.

Interestingly, telomere length was not significantly affected neither by transfection with TERT shRNA nor with the mutant DN-TERT during the short period of treatment (Figure 25). Taken together, the dose-dependent stimulation of Pol I by transfection with TERT and the decrease in Pol I transcription by the inhibition of telomerase activity strongly supports the hypothesis that TERT and telomerase directly regulate transcription by RNA polymerase I.

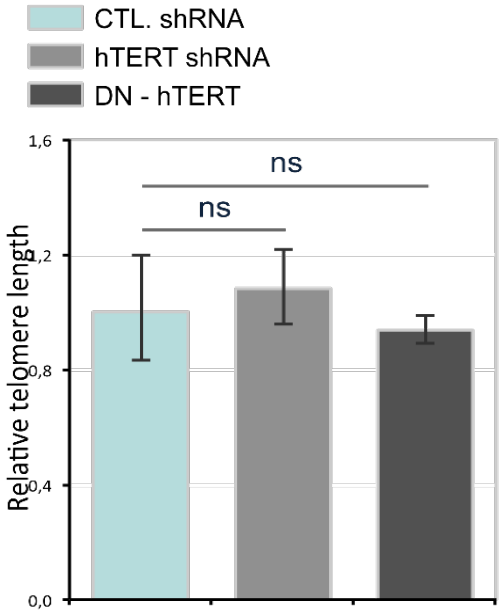


Figure 25: *Quantitative PCR analysis of telomere length of the indicated cells after transfection and antibiotic selection*

3.3.3 The whole functional telomerase holoenzyme is required to bind rDNA and for regulation of Pol I transcription

The telomerase holoenzyme mainly consists of the catalytic subunit (TERT) and a RNA component (TERC). To investigate whether both telomerase components bind as complex to the rDNA, and can also be co-precipitated with Pol I chromatin, we performed RT-PCR reaction to generate cDNA from the telomerase RNA component bound to the immunoprecipitated chromatin.

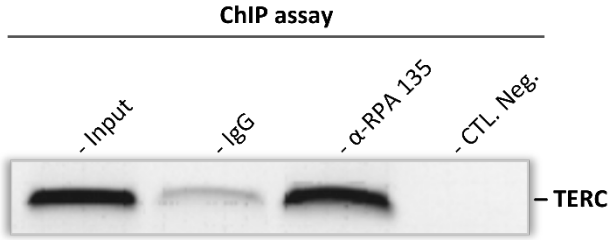


Figure 26-A: *The telomerase RNA component co-precipitates with Pol I antibodies from HEK293 chromatin (upper lane).*

As indicated in Figure 26-A the whole telomerase holoenzyme binds in a complex to the rDNA. This observation is consistent with previous reports showing TERC localization in the nucleoli of HeLa cells *[Tomlinson, RL et al., 2006*[110]*; Lee, JH et al., 2014*[108]*]*. Moreover, when liver tumour cells derived from TERC -/- mice (18) were subjected to ChIP analysis, no binding of TERT to the rDNA promoter was detectable, arguing that the presence of TERC in the telomerase complex is necessary for the association of TERT with the rDNA (Figure 26 – lower graphics).

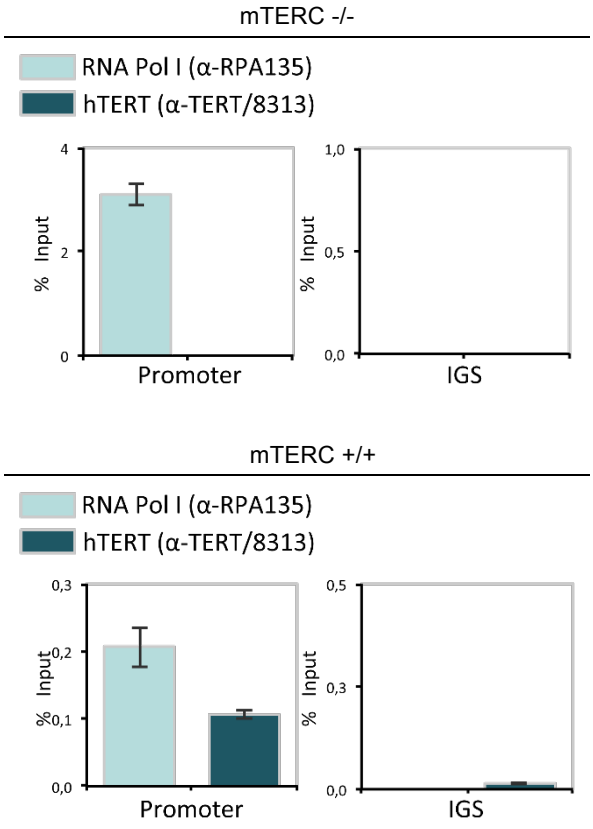


Figure 26-B: *No binding of telomerase to rDNA in liver tumour cells from Terc -/- mice. ChIP analysis with anti-Pol I and anti-TERT specific antibodies and qPCR with the indicated primers.*

In addition, a reduction in TERC expression by siRNA transfection in HCT116 cells impaired RNA polymerase I transcription as monitored by transcription of the 47S pre-rRNA precursor, indicating that telomerase requires both subunits for modulate the ribosomal biogenesis (Figure 27). This data are in line with recent studies showing that functional telomerase complex is required to regulated NF-kB signalling *[Gosh, A et al., 2012[106]]*.

Accumulating evidences are showing that TERT has alternative non-telomeric functions. Our results indicated that an enzymatic activity of the telomerase holoenzyme, other than telomere synthesis was required for the positive effect on rDNA transcription.

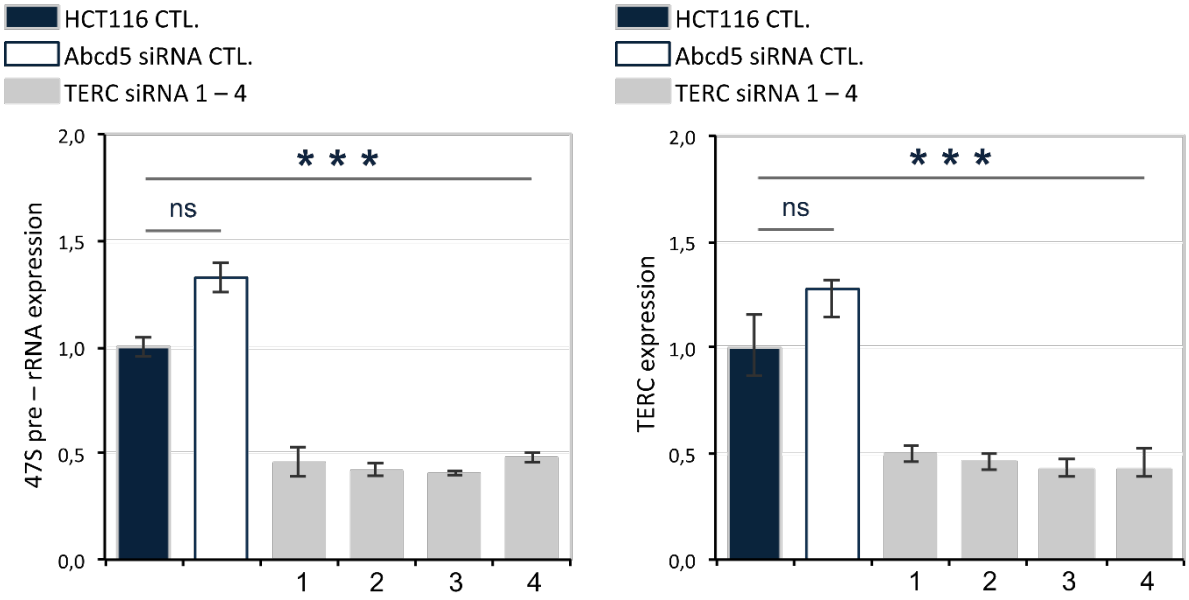


Figure 27: *siRNA against TERC reduces Pol I transcription. HCT116 cells were transfected with two controls and four anti-TERC siRNAs and 47S (left) and TERC (right) expression were monitored by qPCR.*

In order to demonstrate that the described influence of telomerase in the ribosomal DNA transcription is a novel mechanism associated with telomerase enzymatic activity beyond of the telomere lengthening, we used a C-terminally HA-tagged TERT protein that fails to elongate telomeres despite demonstrating enzymatic activity *[Counter, CM et al., 1998 [80]]*. This mutant was expressed in HEK cells, purified and assayed in Pol I in vitro transcription with nuclear extract from TERT

repressed HCT116 cells. As shown in Figure 28, the mutated version of TERT was able to stimulate Pol I transcription in a telomere-lengthening independent manner.

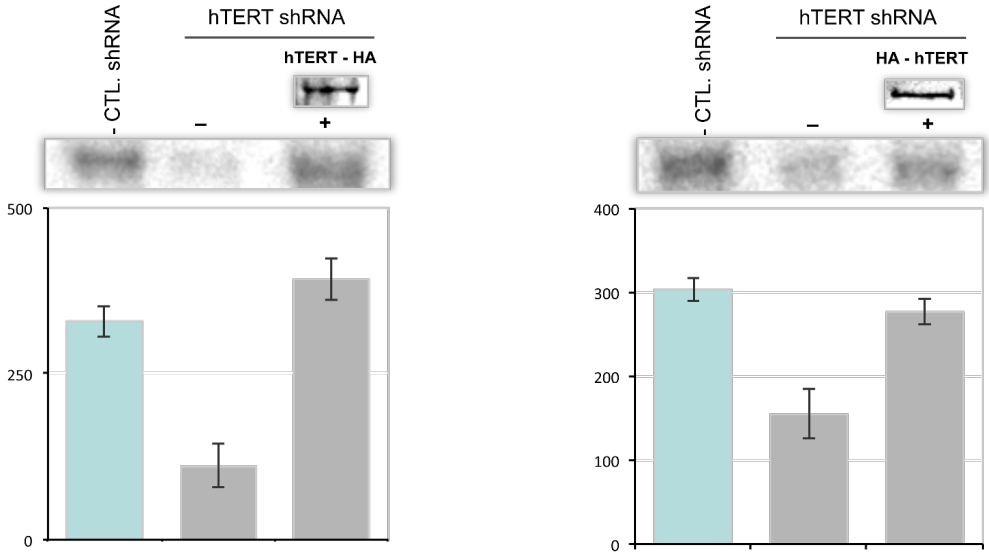


Figure 28: *We expressed both constructs HA-TERT and TERT-HA in HEK cells and tested the purified TERT proteins by the in vitro transcription of RNA polymerase I. Both versions of TERT were able to stimulate Pol I transcription.*

3.4 Telomerase stimulates initiation complex at the rDNA promoter.

In the transcription cycle, the RNA polymerase I progresses through a series of subsequent events, which can be divided into three main stages: initiation, elongation and termination. During transcription initiation the formation of pre-initiation complexes at the promoter region takes place, involving the RNA polymerase I with help of different transcription factors. Once Pol I clears the promoter, the elongation step begins and the rDNA can be transcribed into a corresponding rRNA sequence. Finally, transcription termination elements at the 3´ end of the transcribed region, forces Pol I to pause and to dissociate the transcription complex from the rDNA template and the new transcript.

3.4.1 Telomerase does not leave the rDNA promoter during transcription

Given the association of the telomerase holoenzyme throughout promoter and gene internal regions of rDNA, we supposed that TERT could modulate rRNA synthesis at any stage during the transcription cycle. Template immunoprecipitation assays (TIP) were used to further gain mechanistic insights into the action of

telomerase at the rDNA promoter. The combination of in vitro transcription and chromatin immunoprecipitation methods *[Assfalg, R et al., 2012[49]]* were preformed by pre-incubating HeLa nuclear extract with linearized pHrP2 rDNA template. The in vitro transcription reaction was started by addition of nucleotides and stopped at different time points by cross-linking with formaldehyde. Telomerase and Pol I were precipitated with specific antibodies, and the associated template to both proteins of interest was detected by semiquantitative PCR.

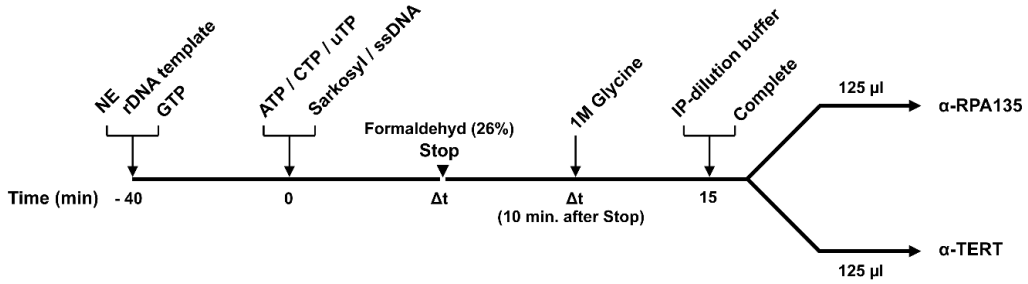


Figure 29-A: *After pre-incubation of HeLa nuclear extract with rDNA template, the in vitro transcription reaction was started by the addition of nucleotides and stopped after the indicated time points by cross-linking with formaldehyde. Pol I and telomerase were precipitated with specific antibodies and the associated template was detected by PCR*

The binding kinetic of telomerase at the rDNA promoter after transcription initiation, revealed that whereas RNA polymerase I left the template approximately 10 minutes after transcription start, TERT does not leave the rDNA promoter, staying bound to the rDNA template during transcription (Figure 29 – B).

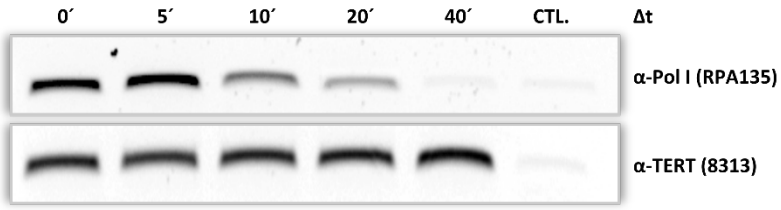


Figure 29-B: *Template immunoprecipitation experiment revealed that telomerase does not leave the rDNA promoter during transcription.*

3.4.2 rDNA promoter occupancy of TERT is impaired by telomerase inhibition leading to a reduction of Pol I activity in vitro.

However, telomerase inhibition significantly reduced occupancy of TERT to the rDNA promoter, either by transfection with TERT shRNA or expression of a DN-

TERT mutant, as exhibited in ChIP experiments with telomerase specific antibodies and quantification of TERT association to the rDNA promoter region (Figure 30).

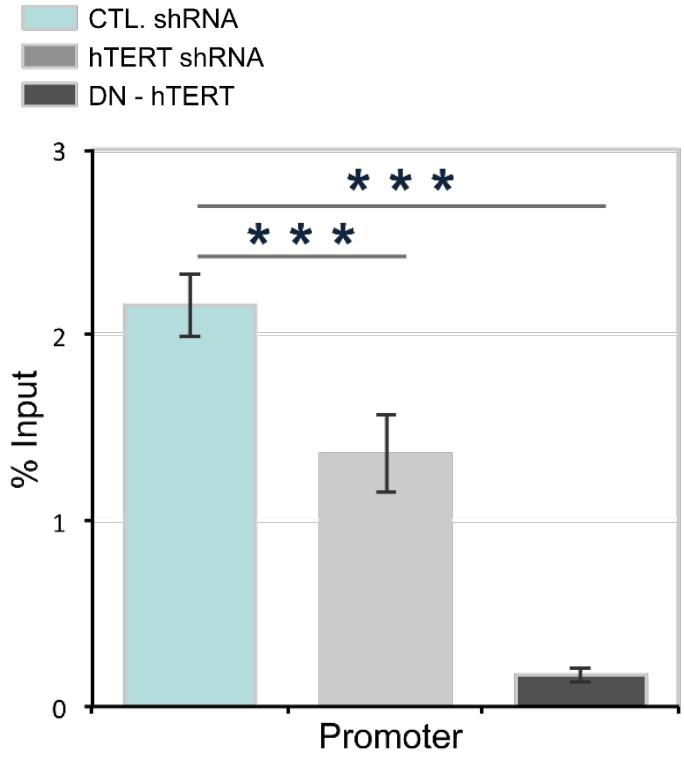


Figure 30: *ChIP experiments with telomerase specific antibodies and analysis of TERT binding to the rDNA promoter in HCT116 cell demonstrated that inhibition of telomerase reduces rDNA promoter occupancy of telomerase.*

Next, we wondered whether the reduced rDNA promoter occupancy of TERT caused by telomerase inhibition might influence the RNA polymerase I activity. For this end, parallel to multiple-round transcription assay we also limited the transcription reaction to a single round by adding Sarkosyl to 0.025%. This detergent has been used to approach the formation of transcription initiation complexes by RNA polymerase I.

As illustrated in Figure 31, besides of multiple-round transcription also the single-round in vitro transcription was severely affected by telomerase inhibition in nuclear extracts harvested from the treated HCT116 cells. Thus, the decreased rDNA promoter occupancy of TERT caused by telomerase inhibition resulted in a marked reduction of in vitro Pol I transcription activity.

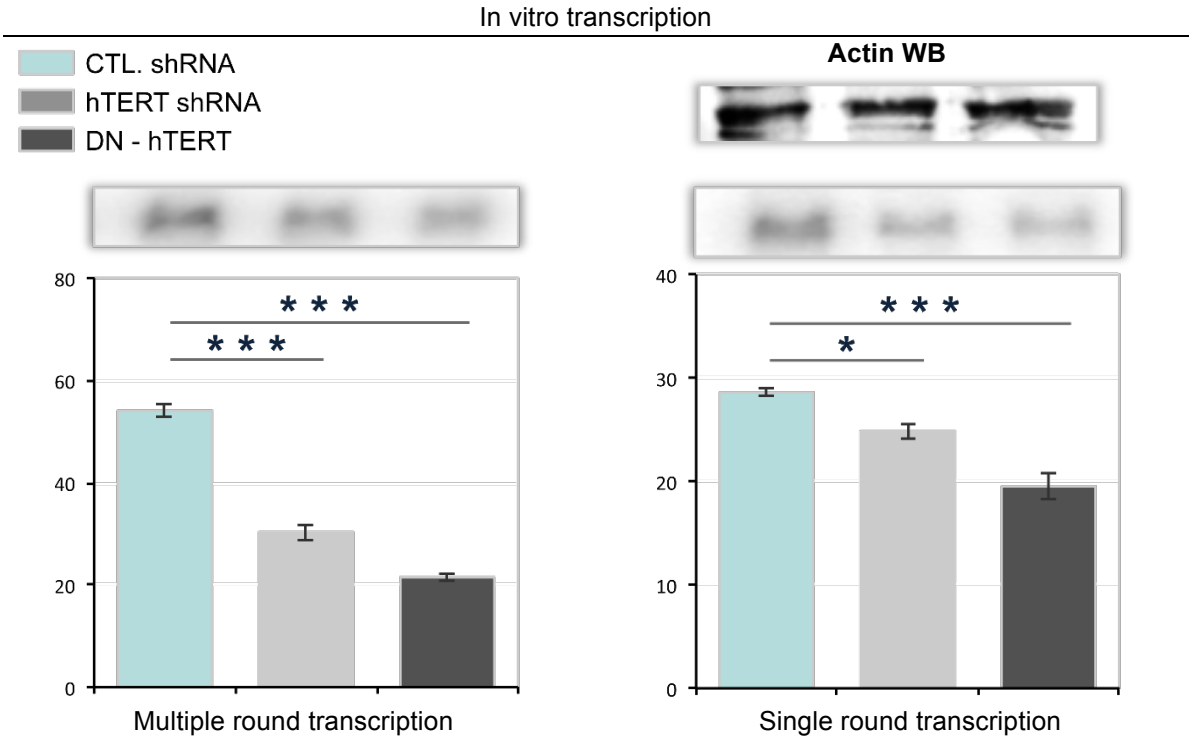


Figure 31: *In vitro transcription of Pol I with nuclear extracts of shRNA treated HCT116 cells. A representative RNA gel electrophoresis image is shown (upper lanes. Actin served as a loading control for western blotting.*

3.4.3 Binding of telomerase to rDNA gene internal regions might not directly influence the Pol I transcription elongation stage.

Given that TERT have been also detected binding to internal positions on the rDNA, it should also be assumed that in addition to stimulating formation of initiation complexes at the rDNA promoter, telomerase might also play a role in a step subsequent to transcription initiation. To address this question, a promoterless in vitro transcription assay was performed using nuclear extracts from telomerase repressed HCT116 cells and an unspecific DNA template excised from pUC18 with NdeI and EcoRI restriction enzymes (Figure 32 – left). The lack of the promoter region in the restricted pUC18 template allows investigating promoter-independent elongation activity of the RNA polymerase I. With the same aim, nuclear run-on experiments were performed with isolated nuclei from the indicated cells and the elongated rRNA was quantified after cDNA synthesis by qPCR with primers against gene-internal sequences of the rDNA. Both experiments were carried out in the presence of α-Amanitin, a specific inhibitor of both RNA polymerases II and III

(Figure 32 – right). As demonstrated by both experiments, the transcription elongation capacity of Pol I was not reduced by telomerase repression, indicating that binding of telomerase to internal positions on the rDNA might not directly influence transcription elongation

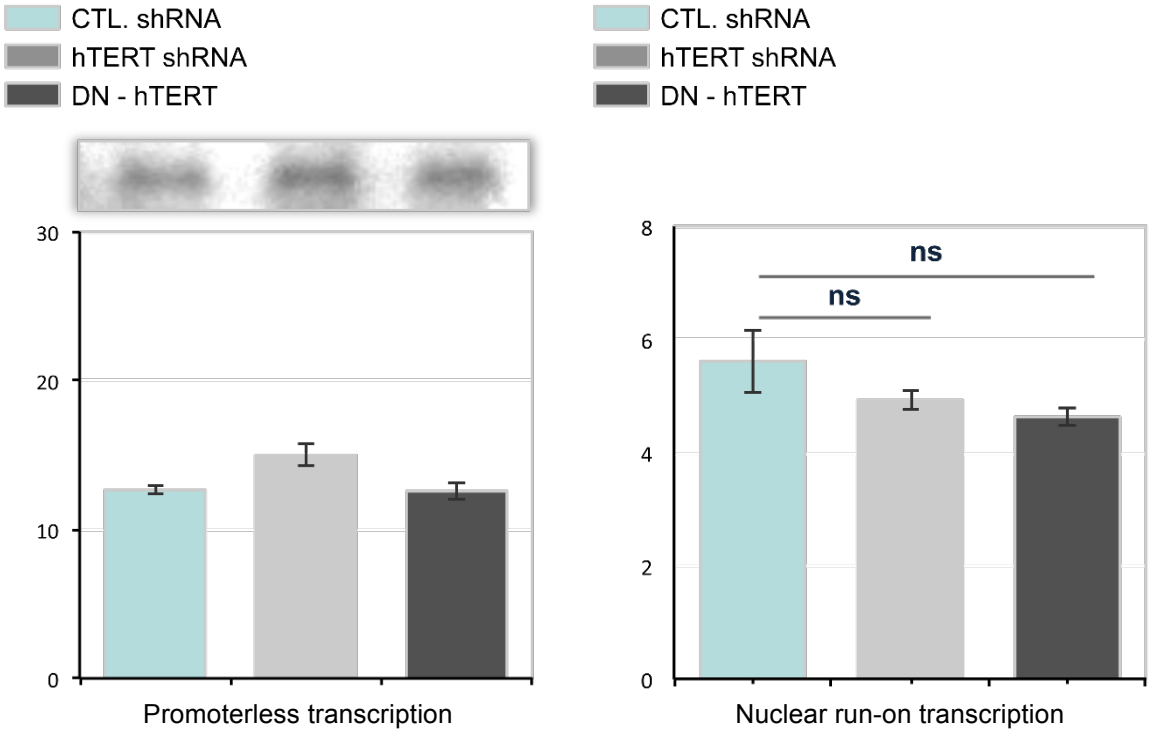


Figure 32 (left): *The transcription elongation capacity of Pol I was not affected by telomerase repression. As demonstrated by promoterless in vitro transcription experiment.* ***Figure 32 (right):*** Nuclear run-on experiments were performed with isolated nuclei of the indicated cells and elongated rRNA was quantified after cDNA synthesis by qPCR with primers against gene-internal sequences of the rDNA

To further investigate the function of TERT as transcription factor of RNA polymerase I, HA-tagged hTERT fusion protein was overexpressed in two different mouse cell types and purified by isolation with monoclonal anti-HA agarose beads (SIGMA). After elution under gentle conditions with HA peptide the purified proteins were subjected to western blot analysis and were found to migrate with molecular weight of approximately 127kDa, the expected size of hTERT – HA recombinant protein (Figure 33). The purified proteins were tested in a defined RNA polymerase I transcriptional in vitro system using nuclear extracts from telomerase-shRNA knockdown HCT116 cells. We show that the rRNA synthesis in the mentioned nuclear extracts was only successful reconstituted when hTERT was expressed in TERC competent cells derived from telomerase-positive mouse liver tumour *[Katz, SF et al., 2012*[114]*]*, whereas expression of hTERT in cells from mouse liver tumour

lacking the telomerase RNA component (TERC -/-) failed to transcribe the rDNA template (Figure 34). These results clearly demonstrate that the telomerase holoenzyme (TERT/TERC) activates RNA polymerase I transcription rather than the catalytic subunit (TERT) alone.

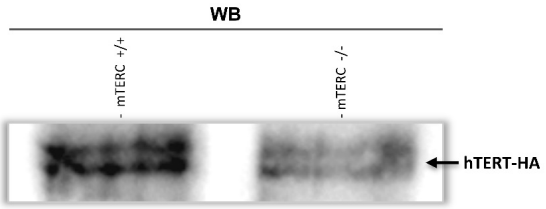


Figure 33: *HA-TERT was expressed in TERC competent mouse liver cells or those lacking the telomerase RNA component TERC, purified and used for in vitro transcription.*

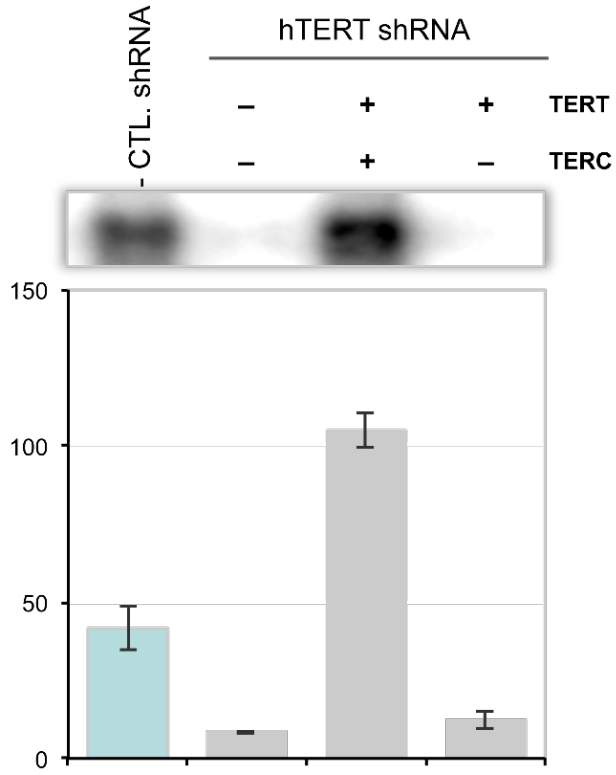


Figure 34: *Purified TERT from TERC -/- cells does not stimulated in vitro transcription by Pol I.*

3.4.4 Telomerase stimulates initiation complex formation at the rDNA promoter.

We then tested the telomerase ability to stimulating the formation of initiation complexes. HA-hTERT fusion protein was expressed and purified in HEK293 cells and used for function applications in reconstitution experiments in vitro.

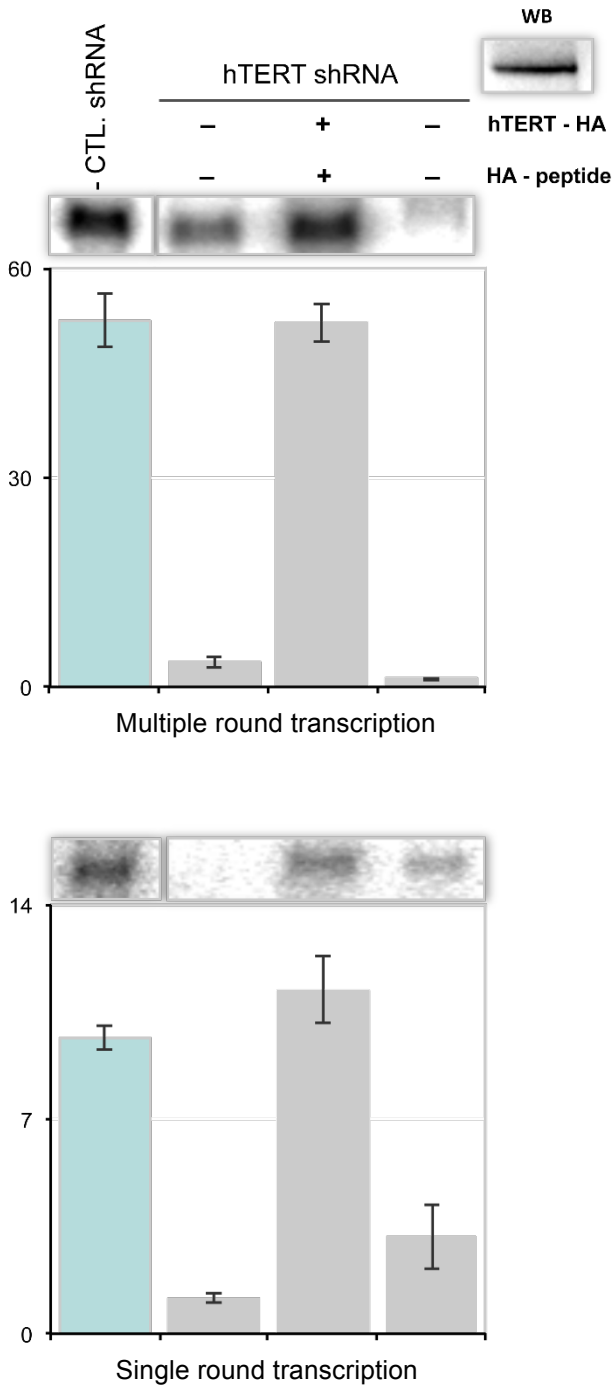


Figure 35: *Reconstruction of RNA polymerase I in vitro transcription with purified telomerase (hTERT- HA Western blotting with anti-HA antibody) demonstrated that telomerase stimulates initiation complex formation at the rDNA promoter*

Pre-incubation of recombinant hTERT with nuclear extract from HCT116 shRNA-treated cells and rDNA template was followed by either addition of only rNTPs to initiate multiple round transcriptions or by adding together rNTPs and Sarkosyl limiting the transcription reaction to a single round. The strength of the resulting signal is proportional to the amount of newly formed initiation complexes as shown

by the data quantification performed through phosphorimager analysis. Thus, we observed a strong stimulation of RNA polymerase I transcription by the recombinant TERT, either in the absence or presence of Sarkosyl (Figure 35). Our data indicated that telomerase stimulates initiation complex formation at the rDNA promoter.

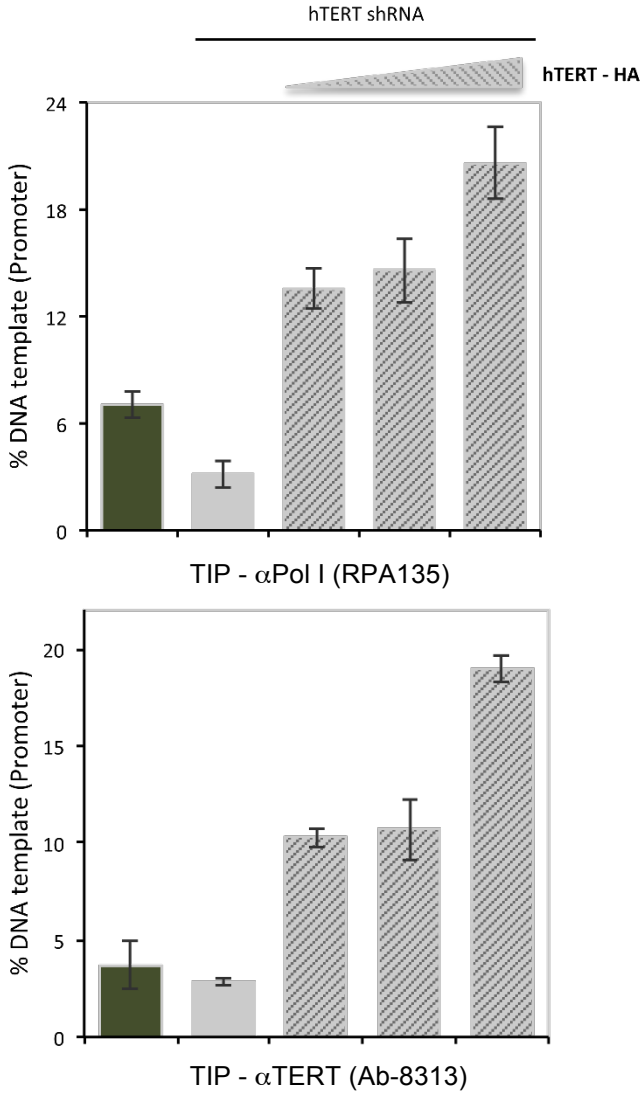


Figure 36: *Template-immunoprecipitation experiment showing that titration of purified telomerase to nuclear extracts of TERT-repressed cells enhances binding of Pol I to the rDNA promoter.*

Moreover, template immunoprecipitation (TIPs) experiments displayed an enhanced formation of RNA polymerase I complexes at the rDNA promoter after addition of purified recombinant telomerase, in a concentration-dependent manner. Combining in vitro transcription and immunoprecipitation protocols this newly developed method was performed by incubating increasing amounts of purified TERT-HA fusion protein with rDNA template and TERT-shRNA-derived nuclear extracts. The

proteins of interest were immunoprecipitated with specific antibodies and the crosslinked rDNA template was isolated and quantified. The TIP experiment revealed that more RNA polymerase I complexes are formed at the rDNA promoter in the presence of telomerase (Figure 36). The qPCR analysis reveals that telomerase titration increased the number of active initiation complexes at the rDNA promoter.

3.5 Inhibition of telomerase or Pol I retards proliferation.

Cell growth is a prerequisite for proliferating cells, which is characterized by an increased demand of protein synthesis, therefore the ribosomal biogenesis is a limiting factor for both growth and proliferation processes. However, telomerase is not expressed in most normal human somatic cells, but a high telomerase activity is found in 80 – 90% of human cancers, it seem to be essential for the continued growth and proliferation of malignant cells. In addition, TERT may act as hyperproliferative and growth-promoting factor *[Shi, S et al., 2002[115]; Forsythe, HL et al., 2002[116]; Gorbunova, V & Seluanov, A 2003[117]]*.

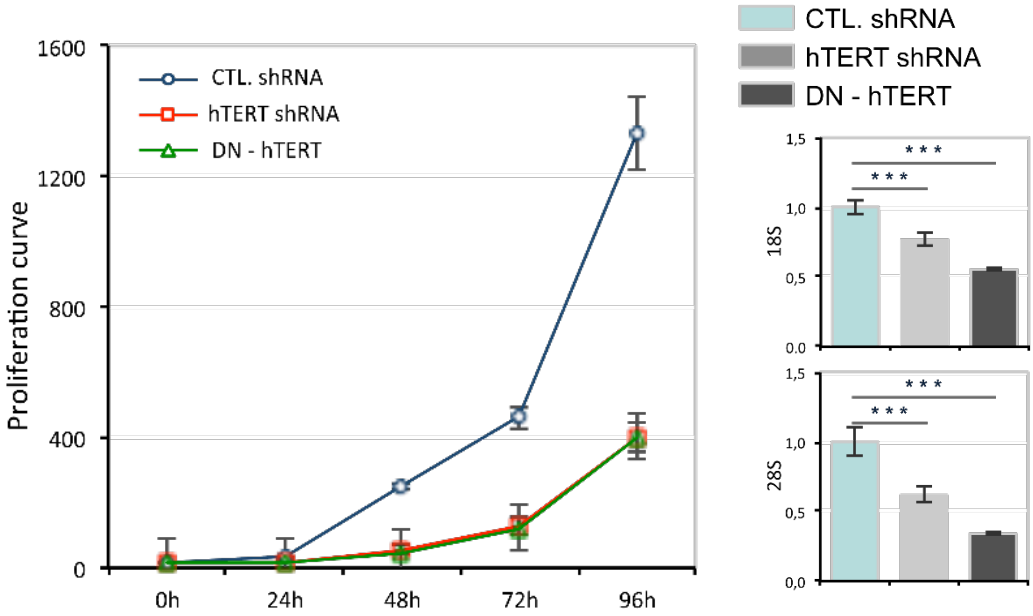


Figure 37: *Telomerase inhibition retards proliferation and ribosomal biogenesis. HCT116 cells stably transfected with the indicated shRNA constructs were monitored for proliferation (upper graphic) and by qPCR for their mature rDNA content (18S and 28S) lower panel*

Here we have shown that telomerase inhibition by expression of a mutant catalytic subunit (DN-hTERT) or by knockdown with specific short harping RNA, significantly

impaired Pol I transcription in HCT116 cells. We then addressed the question whether disruption of Pol I transcription by knockdown of TERT may affect cellular proliferation in the stably transfected carcinoma cells. The figure 37 (left) shows that telomerase knockdown is followed by a strong inhibition of cellular proliferation. Furthermore, the significant reduction in Pol I transcription due telomerase inhibition, results in a decrease in mature 18S and 28S ribosomal RNA subunits (Figure 37 - right). These results indicate that the level of transcription by Pol I may be a critical factor affecting cell cycle dynamics.

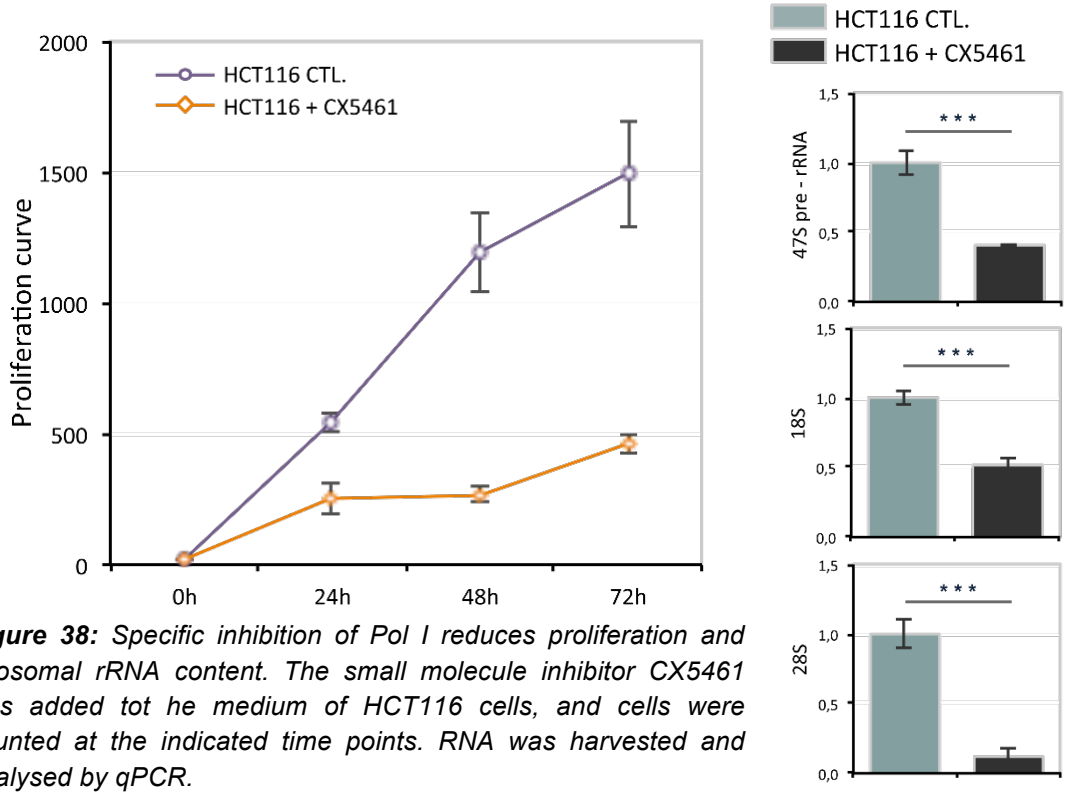


Figure 38: *Specific inhibition of Pol I reduces proliferation and ribosomal rRNA content. The small molecule inhibitor CX5461 was added tot he medium of HCT116 cells, and cells were counted at the indicated time points. RNA was harvested and analysed by qPCR.*

Because cell growth is tightly related to cell proliferation, an adequate ribosome biogenesis appears to be the regulatory key controlling cell cycle progression *[Volarevic, S et al., 2000*[118]*; Derenzini, M et al., 2005*[119]*]*. In the present study we evaluated the effect of Pol I transcription inhibition in the rRNA synthesis, the proliferation dynamics and ribosomal content. For this purpose HCT116 cells were subjected to specific RNA polymerase I inhibition by the small molecule inhibitor CX5461, which may act by disrupting the binding of the transcription factor SL1 to rDNA promoter, and thereby inhibiting the initiation of rRNA synthesis by the Pol I transcription complex *[Drygin, D et al., 2009* [120]*]*. As demonstrated in the Figure 38 (left), the growth kinetic of HCT116 cells treated with the specific Pol I inhibitor

clearly revealed a severely reduced proliferation rate. In addition, loss of the 47S pre-rRNA transcription is accompanied by a significant reduction in mature 18S and 28S rRNA (Figure 38 – right).

Deregulation of protein synthesis has been implicated in cell cancer and senescent decline. Metabolic labelling assays are often used to follow the protein biosynthesis. We assessed the potential of mRNA translation in HCT116 cells followed telomerase or Pol I inhibition by metabolic labelling experiments monitoring the incorporation of radioactive amino acids (35S-methionine/cysteine) into nascent polypeptides. The inactivation of TERT or Pol I did not influence the protein translation (Figure 39). These results indicate that protein biosynthesis is critically regulated even when ribosomal content is decreased.

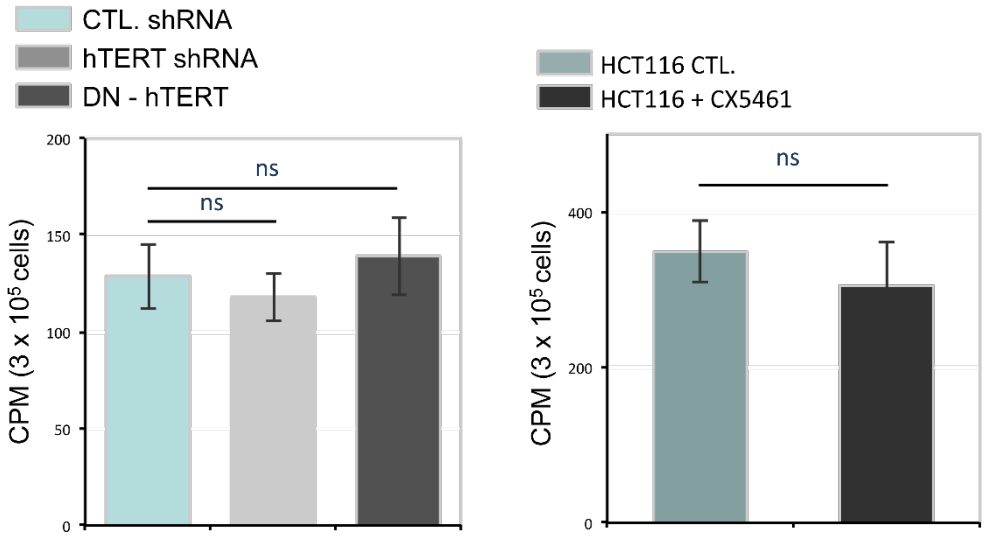
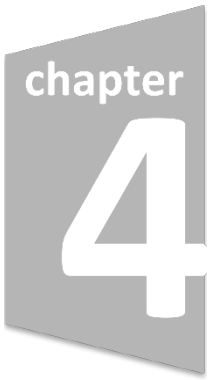


Figure 39: *HCT116 cells stably transfected with the indicated constructs (left) or Pol I inhibited by CX5461 (right), were starved for methionine/cystheine and subsequent labeled with radioactive amino acids for 3h. Synthesized proteins were TCA precipitated, spotted on glass filters and quantified by scintillation counting.*



4. DISCUSSION

4.1 Telomerase beyond telomeres: New roles for an enzyme

The first described and best-analysed function of the telomerase enzyme is its capacity to elongate and maintain the functional telomeres *[Greider, C & Blackburn, L 1985[121]]*. This telomere-interacting function is known as the canonical function of telomerase and is crucial for cell proliferation and genomic stability. While in unicellular organisms the enzyme is constitutively active, in multicellular organism it is differentially expressed. In many adult tissues and most somatic cells in human, telomerase is down regulated during early embryonic development leading to telomere shortening with cell divisions. When the telomere length is critically reduced to lower than 3 kbp, massive genomic instability is triggered; the cells stop dividing and eventually die *[Saretzki, G et al., 1999[69]; Maser, RS & DePinho, RA 2002[70]]*. Only germ line cells, stem cells and highly proliferative tissues, such as activated B and T – cells show high telomerase activity maintaining long and stable telomere length, but they still undergo slowly telomere shortening. In contrast to normal somatic cells, the enzyme is re-activated during tumorigenesis and a vast majority of tumour cells exhibit high telomerase activity; therefore telomerase is often associated and tightly correlated with cellular immortality and the infinite proliferation capacity of tumour cells *[Wright, WE et al., 1996[66]; Martin-Rivera L et al., 1998[67]; Greenberg, RA & Rudolph, KL 2005[68]]*. This important role is based on the (canonical) function of the enzyme that is the maintenance and elongation of telomeres. However, it is emerging recently that telomerase has

other functions, which seem to be independent of its classical, canonical role at telomeres *[Parkinson, EK et al., 2008[75]]*. These new functions have been described inside and outside the nucleus. Many of those telomere-independent functions seem to require both the telomerase catalytic activity and the RNA component TERC, other non-canonical functions can be independent of catalytic activity or the association of the two main telomerase components *[Bednarek, A et al., 1995[76]; Chadeneau, C et al., 1995[77]; Broccoli, D et al., 1996[78]]*.

Recent data support the emerging idea that TERT promotes tumorigenesis independently of its telomere elongating function *[Stewart, SA et al., 2002[82]]*. In addition, several groups have demonstrated that telomerase expression is linked to increased cell survival. For example, the transformation of murine cells correlates with increased mTERT expression, and transgenic mice expressing high levels of mTERT are more susceptible to tumours, suggesting that increased telomerase activity in cells with already-long telomeres increases transformation *in vivo*, indicating that Telomerase expression is also implicated in a number of other functions that would appear to be unrelated to telomere elongation *[Broccoli, D et al., 1996[78]; Stewart, SA et al., 2002[82]; Gonzalez-Suarez, E et al., 2002[122]; Artandi, S et al., 2002[123]]*. More recent studies demonstrated an influence of telomerase on chromatin structure, gene expression *[Smith, LL et al., 2003[83]; Bagheri, S et al., 2006[124]; Park, JI et al., 2009[94]]*, sensitivity to DNA damage, and susceptibility to apoptosis *[Del Bufalo, D et al., 2005[86]]*. In addition, despite its predominant localisation to the Cajal bodies *[Zhu, Y et al., 2004[89]; Zhong, F et al., 2011[6]]*, distinct telomerase functions have been described in the cytoplasm, nucleolus, nucleus and mitochondria *[Tomlinson, RL et al., 2006[110]; Chiodi, I & Mondello, C 2012[107]; Lee, JH et al., 2014[108]]*. Notably, a recent report identified a nucleolar localization signal of hTERT, which appears to be unrelated to its telomere elongating function *[Lin, J et al., 2008[125]]*.

4.2 Binding capacity of telomerase to ribosomal DNA repeats

In recent studies interaction of hTERT with the chromatin status in normal or hTERT overexpressing cells have been reported *[Sharma, GG et al., 2003[126]; Masutomi, K et al., 2005[88]]*. This association with nuclear chromatin can have

implications at multiple levels and cellular functions such as gene expression and DNA damage responses. The authors suggest hTERT as a regulator of the DNA damage response pathway through its action on chromatin structure that is different from its known role on telomeres. In addition overexpression of TERT induced specific changes in Wnt and Myc signalling pathways. The authors discovered that TERT acts as a transcriptional co-factor for the β-catenin transcriptional complex by interacting with the chromatin remodelling protein BRG1 *[Park, JI et al., 2009* [94]*]* and changing the chromatin conformation. This is the first study that reveals a molecular mechanism for transcriptional modulation of gene expression by TERT in mammalian cells.

Cellular trafficking of telomerase and its components between different subcellular compartments seems to be a natural and highly regulated process. Cytoplasmic localisation has been confirmed for TERT as well as for the RNA component *[Chang, LY et al., 1999*[127]*; Teixeira, MT et al., 2002*[128]*; Santos, JH et al., 2004*[129]*]*. Moreover, localisation of telomerase to the nucleolus has been described in humans *[Wong, JM et al., 2002*[130]*]*. Since the new TERT functions as modulator of gene expression and as transcriptional co-regulator of Wnt signalling implay gene regulatory functions of telomerase, and given that nucleoli are the sites of rDNA transcription it was tempting to speculate that telomerase directly binds to rRNA genes and displays a similar function in regulating the RNA polymerase transcription in nucleolus.

Different experimental approaches during the course of this thesis work have identified binding of TERT to rDNA-associated chromatin specially harbouring the promoter and coding regions. The data from subsequently chromatin immunoprecipitation experiments revealed that an association of endogenous or HA-tagged hTERT to the rDNA repeat was detected by using different specific telomerase antibodies in chromatin preparations from HEK293 cells either untransfected or transiently overexpressing HA-tagged hTERT.

Despite rDNA transcription is limiting for cell growth, less than 50% of the rRNA genes are transcriptionally active. The proportion of active and silent rRNA genes can be identified by their different DNA methylation status. Whereas transcribed

rDNA is unmethylated, silent rDNA harbours methylated cytosine residues at CpG sites *[Santoro, R et al., 2005[131]]*. The association of telomerase with active or silent rRNA genes was monitored by methylation analysis of rDNA that was precipitated in ChIP assays followed by subsequent PCR amplification of the rDNA promoter region. Our data revealed the targeting of hTERT preferentially bind to unmethylated, active rRNA genes. The specificity of TERT – rDNA interaction was also demonstrated by ChIP assays, showing that TERT is able to yield DNA region containing the TCF binding element 2 (TBE2) of the c-myc promoter, whereas the 3´untranslated region was not bound by TERT.

The telomerase RNA component seems to bind to rDNA together with the catalytic reverse transcriptase subunit and ChIPs performed using liver carcinoma cells established from mTERC -/- mice indicated a lack of binding of telomerase at rDNA, suggesting that the telomerase holoenzyme would bind to rDNA rather than the catalytic subunit alone. We then compared the level of TERT-rDNA association under two physiological conditions in cultivated cell lines and tissues. We found that binding of telomerase to the rDNA is higher in tumour and transformed cells than in untransformed or resting cells. Our results are also consistent with recent findings on the binding of TERT to extratelomeric motifs, suggesting a novel function of telomerase as a transcriptional co-regulator of Wnt or NF-κB signalling *[Park, JI et al., 2009[94]; Ghosh, A et al., 2012[106]]*. TERT binding to the TCF3 sites was also confirmed in our study, as reported by Park and co-workers in HeLa cells and proliferating intestinal epithelium. Thus, the binding dynamics of TERT to the TCF sites may also be influenced by oncogenes or growth signals.

4.2.1 TERT binds to rDNA in regenerating hepatocytes

Telomerase in liver is mainly studied due to its association with hepatocellular carcinoma as a cancer marker and treatment target. Although there have been many reports on the relationship between activation of telomerase and carcinogenesis, the role of telomerase in normal cellular growth is still unclear. TERT has been investigated in studies on liver regeneration in correlation with insulin signalling. A recent study has shown that in insulin-deficient rats after hepatectomy, *rTert* mRNA level and telomerase activity was lower than in normal

rats. Furthermore, liver restitution mass was lower in insulin-deficient rats, this may suggest a relation between insulin signalling and telomerase in this organ *[Tseng, JH et al., 2009[132]]*. It suggest that RNA polymerase I transcription might be impaired under these conditions. It is interesting that mice with a hypomorphic dyskeratosis congenita 1 (DKC1) mutant protein show deregulated ribosome function *[Ruggero, D et al., 2003[133]]*. DKC1, which is necessary for proper rRNA processing, is part of the telomerase complex, and a subset of dyskeratosis congenita patients have mutations in TERT or TERC, the core components of telomerase. Thus, extratelomeric functions of telomerase may contribute to the pathology of dyskeratosis congenita.

In our study, we analysed the relationship between the rDNA occupancy capacity of TERT and upregulation of Pol I activity during the liver regeneration process by using an in vivo mouse partial hepatectomy (PH) model. Interestingly, we observed a non-oncogenic stimulation of telomerase recruitment to rDNA array when resting hepatocytes enter a proliferative mode. This observation may reflect a physiological response to an elevated need for ribosomes, indicating a physiological role for telomerase in gene regulation *in vivo*. Although a final conclusion regarding the extra-telomeric functions of telomerase is still elusive and unresolved, our data support the notion that the nucleolar-localised telomerase stimulates rRNA transcription.

4.3 Telomerase stimulates ribosomal transcription

Although elongation of telomeres is thought to be the prime function of reactivated telomerase in cancers, this activity alone does not account for all of the properties that telomerase reactivation attributes to human cancer cells. Functional telomerase was found regulating NF-κB signalling *[Ghosh, A et al. 2012[106]]*. The authors observed that the telomerase holoenzyme is important to directly regulate NF-κB-dependent gene expression by binding to the NF-κB p65 subunit and recruitment to a subset of NF-κB promoters. However, recent reports have shown that TERT can regulate the NF-κB transcription independently of its enzymatic activity *[Ding, D et al., 2013[134]]*. Thus, it appears as telomerase might play an important role as a central regulator of proliferation and cell cycle progression, an

idea that specially fits with its role in the regulation of rDNA transcription described in this study. To further understand the implication of TERT in Pol I transcription transfection of a Pol I-specific reporter plasmid was performed in transformed human fibroblasts with parallel overexpression of different amounts of hTERT. Curiously, increased over-expression of hTERT leads to a dose-dependent increase of rDNA transcription, indicating an oncogene/growth signal-dependent stimulation of Pol I transcription by TERT. A similar stimulatory effect of TERT on genes of the Wnt pathway was observed by Park and collaborators in tumour cells; thus, they do not exclude the possibility that the described effects of TERT are also dependent on a tumour environment. Conversely, we observed a decreased rDNA transcription after telomerase inhibition by Imetelstat, shRNA-mediated depletion of hTERT or ectopic expression of a dominant-negative TERT mutant, which carries a point mutation inactivating the telomere elongation capacity *[Hahn, WC et al., 1999[81]]*, possibly by the export and degradation of the endogenous telomerase *[Nguyen, BN et al., 2009[113]]*. Taken together, these results demonstrate that the enzymatic activity of telomerase is required for stimulating rDNA transcription.

The activity of rRNA gene transcription in a given cell is the major determinant of cell proliferation and cell growth, and transcription is tightly regulated to match ribosome amounts with the physiological needs of the cell. The data achieved during this doctoral thesis provided the first mechanistic insight into the action of telomerase regulating rRNA gene transcriptional activity. Template immunoprecipitation experiments showed that TERT remains bound to the rDNA template during transcription and stimulates initiation complex formation at the promoter, implying that telomerase is able to recruit or stabilise the binding of Pol I initiation factors or the polymerase at the promoter. Because this feature depends on growth signals or proliferation, telomerase stabilises Pol I transcription initiation when there is a high demand for ribosomal transcription.

Since telomerase can exert its action via telomere-dependent and telomere-independent mechanisms, not all these functions or their mechanisms are well understood and might co-exist together within a cell. This sometime confuses and creates controversies, in particular to functions of TERT that are exerted within the

same compartment as telomere maintenance: the nucleus. It was possible to dissect this concern with the help of two different TERT constructs, which were transiently overexpressed, purified and tested by *in vitro* transcription experiments. We used a HA-TERT construct and a C-terminal tagged TERT-HA mutant, which is catalytically active but cannot elongate telomeres *[Counter, CM et al., 1998[80]]*. Both versions of TERT were able to stimulate rDNA transcription. Together with the observation that TERC is also required for the telomerase binding to rDNA promoter as well as for their stimulation of the Pol I transcription, these results indicate that an enzymatic activity of telomerase other than the canonical activity may be required for the positive effect on rDNA transcription. This novel telomere-independent function here described seems to require catalytic activity and the involvement of the RNA component TERC.

Telomerase expression is often associated with enhanced cell survival under conditions of cellular stress. In contrast, telomerase knockdown in various cancer cells lines resulted in changes of gene expression. Human cancer cells show a rapid and defined growth-inhibitory response to telomerase inhibition independent of bulk telomere shortening *[Li, S et al., 2005[135]]*. In addition, almost all processes leading to mature ribosomes are organised within one specialised cellular compartment, the nucleolus. Pol I transcription, rRNA processing and ribosome assembly are therefore in close proximity *[Schwarzacher, HG & Mosgoeller, W 2000[136]]*. Before finally concluding the discussion on the regulatory mechanism of telomerase with respect to rRNA gene transcription, it is still important to briefly focus on the potential role of telomerase in ribosome biogenesis. One of the most interesting observations in our study is that inhibition of telomerase activity leads to repression of the ribosomal biogenesis and cellular proliferation. This analysis revealed that rRNA production was defective with decreased levels of newly matured rRNAs. It is tempting to speculate that the reduction in ribosomal biogenesis due to altered cell cycle dynamics *[Volarevic, S et al., 2000[118]; Derenzini, M et al., 2005[119]]*, contributes to the growth inhibition observed after telomerase depletion in cancer cells.

The experimental results of this thesis work have clearly shown how non-canonical features of telomerase may direct Pol I transcription in oncogenic and regenerative

hyperproliferation. Taken together, our data indicate the existence of a novel mechanism associated with telomerase enzymatic activity, which differs from previous findings describing other telomere length independent functions of TERT.



5. SUMMARY

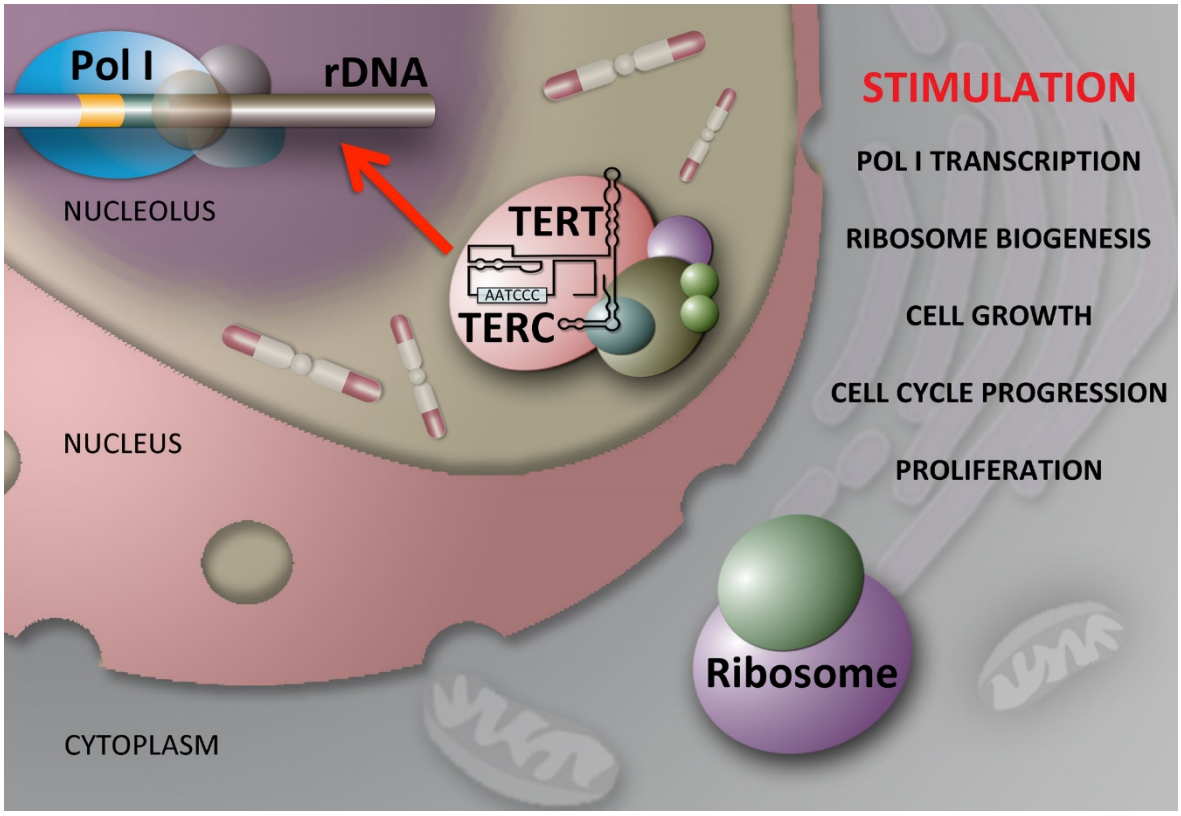
Telomerase is a ribonucleoprotein with reverse transcriptase activity, well known for protecting the ends of chromosomes (telomeres) during cell proliferation and for preventing senescence. Telomerase contains the catalytic subunit TERT and an RNA component TERC including the template region for telomere synthesis. Both TERT and TERC elements are required and sufficient for its canonical function, which is the maintenance and elongation of telomeres (catalytic activity). In addition to performing its canonical function, telomerase has been shown to participate in several cellular processes beyond the telomeric level. The results obtained in this study have provided solid evidence about the existence of a novel non-canonical mechanism associated with telomerase enzymatic activity. The here-identified new function of telomerase differs from previous findings, describing telomere length and telomerase-independent functions of TERT.

The first part of our study was focussed on demonstrate that telomerase binds to the rDNA locus and stimulates the transcription of ribosomal gene. In this concern our work was in line with recent findings reporting association of TERT to extra-telomeric motives and making evident novel a feature of telomerase as a transcriptional co-regulator of Wnt - or NF-κB signalling. Our experimental outcomes indicated that the telomerase holoenzyme is associated with different rDNA domains. Methylation sensitive restriction analysis of chromatin immunoprecipitated DNA revealed that TERT is associated with the active fraction

of the rRNA genes. ChIP re-ChIP experiments showed that telomerase catalytic subunit (TERT) and RNA polymerase I occupy identical rDNA molecules. The RNA component of telomerase (hTERC) was also found binding to rDNA chromatin together with hTERT and ChIPs performed using liver carcinoma cells established from mTERTC -/- mice indicated a lack of binding of telomerase at rDNA, suggesting that the telomerase holoenzyme would bind to rDNA rather than the catalytic subunit alone. We also showed that the level of TERT associated with the rDNA promoter and rDNA internal sequences, is higher in tumour and transformed cells than in resting control cells both in culture and liver tissue. In addition, ChIP /dot blot revealed that telomere binding of telomerase can only be detected in liver carcinoma (i.e. proliferating hepatocytes) but not in normal non-proliferating hepatocytes. These results exposed solid evidence that telomerase is specific recruited to telomeric regions as well as to rDNA regions under hyperproliferative conditions. Furthermore, our data have also proved non-oncogenic rDNA-binding features of telomerase when resting hepatocytes enter a proliferative mode. This contribution to the transcriptional process by Pol I is growth-signal dependent and might be a physiological response to an elevated need for ribosomes.

The second part of this thesis investigated the role of telomerase in ribosomal DNA transcription. The implication of TERT in Pol I transcription was demonstrated by reporter assays. Here we showed that increased over-expression of telomerase (hTERT) led to progressive increase of rDNA transcription in Ras transformed cells. Moreover, in vitro experiments with cells extracts from telomerase-compromised cells and using partially purified hTERT expressed in HEK 293 or TERC deficient mice cells suggested that telomerase holoenzyme stimulates the Pol I transcription. As observed by in vitro transcription experiments, a purified C-terminally tagged TERT-HA construct, which is not able to elongate telomeres could rescue the impaired transcription activity of TERT depleted cells revealing a telomere length independent function of telomerase. We also provided the first mechanistical insight into the action of telomerase as a transcription factor: template immunoprecipitation assays showed that TERT remains bound to the template during transcription and stimulates initiation complex formation at the rDNA promoter, implying that telomerase is able to recruit or stabilize the binding

of Pol I initiation factors or the polymerase at the promoter when there is a high demand for ribosomal transcription.



Conversely, we have shown a regulation of Pol I activity mediated telomerase inhibition and its effect on cellular proliferation in tumours. It is well known that cancer cells have a rapid and defined growth-inhibitory response to telomerase inhibition in a telomere-lengthening independent manner. This is in line with our observations that shRNA-mediated depletion of hTERT or ectopic expression of a dominant-negative TERT mutant impaired rDNA transcription and therefore inhibiting the ribosomal biogenesis. It is tempting to speculate that reduction in ribosomal biogenesis could contribute to the growth-inhibition observed after telomerase depletion in cancer cells. Furthermore, siRNA knockdown experiments against the telomerase RNA component (TERC) indicated that the reduction of TERC expression was accompanied by a significant reduced Pol I transcription.

In summary, here we have discussed about the data obtained during this thesis work providing a novel role of telomerase in the nucleolus that seem independent of its canonical function. The major focus of our study have been to evaluate the

mechanism underlying telomerase influence in the RNA polymerase I transcription. Finally, with this study we have proved that by modulating ribosomal biogenesis, telomerase influences the dynamics of cell growth, cell cycle progression and proliferation.



6. REFERENCES

1. Drygin, D., Rice, W. G. & Grummt, I. The RNA polymerase I transcription machinery: an emerging target for the treatment of cancer. *Annu. Rev. Pharmacol. Toxicol.* 50, 131–56 (2010).

2. Derenzini, M. *et al.* Nucleolar size indicates the rapidity of cell proliferation in cancer tissues. *J. Pathol.* 191, 181–6 (2000).

3. Maggi, L. B. & Weber, J. D. Nucleolar adaptation in human cancer. *Cancer Invest.* 23, 599–608 (2005).

4. Holt, S. E., Wright, W. E. & Shay, J. W. Regulation of telomerase activity in immortal cell lines. *Mol. Cell. Biol.* 16, 2932–9 (1996).

5. Greenberg, R. A., Allsopp, R. C., Chin, L., Morin, G. B. & Depinho, R. A. Expression of mouse telomerase reverse transcriptase during development , differentiation and proliferation. (1998).

6. Zhong, F. *et al.* Disruption of telomerase trafficking by TCAB1 mutation causes dyskeratosis congenita. *Genes Dev.* 25, 11–6 (2011).

7. Etheridge, K. T. *et al.* The nucleolar localization domain of the catalytic subunit of human telomerase. *J. Biol. Chem.* 277, 24764–70 (2002).

8. Wu, Y.-L. *et al.* Immunodetection of human telomerase reverse-transcriptase (hTERT) re-appraised: nucleolin and telomerase cross paths. *J. Cell Sci.* 119, 2797–806 (2006).

9. Grummt, I. Life on a planet of its own: regulation of RNA polymerase I transcription in the nucleolus. *Genes Dev.* 17, 1691–702 (2003).

10. Moss, T., Langlois, F., Gagnon-Kugler, T. & Stefanovsky, V. A housekeeper with power of attorney: the rRNA genes in ribosome biogenesis. *Cell. Mol. Life Sci.* 64, 29–49 (2007).

11. Warner, J. R. The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* 24, 437–440 (1999).

12. Frank, J. The ribosome – a macromolecular machine par excellence. *Chem. Biol.* 7, R133–R141 (2000).

13. Sommerville, J. Nucleolar structure and ribosome biogenesis. *Trends Biochem. Sci.* 11, 438–442 (1986).

14. Verschoor, A., Warner, J. R., Srivastava, S., Grassucci, R. A. & Frank, J. Three-dimensional structure of the yeast ribosome. *Nucleic Acids Res.* 26, 655–61 (1998).

15. Moss, T. & Stefanovsky, V. Y. Promotion and regulation of ribosomal transcription in eukaryotes by RNA polymerase I. *Prog. Nucleic Acid Res. Mol. Biol.* 50, 25–66 (1995).

16. Warner, J. R. Minireview: Nascent Ribosomes. 107, 133–136 (2001).

17. Laferté, A., Favry, E., Sentenac, A., Riva, M. & Carles, C. The transcriptional activity of RNA polymerase I is a key determinant for the level of all ribosome components. 3, 2030–2040 (2006).

18. Chédin, S., Laferté, A., Riva, M. & Carles, C. Is Ribosome Synthesis Controlled by Pol I Transcription ? 11–15 (2007).

19. Grozdanov, P., Georgiev, O. & Karagyozov, L. Complete sequence of the 45-kb mouse ribosomal DNA repeat: analysis of the intergenic spacer☆. *Genomics* 82, 637–643 (2003).

20. James E Sylvester, Iris L Gonzalez, E. B. M. Structure and organization of vertebrate ribosomal DNA. (2004).

21. Haltiner, M. M., Smale, S. T. & Tjian, R. Two distinct promoter elements in the human rRNA gene identified by linker scanning mutagenesis. 6, 227–235 (1986).

22. Learned, R. M., Learned, T. K., Haltiner, M. M. & Tjian, R. T. Human rRNA transcription is modulated by the coordinate binding of two factors to an upstream control element. *Cell* 45, 847–857 (1986).

23. Grummt, I., Maier, U., Ohrlein, A., Hassouna, N. & Bachellerie, J. P. Transcription of mouse rDNA terminates downstream of the 3’ end of 28S RNA and involves interaction of factors with repeated sequences in the 3' spacer. *Cell* 43, 801–10 (1985).

24. Grummt I, Kuhn A, Bartsch I, R. H. A transcription terminator located upstream of the mouse rDkJA initiation site affects rRNA synthesis. 47, 901–911 (1986).

25. Henderson, S. & Sollner-Webb, B. A transcriptional terminator is a novel element of the promoter of the mouse ribosomal RNA gene. *Cell* 47, 891–900 (1986).

26. McStay, B. & Reeder, R. H. A termination site for Xenopus RNA polymerase I also acts as an element of an adjacent promoter. *Cell* 47, 913–20 (1986).

27. Gerbi, S. A. The evolution of eukaryotic ribosomal DNA. *Biosystems* 19, 247–258 (1986).

28. Xie, WQ and Rothblum, L. Mol Cell Biol. *Mol Cell Biol. 1992 Mar;12(3)1266-75.* (1992). doi:10.1007/SpringerReference_93033

29. Hannan, RD., Luyken, J., Rothblum, L. Regulation of rDNA transcription factors during cardiomyocyte hypertrophy induced by adrenergic agents.pdf. (1995).

30. Kuhn, C.-D. *et al.* Functional architecture of RNA polymerase I. *Cell* 131, 1260–72 (2007).

31. Werner, M., Thuriaux, P. & Soutourina, J. Structure-function analysis of RNA polymerases I and III. *Curr. Opin. Struct. Biol.* 19, 740–5 (2009).

32. Goodfellow, S. J. & Zomerdijk, J. C. B. M. Epigenetics: Development and Disease. 61, (2013).

33. O’Sullivan, A. C., Sullivan, G. J. & McStay, B. UBF binding in vivo is not restricted to regulatory sequences within the vertebrate ribosomal DNA repeat. *Mol. Cell. Biol.* 22, 657–68 (2002).

34. Chen, D., Belmont, A. S. & Huang, S. Upstream binding factor association induces large- scale chromatin decondensation. 101, (2004).

35. Mais, C., Wright, J. E., Prieto, J., Raggett, S. L. & Mcstay, B. UBF-binding site arrays form pseudo-NORs and sequester the RNA polymerase I transcription machinery. 50–64 (2005). doi:10.1101/gad.310705.quences

36. Sanij, E. *et al.* UBF levels determine the number of active ribosomal RNA genes in mammals. *J. Cell Biol.* 183, 1259–74 (2008).

37. Beckmann, H., Chen, J.-L., O'Brien, T. & Tjian, R. Coactivator and Promoter-Selective Properties of RNA Polymerase I TAFs. *Science (80-.).* 270, 1506–1509 (1995).

38. Friedrich, J. K., Panov, K. I., Cabart, P., Russell, J. & Joost, C. B. M. TBP-TAF complex SL1 directs RNA polymerase I pre-initiation complex formation and stabilizes upstream binding factor at the rDNA promoter ||. 280, 1–18 (2013).

39. Peyroche, Â. *et al.* The recruitment of RNA polymerase I on rDNA is mediated by the interaction of the A43 subunit with. 19, 5473–5482 (2000).

40. Panov, K. I., Friedrich, J. K., Russell, J. & Zomerdijk, J. C. B. M. UBF activates RNA polymerase I transcription by stimulating promoter escape. *EMBO J.* 25, 3310–22 (2006).

41. Panov, K. I. *et al.* RNA polymerase I-specific subunit CAST/hPAF49 has a role in the activation of transcription by upstream binding factor. *Mol. Cell. Biol.* 26, 5436–48 (2006).

42. Russell, J. & Zomerdijk, J. C. B. M. RNA-polymerase-I-directed rDNA transcription, life and works. *Trends Biochem. Sci.* 30, 87–96 (2005).

43. Stefanovsky, V., Langlois, F., Gagnon-Kugler, T., Rothblum, L. I. & Moss, T. Growth factor signaling regulates elongation of RNA polymerase I transcription in mammals via UBF phosphorylation and r-chromatin remodeling. *Mol. Cell* 21, 629–39 (2006).

44. Denissov, S. *et al.* A model for the topology of active ribosomal RNA genes. *EMBO Rep.* 12, 231–7 (2011).

45. Conconi, A., Bespalov, V. a & Smerdon, M. J. Transcription-coupled repair in RNA polymerase I-transcribed genes of yeast. *Proc. Natl. Acad. Sci. U. S. A.* 99, 649–54 (2002).

46. Bradsher, J. *et al.* CSB is a component of RNA pol I transcription. *Mol. Cell* 10, 819–29 (2002).

47. Iben, S. *et al.* TFIIH plays an essential role in RNA polymerase I transcription. *Cell* 109, 297–306 (2002).

48. Lebedev, A., Scharffetter-Kochanek, K. & Iben, S. Truncated Cockayne syndrome B protein represses elongation by RNA polymerase I. *J. Mol. Biol.* 382, 266–74 (2008).

49. Assfalg, R. *et al.* TFIIH is an elongation factor of RNA polymerase I. *Nucleic Acids Res.* 40, 650–659 (2012).

50. Haaf, T., Hayman, D. L. & Schmid, M. Quantitative determination of rDNA transcription units in vertebrate cells. *Exp. Cell Res.* 193, 78–86 (1991).

51. Grummt, I. & Pikaard, C. S. Epigenetic silencing of RNA polymerase I transcription. *Nat. Rev. Mol. Cell Biol.* 4, 641–9 (2003).

52. McStay, B. Nucleolar dominance: a model for rRNA gene silencing. *Genes Dev.* 20, 1207–14 (2006).

53. Conconi, A., Widmer, R. M., Koller, T. & Sogo, J. Two different chromatin structures coexist in ribosomal RNA genes throughout the cell cycle. *Cell* 57, 753–761 (1989).

54. Saucedo, L. J. & Edgar, B. A. Why size matters: altering cell size. *Curr. Opin. Genet. Dev.* 12, 565–71 (2002).

55. Derenzini, M. *et al.* Nucleolar Function and Size in. 152, 1291–1297 (1998).

56. Blasco, M. a. Telomeres and human disease: ageing, cancer and beyond. *Nat. Rev. Genet.* 6, 611–22 (2005).

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

58. Donate, L. E. & Blasco, M. A. Telomeres in cancer and ageing. 76–84 (2011). doi:10.1098/rstb.2010.0291

59. Counter, C. M. *et al.* Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. 1, (1992).

60. Wyatt, H. D. M., West, S. C. & Beattie, T. L. In TERT preting telomerase structure and function. 38, 5609–5622 (2010).

61. Dragony Fu and Kathleen Collins. Purification of human telomerase complexes identifies factors involved in telomerase biogenesis and telomere length regulation. 28, 773–785 (2007).

62. Venteicher, A. S., Meng, Z., Mason, P. J., Veenstra, T. D. & Artandi, S. E. Identification of ATPases pontin and reptin as telomerase components essential for holoenzyme assembly. *Cell* 132, 945–957 (2008).

63. Blasco, M. A., Rizen, M., Greider, C. W. & Hanahan, D. Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. *Nat. Genet.* 12, 200–4 (1996).

64. Cairney, C. J. & Keith, W. N. Telomerase redefined: integrated regulation of hTR and hTERT for telomere maintenance and telomerase activity. *Biochimie* 90, 13–23 (2008).

65. Wright, W. E., Piatyszek, M. A., Rainey, W. E., Byrd, W. & Shay, J. W. Telomerase activity in human germline and embryonic tissues and cells. *Dev. Genet.* 18, 173–9 (1996).

66. Martín-Rivera, L., Herrera, E., Albar, J. P. & Blasco, M. A. Expression of mouse telomerase catalytic subunit in embryos and adult tissues. *Proc. Natl. Acad. Sci. U. S. A.* 95, 10471–6 (1998).

67. Greenberg, R. A. & Rudolph, K. L. Telomere structural dynamics in genome integrity control and carcinogenesis. *Adv. Exp. Med. Biol.* 570, 311–41 (2005).

68. Saretzki, G., Sitte, N., Merkel, U., Wurm, R. E. & von Zglinicki, T. Telomere shortening triggers a p53-dependent cell cycle arrest via accumulation of G-rich single stranded DNA fragments. *Oncogene* 18, 5148–58 (1999).

69. Maser, R. S. & DePinho, R. A. Connecting chromosomes, crisis, and cancer. *Science* 297, 565–9 (2002).

70. Park, Y. P. *et al.* Human telomerase reverse transcriptase (hTERT): a target molecule for the treatment of cisplatin-resistant tumors. *Korean J. Lab. Med.* 28, 430–7 (2008).

71. Ruden, M. & Puri, N. Novel anticancer therapeutics targeting telomerase. *Cancer Treat. Rev.* 39, 444–56 (2013).

72. Vulliamy, T. *et al.* The RNA component of telomerase is mutated in autosomal dominant dyskeratosis congenita. *Nature* 413, 432–5 (2001).

73. Young, N. S. Bone marrow failure and the new telomere diseases: practice and research. *Hematology* 17 Suppl 1, S18–21 (2012).

74. Parkinson, E. K., Fitchett, C. & Cereser, B. Dissecting the non-canonical functions of telomerase. *Cytogenet. Genome Res.* 122, 273–80 (2008).

75. Bednarek, A; Budunova, I; Slaga, TJ; Aldaz, C. Increased telomerase activity in mouse skin premalignant progression. *Cancer Res.* Oct 15;55(, 4566–4569 (1995).

76. Chadeneau, C., Siegel, P., Harley, C. B., Muller, W. J. & Bacchetti, S. Telomerase activity in normal and malignant murine tissues. *Oncogene* 11, 893–8 (1995).

77. Broccoli, D., Godley, L. A., Donehower, L. A. & Varmus, H. E. Telomerase activation in mouse mammary tumors : lack of detectable telomere shortening and evidence for regulation of telomerase RNA with cell proliferation. 16, 3765–3772 (1996).

78. González-Suárez, E., Samper, E., Flores, J. M. & Blasco, M. a. Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis. *Nat. Genet.* 26, 114–7 (2000).

79. Counter, C. M. *et al.* Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase. *Oncogene* 16, 1217–22 (1998).

80. Hahn, W. C. *et al.* Inhibition of telomerase limits the growth of human cancer cells. *Nat. Med.* 5, 1164–70 (1999).

81. Stewart, S. a *et al.* Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *Proc. Natl. Acad. Sci. U. S. A.* 99, 12606–11 (2002).

82. Smith, L. L., Coller, H. A. & Roberts, J. M. Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. *Nat. Cell Biol.* 5, 474–9 (2003).

83. Geserick, C., Tejera, A., González-Suárez, E., Klatt, P. & Blasco, M. A. Expression of mTert in primary murine cells links the growth-promoting effects of telomerase to transforming growth factor-beta signaling. *Oncogene* 25, 4310–9 (2006).

84. Dudognon, C. *et al.* Death receptor signaling regulatory function for telomerase: hTERT abolishes TRAIL-induced apoptosis, independently of telomere maintenance. *Oncogene* 23, 7469–74 (2004).

85. Del Bufalo, D. *et al.* Involvement of hTERT in apoptosis induced by interference with Bcl-2 expression and function. *Cell Death Differ.* 12, 1429–38 (2005).

86. Mondello, C. *et al.* Oxidative stress response in telomerase-immortalized fibroblasts from a centenarian. *Ann. N. Y. Acad. Sci.* 1091, 94–101 (2006).

87. Masutomi, K. *et al.* The telomerase reverse transcriptase regulates chromatin state and DNA damage responses. *Proc. Natl. Acad. Sci. U. S. A.* 102, 8222–7 (2005).

88. Zhu, Y., Tomlinson, R. L., Lukowiak, A. a, Terns, R. M. & Terns, M. P. Telomerase RNA accumulates in Cajal bodies in human cancer cells. *Mol. Biol. Cell* 15, 81–90 (2004).

89. Kim, H. R., Christensen, R., Park, N. H., Sapp, P. & Kang, M. K. Elevated expression of hTERT is associated with dysplastic cell transformation during human oral carcinogenesis in situ. *Clin. Cancer Res.* 7, 3079–86 (2001).

90. Mukherjee, S., Firpo, E. J., Wang, Y. & Roberts, J. M. Separation of telomerase functions by reverse genetics. *Proc. Natl. Acad. Sci. U. S. A.* 108, E1363–71 (2011).

91. Sarin, K. Y. *et al.* Conditional telomerase induction causes proliferation of hair follicle stem cells. *Nature* 436, 1048–52 (2005).

92. Choi, J. *et al.* TERT promotes epithelial proliferation through transcriptional control of a Myc- and Wnt-related developmental program. *PLoS Genet.* 4, e10 (2008).

93. Park, J.-I. *et al.* Telomerase modulates Wnt signalling by association with target gene chromatin. *Nature* 460, 66–72 (2009).

94. Barker, N. *et al.* The chromatin remodelling factor Brg-1 interacts with b -catenin to promote target gene activation. 20, 4935–4943 (2001).

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

96. Zhu, H., Fu, W. & Mattson, M. P. The catalytic subunit of telomerase protects neurons against amyloid beta-peptide-induced apoptosis. *J. Neurochem.* 75, 117–24 (2000).

97. Maida, Y. *et al.* An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA. *Nature* 461, 230–5 (2009).

98. Walne, A. J. & Dokal, I. Dyskeratosis Congenita: a historical perspective. *Mech. Ageing Dev.* 129, 48–59

99. Vulliamy, T., Marrone, A., Dokal, I. & Mason, P. J. Association between aplastic anaemia and mutations in telomerase RNA. *Lancet* 359, 2168–70 (2002).

100. Kocak, H. *et al.* Hoyeraal-Hreidarsson syndrome caused by a germline mutation in the TEL patch of the telomere protein TPP1. *Genes Dev.* 28, 2090–102 (2014).

101. Armanios, M. Syndromes of telomere shortening. *Annu. Rev. Genomics Hum. Genet.* 10, 45–61 (2009).

102. Prescott, J., Wentzensen, I. M., Savage, S. A. & De Vivo, I. Epidemiologic evidence for a role of telomere dysfunction in cancer etiology. *Mutat. Res.* 730, 75–84 (2012).

103. Shammas, M. A. *et al.* Telomerase inhibition by siRNA causes senescence and apoptosis in Barrett ' s adenocarcinoma cells : mechanism and therapeutic potential. 14, 1–14 (2005).

104. Sambrook, J. & Russell, D. W. SDS-Polyacrylamide Gel Electrophoresis of Proteins. *CSH Protoc.* 2006, (2006).

105. Ghosh, A. *et al.* Telomerase directly regulates NF-κB-dependent transcription. *Nat. Cell Biol.* 14, 1270–81 (2012).

106. Chiodi, I. & Mondello, C. Telomere-independent functions of telomerase in nuclei, cytoplasm, and mitochondria. *Front. Oncol.* 2, 133 (2012).

107. Lee, J. H. *et al.* Catalytically active telomerase holoenzyme is assembled in the dense fibrillar component of the nucleolus during S phase. *Histochem. Cell Biol.* 141, 137–52 (2014).

108. Taggart, A. K. P., Teng, S.-C. & Zakian, V. A. Est1p as a cell cycle-regulated activator of telomere-bound telomerase. *Science* 297, 1023–6 (2002).

109. Tomlinson, R. L., Ziegler, T. D., Supakorndej, T., Terns, R. M. & Terns, M. P. Cell cycle-regulated trafficking of human telomerase to telomeres. *Mol. Biol. Cell* 17, 955–65 (2006).

110. Xin, H. *et al.* TPP1 is a homologue of ciliate TEBP-beta and interacts with POT1 to recruit telomerase. *Nature* 445, 559–62 (2007).

111. Nguyen, B. N., Elmore, L. W. & Holt, S. E. Mechanism of dominant-negative telomerase function. *Cell Cycle* 8, 3227–33 (2009).

112. Katz, S.-F. *et al.* Disruption of Trp53 in livers of mice induces formation of carcinomas with bilineal differentiation. *Gastroenterology* 142, 1229–1239.e3 (2012).

113. Shi, S. *et al.* Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression. *Nat. Biotechnol.* 20, 587–91 (2002).

114. Forsythe, H. L., Elmore, L. W., Jensen, K. O., Landon, M. R. & Holt, S. E. Retroviral-mediated expression of telomerase in normal human cells provides a selective growth advantage. *Int. J. Oncol.* 20, 1137–43 (2002).

115. Gorbunova, V. & Seluanov, A. Telomerase as a growth-promoting factor. *Cell cycle (Georgetown, Tex.)* 2, 534–537 (2003).

116. Volarevic, S. *et al.* Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. *Science* 288, 2045–7 (2000).

117. Derenzini, M. *et al.* Key role of the achievement of an appropriate ribosomal RNA complement for G1-S phase transition in H4-II-E-C3 rat hepatoma cells. *J. Cell. Physiol.* 202, 483–91 (2005).

118. Drygin, D. *et al.* Anticancer activity of CX-3543: a direct inhibitor of rRNA biogenesis. *Cancer Res.* 69, 7653–61 (2009).

119. Greider, C. W. & Blackburn, E. H. Identification of a Specific Telomere Terminal Transferase Activity in Tetrahymena Extracts. 43, 405–413 (1985).

120. González-Suárez, E., Flores, J. M. & Blasco, M. A. Cooperation between p53 mutation and high telomerase transgenic expression in spontaneous cancer development. *Mol. Cell. Biol.* 22, 7291–301 (2002).

121. Artandi, S. E. *et al.* Constitutive telomerase expression promotes mammary carcinomas in aging mice. *Proc. Natl. Acad. Sci. U. S. A.* 99, 8191–6 (2002).

122. Bagheri, S. *et al.* Genes and pathways downstream of telomerase in melanoma metastasis. *Proc. Natl. Acad. Sci. U. S. A.* 103, 11306–11 (2006).

123. Lin, J. *et al.* Nucleolar localization of TERT is unrelated to telomerase function in human cells. *J. Cell Sci.* 121, 2169–76 (2008).

124. Sharma, G. G. *et al.* Human Heterochromatin Protein 1 Isoforms HP1 Hs ␣ and HP1 Hs NL Interfere with hTERT-Telomere Interactions and Correlate with Changes in Cell Growth and Response to Ionizing Radiation. 23, 8363–8376 (2003).

125. Chang, L. Y. *et al.* Telomerase activity and in situ telomerase RNA expression in oral carcinogenesis. *J. Oral Pathol. Med.* 28, 389–96 (1999).

126. Teixeira, M. T., Forstemann, K., Gasser, S. M. & Lingner, J. Intracellular trafficking of yeast telomerase components. *EMBO Rep.* 3, 652–9 (2002).

127. Santos, J. H., Meyer, J. N., Skorvaga, M., Annab, L. A. & Van Houten, B. Mitochondrial hTERT exacerbates free-radical-mediated mtDNA damage. *Aging Cell* 3, 399–411 (2004).

128. Wong, J. M. Y., Kusdra, L. & Collins, K. Subnuclear shuttling of human telomerase induced by transformation and DNA damage. *Nat. Cell Biol.* 4, 731–6 (2002).

129. Methylation, D. N. A., Santoro, R. & Grummt, I. Epigenetic Mechanism of rRNA Gene Silencing : Temporal Order of NoRC-Mediated Histone Modification, Chromatin. 25, 2539–2546 (2005).

130. Tseng, J.-H., Ouyang, C.-H., Lin, K.-J. & Yeh, T.-S. Significance of insulin signaling in liver regeneration triggered by portal vein ligation. *J. Surg. Res.* 166, 77–86 (2011).

131. Ruggero, D. & Pandolfi, P. P. Does the ribosome translate cancer? *Nat. Rev. Cancer* 3, 179–92 (2003).

132. Ding, D., Xi, P., Zhou, J., Wang, M. & Cong, Y.-S. Human telomerase reverse transcriptase regulates MMP expression independently of telomerase activity via NF-κB-dependent transcription. *FASEB J.* 27, 4375–83 (2013).

133. Li, S., Crothers, J., Haqq, C. M. & Blackburn, E. H. Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA. *J. Biol. Chem.* 280, 23709–17 (2005).

134. Schwarzacher, H. G. & Mosgoeller, W. Ribosome biogenesis in man: current views on nucleolar structures and function. *Cytogenet. Cell Genet.* 91, 243–52 (2000).

135. Gomez, D.E., Armando R.G., Farina, H.G, Menna P.L., Gerrudo, C.S., Ghiringhelli, P.D., Alonso, D.F. Telomere structure and telomerase in health and disease (Review). *Int J Oncol* 41 (5): 1561-9 (2012).



ACKNOWLEDGEMENTS

I am heartily thankful to those who were involved in the process of my PhD study and made this thesis possible over the past four years.

First, I would like to express my gratitude to Prof. Dr. med. Karin Scharffetter-Kochanek for offering me the possibility to perform my PhD studies at the Department of Dermatology and Allergic Diseases of the Ulm University.

I would like to express my sincere thank my supervisor PD. Dr. Sebastian Iben for giving me the opportunity to work under his supervision on an exciting project in a great team. I am grateful for his constant advice and support, for directing all the research work during my time as PhD student. He really helped me to improve my skills, broadened my knowledge and developed my growth in the scientific world.

Further, I would also like to convey my appreciation to all lab members for creating an enjoyable atmosphere during work specially my colleagues and friends Sylvia Koch and Robin Assfalg for all the nice time we spent together, for pleasant company, for sharing thoughts, opinions, moods, easy and hard moments. I am also grateful to our technician Adrian Schelling for his technical support and help with the lab work.

Special thanks to PD Dr. Cagatay Günes for ideas, suggestions and support he gave to this project, for helping with the techniques especially the optimization of

telomerase activity measurement and his supportive encouragement. My warm and sincere gratitude also goes to the people from the Leibniz Institute for Age Research - Fritz Lipmann Institute (FLI), for their collaboration and kind supports, especially to Jitendra K. Meena.

Many thanks to Dr. Andre Lechel and Sarah-Fee Katz from the Department of internal medicine I of University Medical Center Ulm, for providing the mice liver tumour tissue samples.

I would like to thank all present and former members of the Department of Dermatology and Allergic Diseases for permanent help, sharing expertise and the constructive lab environment. Special thanks go to all members from the Research group of Prof. Dr. Hartmut Geiger for sharing protocols and reagents, helpful discussions and the great atmosphere. My respect and thanks to Adelheid Hainzl, you are not only a excellent technician but also a kind person, your active and optimistic attitude to life is what I will always remember. A lot of thanks to Patrick Meyer for IP and computer-wise advices, you are also a nice friend!

Last but not least, I owe my deepest gratitude to my great family in Cuba to whom this thesis is dedicated: my parents, daily inspiration in my life and who always stand by my side, thank you for your unconditional and true love, understanding and supports. To my family and friends from Switzerland, the most extraordinary and precious persons, I've been lucky to know, thank you for always being there for me, for your encouragement, support and help.

My apologies to the people who are not mentioned here, I would like to convey my sincere appreciation to all of you!



APPENDICES

- Curriculum vitae
- Publication list

CURRICULUM VITAE — Omar García González

PERSONAL DATA

Date / place of birth:	11. August.1970 / Havana, Cuba
Nationality:	German / Cuban

EDUCATION

PhD *Jul. 2010 – Feb. 2015*	**Doctor of Human biology (Dr. biol.hum.)** Institute for Dermatology and Allergic Diseases. University of Ulm
Bachelor degree *Sept. 1992 – Jul. 1997*	**Bachelor study in Biochemistry** Faculty of Biology. University of Havana – Cuba
High school certificate *Sept. 1984 – Jul. 1988*	**Pre-college** Luis Augusto Turcios Lima – School. Havana – Cuba

PROFESSIONAL CAREER

Sept. 1997 – Apr. 2004 Havana, Cuba	LABIOFAM: Pharmaceutical company for Veterinary Products.
	FINLAY Institute: Center for research and production of vaccines.
Sept. 2004 – Dec. 2005 Bern, Switzerland	University of Bern / Qualification program for biomedical and scientific research (BNF)
Dec. 2005 – Feb. 2009 Basel, Switzerland	Inpheno AG: Institute f. med. Microbiology, University Basel.
Jul. 2010 – Aug. 2014 Ulm, Germany	Institute for Dermatology and Allergic Diseases. University of Ulm

PUBLICATION LIST

García González O, Assfalg R, Koch S, Schelling A, Meena J. K., Kraus J, Lechel A, Benes V, Scharffetter-Kochanek K, Kestler H. A., Günes C, Iben S. Telomerase stimulates ribosomal DNA transcription under hyperproliferative conditions. Nature Communications 5, Article number: 4599 doi: 10.1038/ncomms5599

Koch S, García González O, Assfalg R, Schelling A, Schaefer P, Iben S, Scharffetter-Kochanek K. Cockayne syndrome protein A is a transcription factor of RNA polymerase I. Cell Cycle. 2014 Apr 29;13(13)

Assfalg R, Lebedev A, García González O, Schelling A, Koch S, Iben S. TFIIH is an elongation factor of RNA polymerase I. Nucleic Acids Res. 2012 Jan; 40 (2): 650-9. doi: 10.1093/nar/gkr746. Epub 2011 Sep 28.