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Transcriptional and post-transcriptional regulation of telomerase reverse transcriptase (hTERT) expression

The role of histone modification and alternative splicing

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Stockholm 2008

Published by Karolinska Institutet. Printed by Larserics Digital Print AB, Sundbyberg.

ISBN 978-91-7357-479-2

To my family

献给我的亲人

ABSTRACT

Telomerase, an RNA-dependent DNA polymerase responsible for telomeric DNA elongation, is silent in most normal differentiated human cells due to the tight repression of its catalytic unit, telomerase reverse transcriptase (*hTERT*), whereas constitutive *hTERT* expression and telomerase activation are prerequisites for cellular immortalization/malignant transformation. As reconfiguration of chromatin acts like a master on/off switch, determining whether particular genes are active or inactive, the project was specifically designed to first elucidate molecular strategies that integrate diverse signalling pathways at the *hTERT* chromatin and consequently result in telomerase activation or repression in normal and malignant human cells. Secondly, we address the role of alternative splicing in the regulation of *hTERT* mRNA expression.

We identified the mitogen-activated protein kinase (MAPK) cascade-mediated histone H3 Ser10 phosphorylation to be a molecular link between proliferation and induction of *hTERT* expression. In normal human T lymphocytes and fibroblasts, growth or stress stimuli induce *hTERT* expression and/or telomerase activity that is preceded by phosphorylated histone H3 Ser10 at the *hTERT* promoter. Blockade of the MAPK-triggered H3 phosphorylation significantly abrogates *hTERT* induction and Ser10 phosphorylation at the promoter. These results define H3 phosphorylation as a key to *hTERT* transactivation induced by proliferation and reveal a fundamental mechanism for telomerase regulation in both normal human cells and transformed T cells (paper I).

We found that histone H3 methyltransferase (HMT) SMYD3 directly activated transcription of the *hTERT* gene by binding to the *hTERT* core promoter and affecting tri-methylation of histone H3-K4 at the *hTERT* chromatin (paper II). In contrast to SMYD3, histone H3 demethylases (HDMs) LSD1 and RBP2 are shown to repress the *hTERT* gene. The inhibition of LSD1 function or expression up-regulated or de-repressed the *hTERT* expression. Similarly, RBP2 depletion led to the *hTERT* mRNA induction in normal human telomerase-deficient fibroblasts. During the differentiation of leukemic HL60 cells, both LSD1 and RBP2 were recruited to the *hTERT* promoter and demethylated the local H3-K4, which was accompanied by the repression of *hTERT* transcription. Taken together, LSD1 and RBP2 are required for the establishment of a stable repression state of the *hTERT* gene in human normal or differentiated malignant cells (papers III and IV).

In addition, we found that human normal and malignant lymphoid cells, like other human cells, express splicing variants of *hTERT* mRNA and require transcriptional activation of the *hTERT* gene to acquire telomerase activity (paper V).

Overall, the present findings provide significant insights into the regulatory mechanisms for *hTERT* transcription and telomerase expression, and may be implicated in manipulation of telomerase activity for anti-aging and anti-cancer therapeutic purposes.

ISBN 978-91-7357-479-2

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numeral throughout the text:

I. Ge Z, Liu C, Björkholm M, Gruber A and Xu D (2006). Mitogen-activated protein kinase cascade-mediated histone H3 phosphorylation is critical for telomerase reverse transcriptase expression/telomerase activation induced by proliferation. Molecular and Cellular Biology, 26, 230-237.

II. Liu C, Fang X, Ge Z, Jalink M, Kyo S, Björkholm M, Gruber A, Sjöberg J and Xu D (2007). The telomerase reverse transcriptase (*hTERT*) gene is a direct target of the histone methyltransferase SMYD3. Cancer Research, 67, 2626-2631.

III. Zhu Q, Liu C, Ge Z, Fang X, Zhang X, Stråat K, Björkholm M and Xu D (2008). Lysine-specific demethylase 1 (LSD1) is required for the transcriptional repression of the telomerase reverse transcriptase (*hTERT*) gene. PLoS ONE (in press)

IV. Ge Z, Li W, Liu C, Zhu Q, Björkholm M, Gruber A and Xu D. The recruitment of the histone demethylase RBP2 by Mad1 for the transcriptional repression of the Myc target gene telomerase reverse transcriptase (*hTERT*). (Submitted)

V. Jalink M, Ge Z, Liu C, Björkholm M, Gruber A and Xu D (2007). Human normal T lymphocytes and lymphoid cell lines do express alternative splicing variants of human telomerase reverse transcriptase (*hTERT*) mRNA. Biochemical and Biophysical Research Communications, 353, 999-1003.

CONTENTS

LIST OF ABBREVIATIONS

CBP	CREB binding protein
ChIP	Chromatin immunoprecipitation
CHX	Cycloheximide
CREB	Cyclic AMP-responsive element binding protein
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDFs	Human diploid fibroblasts
HDM	Histone demethylase
HMT	Histone methyltransferase
HP1	Heterochromatin-associated protein 1
hPOT1	Human protection of telomeres 1
hTERT	Human telomerase reverse transcriptase
hTER	Human telomerase RNA
IP	Immunoprecipitation
LSD1	Lysine specific demethylase 1
MSK	Mitogen- and stress-activated kinase
PCAF	p300/CBP-associated factor
pRB	Retinoblastoma protein
RBP2	Retinoblastoma binding protein 2
siRNA	Small interfering RNA
SMYD3	SET and MYND domain-containing protein 3
SRC	Steroid receptor co-activator
TGF-β1	Transforming growth factor-beta 1
TNF	Tumor necrosis factor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRAP	Telomeric repeat amplification protocol
TRF	Telomere repeat binding factor
TSA	Trichostatin A

INTRODUCTION

1. Telomere

The telomere is a special functional complex at the end of linear eukaryotic chromosomes, consisting of tandem repeat DNA sequences and associated proteins [1].

1.1 Telomere structure

The DNA sequence at telomeres consists of tandem GT-rich repeats (TTAGGG)n in human and mouse. The size of telomeric DNA differs greatly from species to species. For example, the repeat sequences may span up to 150 kilobases (kb) per telomere in mouse, whereas each human telomere is only 5 to 15 kb long [1]. In addition, telomeres terminate with a single-stranded 3′ overhang of the G rich strand [2]. The size of this 3′ overhang is species specific, varying from an exact length of 16 nucleotides in *Oxytricha* to a variable stretch of 50–100 nucleotides in mouse and human telomeres. Furthermore, the telomere binding proteins such as telomere repeat binding factor 1 and 2 (TRF1 and TRF2) and human protection of telomeres 1 (hPOT1) can bind directly to the double- or single-strand regions to regulate telomere length and integrity [3,4].

1.2 Telomere function

One of the important functions of telomeres is that they help to solve the “end replication problem” [5]. Conventional DNA replication machinery utilizes an RNA primer to initiate DNA synthesis, leading to the problem that extreme terminal sequences will not be represented on the 5' end of one daughter DNA strand, after removal of a terminal RNA primer. If it were not for telomeres, this would quickly result in the loss of vital genetic information. In addition, the telomere is also involved in several other essential biological functions. For example, it protects chromosomes from recombination, end-to-end fusion, and recognition as damaged DNA; contributes to the functional organization of chromosomes within the nucleus; participates in the regulation of gene expression. Furthermore, according to the Hayflick Limit theory [6], which

states that cells have a maximum number of population doublings before undergoing senescence, it has been proposed that telomere shortening may serve as a mitotic O'clock that records the history of cellular replication.

1.3 Telomere shortening and cellular life-span

Normal somatic cells experience telomeric attrition at a mean loss of 30-150 base pairs (bp) of telomeric DNA per replication, until a critical minimum telomeric length is reached whereby the cells then undergo a permanent growth arrest known as replicative senescence or mortality stage 1 (M1) [7]. Cells that escape replicative senescence by inactivation of a critical cell cycle checkpoint gene such as p53 continue to divide until mortality stage 2 (M2) is induced. During M2, cells undergo a period of crisis which may be the result of terminally short telomeres [8]. Cells surviving from crisis may maintain telomere length and become immortal.

2. Telomerase

How are immortal cells able to maintain their telomere length? It is known that a ribonucleoprotein enzyme named telomerase is activated in these cells. Telomerase adds telomeric repeats to the ends of telomeres and its activation has been shown to be required for cells to overcome replicative senescence and obtain the ability to divide without limits via stabilizing telomere sizes [9].

2.1 Telomerase components

Telomerase consists of two essential components: one is the functional RNA component (in humans called hTER), which serves as a template for telomeric DNA synthesis; the other is telomerase reverse transcriptase (*hTERT*), which is a catalytic subunit with reverse transcriptase activity [10]. hTER is ubiquitously expressed in all tissues regardless of telomerase activity [11], whereas *hTERT* is only present in cells expressing telomerase activity. Therefore, *hTERT* is a rate-limiting factor for telomerase activity control and in most cases, its down- or up- regulation leads to corresponding changes in the levels of telomerase activity [12]. Telomerase recognizes and elongates telomeres through association with the hTER template region and then translocates to the next available position for hTER binding [5].

2.2 Telomerase activity and cellular proliferation

Telomerase is silent in most normal human differentiated cells due to the tight repression of the *hTERT* gene, while it is expressed in some types of normal cells, including stem/progenitor cells, germ-line cells, endometrial cells, basal layer cells of skin and cervical keratinocytes, epithelial and endothelial cells, and activated lymphocytes [13-16]. The common feature of these telomerase-positive normal cells is their highly regenerative capacity. Telomerase activity in these cells is tightly associated with cellular proliferation.

Importantly, there is considerable evidence documenting that normal cells with high proliferation capabilities exhibit inducible *hTERT*/telomerase expression: up-regulated when actively proliferating whereas repressed during quiescent status. For example, in hematopoietic progenitor cells, telomerase activity is weakly detected in the primitive stem cells, while dramatically increased upon mitogenic stimulation [17]. In addition, it has been observed that the expression of *hTERT* is weakly induced in normal, early passage human fibroblasts during their transit through S phase. This regulated expression of *hTERT* results in functional yet transient expression of active telomerase. The proliferation-induced telomerase activation may play an important part in maintenance of physiological life-span of normal human cells.

2.3 Telomerase activation and cancers

High telomerase activity is observed in up to 90% of human cancer cells [18]. The vast majority of studies have confirmed that the primary mode of repression and derepression of telomerase in normal human cells and in cancers occurs at the level of transcription of *hTERT* [19]. Several lines of evidence have demonstrated the role of *hTERT* in the transformation of human cells. For example, it has been found that direct tumorigenic transformation could be achieved through co-expression of *hTERT*, the SV40 large-T oncoprotein and an oncogenic Ha-*ras* gene (Ha-*rasV12*) in both human diploid fibroblasts and epithelial cells [20]. Furthermore, a significant proportion of transgenic female mice ectopically expressing telomerase developed spontaneously invasive mammary carcinomas when aged [21]. Collectively, these findings provide persuasive evidence that telomerase over-expression promotes carcinogenesis.

The clinical implications of telomerase activity in cancer have been investigated. A growing body of evidence indicates that telomerase can be used in the diagnosis of cancers and higher telomerase activity is associated with poor outcomes. More importantly, telomerase has been suggested to be an attractive target for cancer therapy. When telomerase activity or *hTERT* function is inhibited, tumor cells undergo telomere shortening, loss of tumorigenicity and eventual growth arrest and/or apoptosis [18].

3. Regulation of telomerase activity

3.1 Transcriptional regulation of the *hTERT* gene

As described above, *hTERT* mRNA expression is closely correlated with telomerase activity, and therefore transcriptional regulation of *hTERT* expression represents the primary and rate-limiting step in the activation of telomerase activity in most cells.

3.1.1 hTERT promoter

It has been established that the minimum sequence requirement for *hTERT* promoter activity is contained within the 330 bp upstream of the ATG (the translation start site) [22,23] and the transcription initiation sites map 60 to 120 bp upstream of the ATG [23-25]. Sequence analysis indicates that the *hTERT* promoter has no TATA or CAAT boxes but is highly GC-rich. Importantly, the *hTERT* promoter contains binding sites for many transcription factors. For example, the *hTERT* proximal promoter harbors two E-boxes (CACGTG) and five GC-boxes, the consensus binding motifs for the Myc network and Sp1 family proteins, respectively [23,26]. The presence of these potential transcription factor binding sites suggests that the regulation of *hTERT* expression may be subject to multiple levels of control by different factors in different cellular contexts.

3.1.2 Positive regulators of hTERT gene transcription

The *hTERT* promoter contains the Myc binding site (E-box). It is clear that over-expression of c-Myc protein results in an E-box-dependent increase in the *hTERT* promoter activity and that *hTERT* gene is a direct transcriptional

target of *c*-Myc [25,27]. Moreover, c-Myc can interact with other proteins, such as histone acetyltransferase (HAT) to activate *hTERT* expression.

In addition, the GC-rich *hTERT* promoter contains multiple binding sites for a ubiquitously expressed, zinc finger transcription factor Sp1 [23,24,26]. It has been known that Sp1 cooperates with c-Myc to activate *hTERT* transcription in a cell type-specific manner [26]. Sp1 is also found in a complex containing the coactivator protein p300 with HAT activity [28,29]. Interestingly, Sp1 seems to have a dual role in the control of *hTERT* expression. On one hand, Sp1 can interact with c-Myc or HAT to activate *hTERT* expression, and on the other hand, it cross-talks with histone deacetylases (HDACs) to negatively regulate the *hTERT* transcription.

Moreover, many other factors have been identified to up-regulate *hTERT* expression and telomerase activity, including estrogen, viral oncoproteins (exemplified by human papillomavirus E6), epidermal growth factor (EGF), HDAC inhibitor trichostatin A (TSA), upstream stimulatory factor (USF) and Ets family of transcription factors, etc [30,31].

3.1.3 Negative regulators of hTERT gene transcription

It has been well known that upon the *hTERT* repression during differentiation of HL60 and U937 leukemic cells, the Mad1/Max complex replaces the c-Myc/Max complex at the *hTERT* promoter E-boxes [32,33]. In addition, several lines of evidence indicate that HDAC complexes can be recruited to the *hTERT* promoter by Mad1 [34-36] for transcriptional repression of *hTERT* expression.

In addition, *hTERT* expression and telomerase activity are down-regulated by some other negative regulators, such as transforming growth factor ß (TGF-ß), p53, Interferon-α (IFN-α), retinoblastoma protein (pRB) and Wilms' tumor 1 (WT1), etc [30,31].

3.2 Post-transcriptional regulation of *hTERT* expression: The role of alternative splicing

Although, as discussed above, the transcriptional regulation of the *hTERT* gene is a significant determinant for controlling telomerase activity, recent studies demonstrated the presence of multiple pathways that affect *hTERT* expression

and telomerase activity. For instance, a post-transcriptional modification mechanism, contributing to an alternative splicing of *hTERT* mRNA, plays a part role in regulating telomerase activity in many types of human cells and tissues [37].

Alternative splicing is the process that occurs in eukaryotes in which the splicing process of a pre-mRNA transcribed from one gene can lead to different mature mRNA molecules and therefore to different proteins.

In addition to a full-length *hTERT* mRNA that leads to a functional *hTERT* protein, the telomerase transcript has been shown to have at least six alternate splicing sites (four insertion sites and two deletion sites) [38]. The α-deletion lacks 36 nucleotides from the 5' end of exon 6 and lies within the reverse transcriptase (RT) motif A [38] and the β-deletion entirely lacks exon 7 and exon 8, resulting in a loss of 182 bp [39,40]. It has been shown that the smaller splice variants (ß and α+ß) were inactive whereas the α variant could act in a dominant-negative manner [38,41].

The specific pattern of *hTERT* mRNA variants in human development provides evidence that alternative splicing is non-random and participates in the regulation of telomerase activity. For example, fetal heart expresses the original coding sequence and the β spliced transcript. By the 13th week of heart development, all expression of *hTERT* is suppressed and telomerase activity repressed [42]. In addition, the splicing variants of the *hTERT* mRNA, especially the β-deletion type of splicing variant, are basally expressed in the uterus [43]. Moreover, telomerase activity and the full-length *hTERT* transcript were observed in endometrial samples from the proliferative and early secretory phases, but not in those from the late secretory phase [43]. Furthermore, our previous study has shown a striking correlation between full-length *hTERT* mRNA expression and telomerase activation in renal cell carcinoma (RCC). It indicates that telomerase activation is achieved via induction of or switch to expression of full-length *hTERT* mRNA during the oncogenic process of kidneys [44].

However, regulatory pathways governing full-length *hTERT* expression are not fully understood. It has been reported that TGF-ß1 is able to modulate the splicing pattern of *hTERT* by shifting its expression from the full-length active

hTERT splice form to the inactive smaller ß variant [45]. In addition, our previous study demonstrated that the induction of the oncogene c-Myc was closely associated with the expression of full-length *hTERT* transcripts and c-Myc may actively participate in the selective up-regulation of full-length *hTERT* mRNA expression in RCCs [44].

3.3 Post-translational modification of *hTERT*: Phosphorylation of *hTERT* protein

The transcriptional regulation of *hTERT* is undoubtedly the primary mechanism in controlling telomerase activity in cells. However, as mentioned above, the expression of *hTERT* is not always sufficient to produce active telomerase in some cell types and therefore post-translational modification of *hTERT* may play a role in modulating the active and inactive states of telomerase activity [46-48].

Several lines of evidence indicate that telomerase activity can be regulated by *hTERT* phosphorylation. Consistent with this, some protein kinases, such as Akt protein kinase (also known as protein kinase B, PKB) and protein kinase C (PKC) have been reported to mediate phosphorylation of *hTERT* protein, leading to telomerase activation [47,48]. Therefore, post-translational modifications of the *hTERT* protein might provide an additional layer of control of telomerase activity.

4. Histone modifications: Critical roles in transcriptional regulation

There is now considerable evidence that epigenetic regulation plays a very important role on gene transcription. Epigenetic regulation of transcription refers to reversible, heritable changes in gene expression that occur in the absence of changes in DNA sequence. A major epigenetic mechanism involves the covalent modification of histones, DNA methylation and ATP-dependent chromatin remodelling complexes that mediate activation or repression of transcription [49].

In eukaryotes, the DNA is wrapped around the histone octamer, which consists of two copies each of H2A, H2B, H3 and H4, to form nucleosome.

Nucleosomes, the basic subunit of chromatin structure, are in turn folded into progressively higher-order structures which are necessary to package eukaryotic genomes within nuclei [50]. It is clear that each core histone is composed of a structured domain and an unstructured amino-terminal ‘tail' of 25-40 residues. These tails protrude from the nucleosomes and provide sites for a variety of post-translational modifications, such as acetylation, phosphorylation and methylation. It is becoming increasingly apparent that such modifications of histone tails determine the interactions of histones with other proteins, which in turn regulate chromatin structure [51].

Chromatin remodelling is the physical act of moving DNA relative to the histone octamer. Remodelling of chromatin and nucleosome organization is a key factor in the physiological control of transcription. Post-translational modifications of histones have been implicated in the physiological control of chromatin structure [52]. Increasing evidence indicates that both covalent histone modifications and ATP-dependent chromatin remodelling complexes can facilitate access of DNA binding factors to chromatin and, thereby, regulate expression of a wide range of genes [53,54]. More importantly, the growing list of diverse histone-modifying enzymes has highlighted the importance of these chromatin remodelling modifiers [55,56] (Table 1 and 2).

4.1 Histone phosphorylation

Phosphorylation of histone H3 was initially linked to chromosome condensation and segregation during mitosis and meiosis [57]. Earlier studies found that mitosis-specific phosphorylation of histone H3 occurs at serine 10 (Ser10), serine 28 (Ser28) and threonine 11 (Thr11) [58]. However, studies found that the phosphorylation of histone H3 at Ser10 has an important role in transcriptional induction of immediate-early genes in mammalian cells, such as the *c-Fos* gene [59]. Therefore, histone H3 Ser10 phosphorylation occurs in two different cell cycle phases with opposite functions. During mitosis, H3 Ser10 is globally phosphorylated, which is required for chromosome condensation and segregation, while in G_0/G_1 transition, a small fraction of transient H3 Ser10 phosphorylation at specific loci leads to chromatin relaxation. It is important to note that this stimulation-dependent phosphorylation of histone H3 is a rapid and transient event and affects a

Table 1. Histone-modifying enzymes

Histone H3 and H4 acetyltransferases (HATs)

P300, CBP, GCN5, PCAF, Tips, ACTR, SRC-1

Histone H3 and H4 deacetylases (HDACs): HDAC1-7

Histone H3 methyltransferases (HMTs)

HMTs (lysine 4): MLL1-4, hSET1, SMYD3, SET7/9, WDR5, ASH1, NSD3/Whistle, Meisetz/PRDM7

HMTs (lysine 9): Suv39h1, Suv39h2, G9a, GLP-1/EuHMT1, SETDB1/ESET, RIZ1/PRDM2, ASH1, EZH2

HMTs (lysine 27): EZH1/2, Eed1, NSD3/Whistle

HMTs (lysine 36): HYPB/ySET2, NSD1, SMYD2

HMTs (lysine 79): DOT1

Histone H3 demethylases (HDMs)

HDMs (lysine 4): LSD1, JARID1(RBP2, PLU,SMCX,SMCY)

HDMs (lysine 9): LSD1, JHDM2A/JMJD1A, JHDM3/JMJD2A, JMJD2B, JMJD2C, JMJD2D

HDMs (lysine 36): JHDM1/FBXL11, FBXL10, JMJD2A, JMJD2C

Histone H4 methyltransferases (HMTs)

NSD1, PR-SET7/SET8, SUV4-20H1, SUV4-20H2, ASH1

Histone H3 serine 10 phosphorylation

MSK1/2, AKT, Aurora B

population of phosphorylated histone H3 distinct from that normally detected in dividing cells [58].

The mechanism by which phosphorylation contributes to transcriptional activation is not well understood. It has been known that the addition of negatively charged phosphate groups to histone tails reduce their affinity for DNA. More importantly, phosphorylation may contribute to transcriptional

Table 2. The effect of the different modifications of histone H3 and H4 tails on gene transcription

Mark	Transcriptionally relevant sites	Transcriptional role
Acetylated lysine (Kac)	H3 (9, 14, 18, 56), H4 (5, 8, 13, 16), H2A, H2B	Activation
Phosphorylated serine/ threonine (S/T ph)	H3 (3,10, 28), H2A, H2B	Activation
Methylated arginine (Rme)	H3 (17, 23), H4 (3)	Activation
Methylated lysine (Kme)	H3 (4, 36, 79)	Activation
	H3 (9, 27), H4 (20)	Repression

activation through the stimulation of HAT activity on the same histone tail. Accumulated evidence has suggested that histone phosphorylation and acetylation might be coupled during the activation of transcription. In support with this, it has been shown that the phosphorylated N-terminal tail is bound preferentially by GCN5 in vitro, which acetylates the same histone H3 tail at the Lys14 position. Therefore, these two modifications may be sequentially linked such that phosphorylation occurs preferentially on unmodified H3 and a fraction of this phosphorylated H3 is subsequently acetylated [60,61].

Importantly, Ser10 residue in the histone H3 tail appears to be the target of a number of signalling kinases [59,60,62]. Members of the aurora AIR2–Ipl1 kinase family have been found to govern histone H3 phosphorylation at Ser10 during mitosis in several organisms [58]. In addition, the sucrose non-fermented 1 (Snf1) kinase is identified as a transcriptionally-linked histone H3 kinase in yeast [63]. As described above, transient inducible phosphorylation is distinct from the more quantitative phosphorylation of bulk histone H3 during mitosis. Consistent with this, it has been demonstrated that distinct signalling pathways govern these two different events in mammalian cells. The mitogen- and stress-activated kinase (MSK) 1 and 2, particularly MSK2 have been identified as the mitogen- and stress-activated histone H3 kinase

[64], while mammalian Aurora B is found to phosphorylate H3 at Ser10 and Ser28 during mitosis [65].

Collectively, phosphorylation of histone H3 at Ser10 has an important role in transcriptional induction of an increasing number of genes that are activated as a consequence of a variety of cell-signalling events.

4.2 SET and MYND domain-containing protein 3 (SMYD3)

As shown in table 1, diverse HMTs catalyse site-specific histone lysine methylation and the characterization of these enzymes has revealed important functions of histone methylation in many different biological processes. Among them, SMYD3, which is over-expressed in the majority of colorectal carcinomas, hepatocellular carcinomas and breast cancers, has been identified as histone H3 lysine 4 (H3-K4) specific di-, tri-methyltransferase and plays an important role in transcriptional regulation as a member of an RNA polymerase complex [66-68].

4.3 Lysine specific demethylase 1(LSD1)

LSD1, also known as KIAA0601 and BHC110, is the first discovered histone lysine demethylase [69]. It belongs to the class of flavin-dependent amine oxidases and uses an amine oxidase reaction to catalyze the removal of methylation [70].

LSD1 target specificity is altered by associated proteins. Specifically, when complexed with the co-Rest corepressor complex, LSD1 demethylates mono- and dimethylated H3-K4 [69], whereas it targets H3-K9 when associated with the androgen receptor (AR) [71]. Moreover, a recent genome-wide localization study in human breast cancer MCF7 cells also identified a general activator role of LSD1 at estrogen receptor (ER)α-target promoters in a ligand-dependent manner, which is in part through its H3-K9 demethylase activity [72]. These findings demonstrate that protein-protein interactions play an important role in the regulation of the activity and substrate specificity of LSD1.

In addition to protein-protein interactions, the surrounding modifications on the histone tails also affect LSD1 activity. It has been demonstrated that Ser10 dephosphorylation is a prerequisite for LSD1 function, whereas K9

deacetylation by HDAC1/2 makes histone H3 more susceptible to LSD1-catalyzed demethylation [70].

In short, LSD1 is a chromatin-modifying enzyme, which is able to read different epigenetic marks on the histone N-terminal tail and can serve as a docking module for the stabilization of the associated cofactor complexes on chromatin.

4.4 Retinoblastoma binding protein 2 (RBP2)

As mentioned above, LSD1 can only catalyze the demethylation of mono- and dimethyl H3-K4 but lacks the ability to demethylate trimethylated substrates. This gives rise to the question as to whether there is a different class of enzymes dedicated to the reversal of trimethylation. Several recent studies highlighted the importance of RBP2, the Jumonji C (JmjC) domain containing HDMs, specific for tri- and dimethylated H3-K4 [73].

RBP2, the member of the JARID1 protein family, is a nuclear phosphoprotein that was initially identified as a potential retinoblastoma protein (pRB) binding protein [74]. Because trimethylated H3-K4 is found surrounding the start site of transcriptionally active genes [75], the trimethyl H3-K4 demethylase activity of JARID1 proteins predicts that these proteins act as transcriptional repressors. Indeed, it has been reported that RBP2 binds to the promoter region of the Hox genes and represses their transcription in embryonic stem (ES) cells. When these cells are stimulated to differentiate, it was observed that RBP2 binding decreases upon gene activation, which is concomitant with an increase in trimethylated H3-K4 levels on the promoter region [73]. In addition, it has also been found that RBP2 physically interacts with a complex that contains HDAC activity, suggesting that transcriptional repression by RBP2 might also involve additional mechanisms [76].

It is very important to determine how RBP2 is recruited to certain chromatin areas. It is possible that RBP2 binds directly to certain chromatin areas or interact with other proteins. In fact, it has been demonstrated that JmjC domain of RBP2 is correspond to the catalytic domain of HDAC [77]. Consistent with this, it has been reported recently that RBP2 specifically associated with sin3B-HDAC complexes, while not with Tip60-containing HAT complexes [78]. However, another study reported that Drosophila orthologs of RBP2, little

imaginal discs (Lid), binds to dMyc. In this study, it was proposed that the binding of dMyc to the catalytic JmjC domain of Lid may directly inhibit Lid's demethylase activity, which could maintain trimethylated H3-K4 surrounding the Myc binding site, allowing chromatin remodelling factors such as nucleosome remodelling factor (NURF) to activate transcription [79].

Taken together, how RBP2 interactes with other proteins and how it is recruited to certain chromatin areas remains an important and interesting question to be addressed.

4.5 Histone code

It is a hypothesis predicting that histone modifications could act as specific "receptors" to recruit unique biological complexes that mediate downstream function. It indicates that covalent modification of a histone tail by one enzyme influences the rate or efficiency with which a second enzyme follows using the now-modified histone tail as substrate. There is now considerable evidence documenting a huge number of possible combinations of histone modifications, and interdependency and crosstalk between the different histone modifications. A striking example is that phosphorylation on H3 Ser10 inhibits methylation of histone H3-K9, and promotes GCN5-mediated acetylation of histone H3-K14 [81].

Recently, more evidence supports the combinatorial reading of multiple "histone code". It has been shown that many chromatin-associated protein complexes contain multiple histone modification recognition modules. These modules can be present within a single protein or in multiple components of the complex [82]. Bromo- and chromodomains bind acetylated and methylated lysine residues, respectively. The binding modules have a high specificity for particular modified histone residues, for example, the chromodomain of HP1 binds exclusively to di/trimethylated H3-K9 and the chromodomain of polycomb protein (PC) binds specifically to trimethylated H3-K27 [83].

Collectively, a specific type of modification on a specific histone residue can provide the signal for the printing or erasing of another mark, either on the same tail or on neighboring tails, within the same or on neighboring nucleosomes to regulate a multitude of cellular processes.

4.6 The MAPK signal transduction pathway and chromatin remodelling

Signal transduction pathways relay information from a variety of different stimuli leading to multiple cellular responses [84]. Mitogen- and stress activated protein kinase (MAPK) pathways transmit and amplify signals involved in cell proliferation, growth and survival. These pathways activate kinases that modify transcription factors, histones and chromatin remodelling factors [85].

Five distinct groups of MAPKs have been characterized in mammals: extracellular signal-regulated kinase 1 and 2 (ERK1/2); c-Jun amino-terminal kinases (JNKs) 1, 2, and 3; p38 isoforms α, β, γ, and δ; ERK3 and 4; and ERK5 [86]. In general, ERK1 and ERK2 are preferentially activated in response to growth factors and phorbol esters (12-O-tetradecanoylphorbol- 13-acetate, TPA), while the JNK and p38 kinases are more responsive to stress stimuli. Each family of MAPKs is composed of a set of three evolutionarily conserved, sequentially acting kinases: a MAPK, a MAPK kinase (MAPKK), and a MAPKK kinase (MAPKKK). MAP kinases are regulated by phosphorylation cascades [86].

The MAPK pathway has been shown to be directly linked to chromatin structure. It has been demonstrated that growth factors and TPA activate the ERK pathway, while stressors such as UV irradiation work through the p38 MAPK pathway. Exposure to these stimulants results in the activation of a series of kinases and transcription factors [87], eventually modifying chromatin proteins such as histone H3 and leading to chromatin remodelling. Compelling evidences suggest that phosphorylation of H3 on Ser10 increase dramatically, coupled with increased acetylated H3 associated with the transcribed chromatin of immediate-early genes following stimulation of the MAPK pathway [60,88].

Importantly, it was found that deregulation of the Ras-MAPK pathway contributes to altered chromatin structure and aberrant gene expression in transformed cells. In oncogene-transformed fibroblasts, for example, elevated H3 Ser10 phosphorylation due to increased MSK1/2 activity may result in the relaxing chromatin structure that in turn promotes transcription factor access to regulatory regions [85].

A previous study demonstrated that MSK1 was associated with Interleukin-6 (IL-6) gene promoter after cells are stimulated with tumor necrosis factor (TNF) [89]. In contrast, however, it was also proposed that MSK1 might be pre-loaded onto the promoters of the immediate-early genes before induction [85]. Furthermore, it has been reported that MSK1 regulates transcription factor ER81-dependent transcription via direct phosphorylation of ER81 as well as via stimulation of coactivators CREB binding protein (CBP)/p300 [90], indicating that MSK1 may be recruited to chromatin by its interaction with transcription factors and coactivators.

In addition to ER81, many other transcription factors have also been identified as MAP kinase substrates. For example, ERK2 has been shown to phosphorylate steroid receptor co-activator-1 (SRC-1), which possesses an intrinsic HAT activity and is a coactivator that enhances the activation of steroid nuclear receptors [91]. Others include cyclic AMP-responsive element binding protein (CREB) [92], signal transducer and activator of transcription 3 (STAT3) [93], p65 subunit of nuclear factor kappa B (NF-κB) [89]. These downstream effects of MAP Kinases could influence chromatin remodelling and activation of gene expression.

Importantly, earlier studies have shown that EGF activates telomerase through the up-regulation of *hTERT* expression. This is a direct action on the *hTERT* promoter through a specific signal transduction pathway in which the Ras/MEK/ERK pathway play major roles [94]. Therefore, it will be of interest to determine the mechanism by which EGF or other mitogenic stimuli activate *hTERT* transcription via the MAP kinase signalling pathway and the direct link between the MAPK pathway, histone modifications and *hTERT* gene expression.

AIMS

The overall aim of this study was to elucidate molecular strategies that integrate diverse signalling pathways at the *hTERT* chromatin and consequently result in telomerase activation or repression in normal and malignant human cells, and to characterize the role of alternative splicing in the regulation of *hTERT* mRNA expression. More specifically we aimed at answering the following questions:

1. How is the *hTERT* chromatin remodelled during a switch from repressive to active status of *hTERT* transcription in human normal cells with high proliferative ability?

2. How is the *hTERT* chromatin maintained in a “closed” state and the *hTERT* gene stably silent in human differentiated cells?

3. How do alterations in the *hTERT* chromatin allow constitutive *hTERT* expression and telomerase activation in cancer cells?

4. What is the role of alternative splicing in the regulation of *hTERT* expression and telomerase activity in human lymphocytes?

METHODOLOGICAL CONSIDERATIONS

1. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay is a powerful tool for monitoring changes in chromatin structure and transcription. It uses a process of formaldehyde fixation, which preserves protein modifications and protein/DNA interactions, followed by specific DNA enrichment using antibodies directed against the protein of interest.

The present study is aimed at delineating molecular strategies that integrate diverse signalling pathways at the *hTERT* chromatin and consequently result in telomerase activation or repression by examining the recruitment of various chromatin-modifiers to the *hTERT* promoter in normal and malignant human cells. For this purpose, ChIP assay is used as a key method in this study.

In this method, intact cells are fixed using formaldehyde, which cross-links and therefore preserves protein/DNA interactions. The DNA is then sheared into small, uniform fragments (usually 200-1000 bp) by sonication. The protein/DNA complexes are then immunoprecipitated using antibodies directed against the DNA-binding transcription factors (e.g., c-Myc, Mad1, Sp1 in papers II and IV), histones and their post-translational modifications (e.g., phosphorylated H3 Ser10, acetylated H3 and H4, mono-, di-, trimethylated H3-K4 and K9 in papers I-IV) and co-factors (e.g., P300, SMYD3, LSD1, RBP2 in papers II-IV). Following immunoprecipitation, the cross-linking is reversed, the proteins are removed by treatment with Proteinase K and the DNA is purified. The DNA is then analyzed by PCR using primers specific for *hTERT* proximal promoter.

It is worth noting that antibodies used for ChIP assay must recognize fixed protein that is bound to DNA and /or complexed with other proteins. Many antibodies that perform well in other applications do not work well in ChIP assay. A further complication is that the antibodies must recognize transcription factor epitopes that are not concealed during DNA binding. In addition, optimal conditions required for shearing cross-linked DNA to fitting length depend on cross-linking time, the cell type, cell concentration per lysis buffer and the sonicator equipment, including the power settings and number of pulses.

2. Western blot and immunoprecipitation (IP)

Western blot (alternatively, immunoblot) is a method to detect a specific protein in a given sample of tissue homogenate or extract. In the present study, it is used to detect the expression at protein level of Ser10 phosphorylated H3 and *hTERT* (paper I), SMYD3 (paper II), methylated histone H3-K4 and K9, acetylated H3 and LSD1 (paper III), Mad1 and RBP2 (paper IV), c-Myc (papers IV and V).

IP is the technique of precipitating an antigen out of solution by specific antigen-antibody interaction. This process can be used to identify protein complexes present in cell extracts by targeting any one of the proteins assumed to be in the complex. Using this method, we found the physical interaction between Mad1 and RBP2 in the differentiated HL60 cells, p493-6 cell line with c-Myc expression shutting-off, a cervical cancer line HeLa and human normal fibroblasts MRC5 cells (paper IV). However, it is very important to note that some protein releasing is salt sensitive. In present study, endogenous Mad1/RBP2 complex could not be detected under standard cell lysis conditions, while it did appear when salt concentration was increased to 250 mM NaCl.

3. Small interfering RNA (siRNA) treatment

Small interfering RNA (siRNA), also known as short interfering RNA or silencing RNA, is a class of 20-25 nucleotide long double-stranded RNA molecules that play a variety of roles in biology. Most notably, siRNA is involved in the RNA interference (RNAi) pathway where the siRNA interferes with the expression of a specific gene.

RNAi describes the phenomenon by which double-stranded (ds) RNA induces potent and specific inhibition of eukaryotic gene expression via the degradation of complementary messenger RNA (mRNA). In eukaryotic organisms, dsRNA produced in vivo or introduced by pathogens is processed into 21-23 nucleotide double-stranded siRNA by an enzyme called Dicer, a member of the RNase III family of dsRNA-specific endonucleases. The siRNA is then incorporated into an RNA-induced silencing complex (RISC), an enzyme complex that serves to

target cellular transcripts complementary to the siRNA for specific cleavage and degradation.

Based on this principle, in present study, we silenced SMYD3 (paper II), LSD1 (paper III) and RBP2 (paper IV) expression by chemically modified Stealth siRNA specific for these targeting genes.

4. Telomeric repeat amplification protocol (TRAP) assay for telomerase activity

The conventional primer-extension based assay for detecting telomerase activity requires large amounts of cells or tissue, and it only detects telomerase with limited sensitivity. Those disadvantages have been overcome by the TRAP assay [95], in which the telomerase reaction product is amplified by using PCR. The original TRAP assay was done with a radioactive label, and it required visualization of results by autoradiography after gel electrophoresis, which is both hazardous and time consuming. The Telomerase PCR ELISA detection provides a way to perform a highly sensitive photometric enzyme immunoassay for the detection of telomerase activity, using nonradioactive ELISA techniques. In present study, this ELISA based TRAP assay is used for detection of telomerase activity (papers I-V).

RESULTS AND DISCUSSION

1. MAPK cascade-mediated histone H3 phosphorylation is critical for *hTERT* expression and telomerase activation induced by proliferation (paper I)

Telomerase activity and *hTERT* expression are highly proliferation-regulated. To further elucidate the underlying mechanisms, we examined whether ConA-stimulated T-cell proliferation would lead to H3 phosphorylation. Both western blot and immunofluorescence results show the rapid accumulation of Ser10 phosphorylated histone H3 in T cells treated with ConA, suggesting that ConA-stimulated T-cell proliferation through the MAPK cascade would lead to H3 phosphorylation.

To further clarify whether histone H3 at the *hTERT* locus was specifically targeted for Ser10 phosphorylation, we performed ChIP assay. We found that in resting T cells, there was no detectable histone H3 Ser10 phosphorylation associated with the *hTERT* promoter, whereas ConA treatment within 30 min led to the accumulation of Ser10 phosphorylated H3 at this promoter, and a maximal level was reached after 1 to 3 h, when *hTERT* mRNA was starting to appear. Thus, accumulation of histone H3 Ser10 phosphorylation at the *hTERT* promoter preceded *hTERT* mRNA induction and telomerase activation in ConA-treated T cells.

We were then interested in whether H3 Ser10 phosphorylation at the *hTERT* promoter and transcriptional activation of this gene were linked. We incubated T cells with ConA in the presence of either H89, a compound that specifically inhibits MSK activities [64], or PD98095, a specific ERK inhibitor that blocks the MAPK signalling pathway [64]. We found that both of them led to a reduction in both *hTERT* promoter-associated H3 Ser10 phosphorylation and *hTERT* mRNA/telomerase expression, indicating a mechanistic link between histone H3 phosphorylation and transcriptional *hTERT* activation in proliferating T cells.

It has been shown that immortalized and tumor cells exhibit growth-regulated telomerase/*hTERT* expression [96,97]. To determine a potential association

between H3 phosphorylation and reversible *hTERT* expression in malignant cells, T-cell lymphoma-derived Jurkat cells, after 72 h of serum depletion, were re-fed with 20% fetal calf serum (FCS) and then analyzed for *hTERT* mRNA expression, telomerase activity, and H3 phosphorylation at the *hTERT* promoter. The result clearly shows a concomitant accumulation of the *hTERT* promoter-associated H3 phosphorylation and *hTERT* mRNA/telomerase induction in the treated Jurkat cells. This result is almost identical to that seen in ConA-treated T cells, indicating that the H3 phosphorylation signalling governs the proliferation-regulated telomerase activity in both normal and malignant T cells.

It has been well established that H3 Ser10 phosphorylation is induced in normal fibroblasts through the MAPK cascade by either mitogen-stimulated ERK or stress-activated p38 signalling [98]. Given the finding that normal human fibroblasts transiently express *hTERT* mRNA in response to mitogen stimulation [99], we hypothesized that the H3 phosphorylation is likely responsible for transcriptional regulation of the *hTERT* gene in this type of cells. To test this, dermal and lung human diploid fibroblasts (HDFs) under serum starvation for 48 to 72 h were exposed to different stimuli known to result in H3 phosphorylation and then analyzed for *hTERT* mRNA expression. We found that all the tested reagents, including EGF, 20% FCS, TPA, and the protein synthesis inhibitors anisomycin and cycloheximide (CHX), induced detectable *hTERT* mRNA expression in two different strains of HDFs. However, it is worth noting that compared to activated T cells, the abundance of *hTERT* transcripts in the treated HDFs was around 50-fold lower. In addition, ChIP assay revealed the occupancy of phosphorylated Ser10 at the *hTERT* promoter in the treated but not control HDFs, which occurred prior to *hTERT* mRNA expression. These results indicate that histone H3 phosphorylation at Ser10 via the MAPK cascade is a critical event that triggers transient *hTERT* transcription in HDFs.

Taken together, MAPK cascade-mediated histone H3 Ser10 phosphorylation is critical for *hTERT* expression and telomerase activation induced by proliferation in both normal and malignant cells examined.

2. Both phosphorylation and acetylation of histone H3 are required to fully transactivate the *hTERT* gene (paper I)

We and others have previously shown that inhibition of HDACs by TSA leads to the transcriptional activation of the *hTERT* gene in normal and malignant cells [33,35,36]. Given the present finding that CHX or serum alone was capable of stimulating *hTERT* expression through H3 phosphorylation, it is likely that histone H3 phosphorylation and acetylation cooperate to synergistically *trans*-activate the *hTERT* gene. To address this issue, we incubated 48h serum-starved HDFs with either 20% FCS, anisomycin, TSA alone, or a combination of FCS or anisomycin with TSA for 16 h. We observed that the combined treatment of the cells with TSA and serum or anisomycin led to much higher levels of *hTERT* mRNA than those in the cells incubated with any single reagent. In addition, telomerase activity was only detectable in the cells treated with TSA plus FCS. These observations indicate that both histone H3 phosphorylation and acetylation are required for the optimal induction of *hTERT* expression and telomerase activity.

It is known that Ser10 phosphorylated H3 is especially susceptible to hyperacetylation at lys14 [60,100]. We indeed found that histone H3 Ser10 phosphorylation was coupled with lys14 acetylation at the *hTERT* promoter in both activated T cells and Jurkat cells, where constitutively high levels of *hTERT* mRNA and telomerase activity were induced. However, it is worth noting that in serum-stimulated fibroblasts, H3 was only phosphorylated at Ser10 without lys14 acetylation on the *hTERT* promoter, which was concomitant with transient, 50-fold lower *hTERT* expression compared to that in the T cells. The obtained results suggest that the role of HDACs on the *hTERT* promoter is predominant and cannot be overridden by an H3 phosphorylation event in fibroblasts.

Based on the above results, we propose a general model for the *hTERT* transcription and telomerase activity control in normal human cells and malignant lymphocytes (Figure 1).

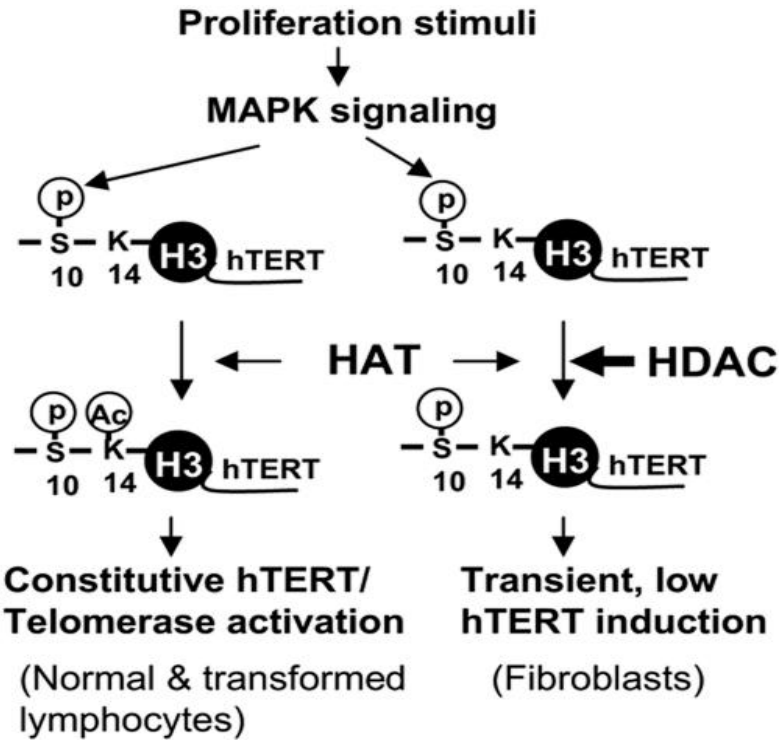

Figure 1. **Working model for histone H3 phosphorylation and acetylation in controlling *hTERT* transcription and telomerase activation.** The model is based on the present finding that both phosphorylation and acetylation of histone H3 are required to fully *trans*-activate the *hTERT* gene. First, growth stimuli trigger H3 Ser10 phosphorylation at the *hTERT* promoter via the MAPK cascade in most, if not all, human cells with proliferation capacities. H3 Ser10 phosphorylation then promotes HATs to acetylate lys14 in the same histone tail. Depending on the status of HDACs on the *hTERT* promoter, local H3 lys14 acetylation may occur, and synergistic effects of phosphorylation and acetylation lead to constitutively high *hTERT* expression and telomerase activation, as seen in normal and malignant T cells. On the other hand, if the role of HDACs on the *hTERT* promoter is predominant, no further lys14 is acetylated, and consequently Ser10 phosphorylation alone induces transient, low levels of *hTERT* expression, as seen in fibroblasts. Thick arrow, the predominant role for HDACs at the *hTERT* promoter.

3. *hTERT* gene is a direct target of the histone methyltransferase SMYD3 (paper II)

To investigate the effect of SMYD3 on *hTERT* expression, we ectopically expressed SMYD3 in human normal fibroblasts and the osteosarcoma cell line Saos2 in which *hTERT* is transcriptionally silent. Our data indicate that SMYD3 positively regulates *hTERT* mRNA expression in both human primary fibroblasts and cancer cells with a repressed *hTERT* gene.

It is known that SMYD3 is over-expressed in the majority of colorectal carcinoma and hepatocellular carcinoma [66], whereas constitutive expression of *hTERT* and telomerase is also observed in most of these tumors [101]. To explore whether SMYD3 was required for constitutive *hTERT* mRNA expression, we knocked down SMYD3 expression with specific SMYD3 siRNAs in human colorectal carcinoma HCT116 and hepatocellular carcinoma Hep3B

cell lines. We found significant reduction in both *hTERT* mRNA and telomerase activity in these cells treated with SMYD3 siRNA, suggesting that SMYD3 is required for constitutive *hTERT* expression and telomerase activity in these cancer cells examined.

To further clarify whether SMYD3 up-regulates *hTERT* expression at the transcriptional level, we examined the effect of SMYD3 on the *hTERT* promoter activity. We found that cotransfection of the SMYD3 expression vector with a luciferase reporter driven by the *hTERT* core promoter sequence (*hTERT*-Luc p181) into the telomerase-negative cell line Saos2 led to the increase in reporter gene activity in a dose-dependent manner. In addition, through substitution mutation of each SMYD3 binding site (MT1–MT5), we found that two of them (MT3 and MT5) were important for the transcriptional activity of the *hTERT* gene. Furthermore, we found that flag-tagged SMYD3 was bound to the *hTERT* promoter by ChIP assay. These data indicate that SMYD3 directly targets the *hTERT* promoter leading to transcriptional activation of the *hTERT* gene.

It has been reported that SMYD3 induces transcriptional activation of its target genes by dimethylating or trimethylating H3-K4 in their promoter regions [66], we sought to investigate whether the level of SMYD3 expression affected methylation patterns of H3-K4 at the *hTERT* promoter. We found that H3-K4 trimethylation was abolished in the *hTERT* core promoter in both HCT116 and Hep3B cells treated with SMYD3 siRNA, suggesting that SMYD3 is responsible for trimethylation of H3-K4 in the *hTERT* promoter region in these cells.

Since H3-K4 methylation alters chromatin folding that in turn contributes to increased accessibility of DNA to transcription factors and provides specific binding sites for certain proteins including HATs, we further examined whether the abolished H3-K4 trimethylation affected the occupancy of the transcription factors c-Myc and Sp1 on the *hTERT* promoter in the SMYD3-silent cells. Our data show that c-Myc, Sp1 and histone H3 acetylation were absent on the *hTERT* promoter in HCT116 cells treated with SMYD3 siRNA.

Taken together, these results indicate that SMYD3 induces *hTERT* transcription by directly binding to the *hTERT* promoter, affecting abundance of trimethylated H3-K4 associated with the *hTERT* chromatin and increasing the

access of positive transcription factors and histone acetylation at the *hTERT* promoter.

4. LSD1 is required for the transcriptional repression of the *hTERT* gene (paper III)

As mentioned in the introduction of the thesis, LSD1 is a HDM belonging to the amine oxidase family. To determine its potential role in the transcriptional regulation of the *hTERT* gene, we incubated normal human MRC5 fibroblasts exhibiting a stable *hTERT* repression in the presence of tranylcypromine, the amino oxidase inhibitor, which has been identified to potently suppress the enzymatic activity of LSD1 [102]. We found that *hTERT* transcripts were not observed in control MRC5 cells while the tranylcypromine treatment induced a weak but detectable expression of *hTERT* mRNA. Similarly, we observed that tranylcypromine up-regulates *hTERT* expression and telomerase activity in the cervical cancer lines HeLa and SiHa. Moreover, we found that *hTERT* mRNA was induced in these cells by using the specific LSD1 siRNA. These data suggest that either pharmacological inhibition of LSD1 activity or depletion of its expression is capable of de-repressing the *hTERT* transcription in normal human fibroblasts.

It has been previously shown that the inhibition of HDAC activity by TSA activates *hTERT* transcription in human fibroblasts and other cell types [34-36,103]. Moreover, an intimate interplay between LSD1 and HDACs has been observed [104]. To explore whether a synergistic induction of *hTERT* expression could be achieved by inhibiting both LSD1 and HDAC activities, MRC5 cells were exposed to either tranylcypromine or TSA alone, or tranylcypromine plus TSA. We found a weak expression of *hTERT* mRNA in the cells treated with tranylcypromine or TSA , whereas the cell exposure to both tranylcypromine and TSA led to a robust increase in *hTERT* transcripts, suggesting that LSD1 and HDACs cooperate together to maintain a repressive state of the *hTERT* transcription in human normal MRC5 cells.

It has been reported that LSD1 interacts with CoREST, a corepressor that exists in the HDAC containing complex [105,106]. We found that depletion of LSD1 but not CoREST using a siRNA approach induced minimal amounts of *hTERT* mRNA whereas the depletion of both of them synergistically activated

hTERT transcription, which was in accordance with those observed in MRC5 cells incubated with both tranylcypromine and TSA. These data indicate that the simultaneous inhibition of both LSD1 and HDACs is required for the optimal induction of *hTERT* mRNA expression in MRC5 cells.

In addition, the presence of LSD1 on the *hTERT* promoter was observed in both HeLa and SiHa cells. Moreover, we demonstrated that tranylcypromine treatment or depletion of LSD1 expression elevated H3-K4 dimethylation and histone H3 acetylation at the *hTERT* promoter in both HeLa and SiHa cells. Similarly, the ChIP result showed that LSD1 recruitment to the *hTERT* promoter did not occur in undifferentiated leukemic HL60 cells while was readily observed in differentiated cells treated with DMSO for 48 hours, concomitant with the loss of H3-K4 dimethylation at the *hTERT* proximal promoter.

Taken together, our results above suggest that LSD1 is required for the transcriptional repression of the *hTERT* gene in both normal and cancerous cells by directly demethylating H3-K4 and indirectly promoting H3 deacetylation associated with the *hTERT* core promoter.

5. HDM RBP2 is required for the transcriptional repression of the *hTERT* gene (paper IV)

RBP2 is a HDM specific for tri- and dimethylated H3-K4 and can act as the transcriptional repressor. Given the fact that leukemic HL60 cells exhibit a stable repression of *hTERT* transcription when induced to undergo terminal differentiation [33,107], we were interested in if RBP2 is required for the transcriptional repression of the *hTERT* gene. We found the down-regulation of *hTERT* expression and telomerase activity was concomitant with the significant up-regulation of Mad1 and RBP2 protein expression in HL60 cells treated with the differentiation-inducer DMSO within 24 hours. In addition, B cell-derived, EBV-immortalized p493-6 cells with an inducible c-Myc expression [108,109] were cultured in the presence of tetracycline for 72 hours. We observed *hTERT* mRNA expression was repressed in the tetracycline-treated cells. Interestingly, however, c-Myc shutting-off did not lead to detectable changes in the level of RBP2 protein expression, which was contrast to that seen in the differentiated

HL60 cells, indicating that the differentiation program rather than c-Myc inhibition induces the enhanced RBP2 expression.

Furthermore, we explored whether the RBP2 depletion could reverse the down-regulation of *hTERT* expression. We did find that knocking-down RBP2 significantly attenuated a decline in *hTERT* mRNA expression resulting from the c-Myc shutting-off in p493-6 cells. Moreover, *hTERT* mRNA was induced by RBP2 siRNA treatment in human normal fibroblasts MRC5 cells, although such an effect was not robust because the inhibition of RBP2 triggered cellular senescence of a significant fraction of MRC5 cells.

Taken together, our results strongly suggest that RBP2 is required for the transcriptional repression of the *hTERT* gene.

6. Mad1 physically interacts with RBP2 and recruits it to the *hTERT* promoter accompanied by the diminished H3-K4 methylation (paper IV)

In *Drosophila*, dMyc was observed to form a complex with the Lid, Drosophila ortholog of RBP2, and to inhibit their demethylating activity toward to histone H3-K4 [79,110]. We thus wanted to explore a potential interaction between RBP2 and Myc/Max/Mad proteins in human cells. However, we could not find the presence of RBP2/c-Myc complex in undifferentiated HL60 cells where c-Myc protein was abundantly expressed. Instead, we did find the physical interaction between Mad1 and RBP2 in the differentiated HL60 cells where both of them were up-regulated. In addition, we further examined the presence of the RBP2/Mad1 and RBP2/c-Myc complexes in other types of cells including the p493-6 cell line, MRC5 cells and a cervical cancer line HeLa. In all these different types of cells, the RBP2/Mad1 rather than RBP2/c-Myc complex was detectable. Thus, c-Myc is not associated with RBP2 while the RBP2/Mad1 interaction is widespread in human cells.

Our previous study showed that the switch of the *hTERT* promoter occupancy from c-Myc/Max to Mad1/Max was concomitant with the dramatically diminished histone H3 and H4 acetylation during the differentiation of HL60 cells [33]. In accordance with this, our present study demonstrated that the HAT p300 and RNA polymerase (pol) II was abundantly present in the *hTERT*

proximal promoter in undifferentiated HL60 cells while substantially diminished in the DMSO-treated cells. Together with these alterations, RBP2 was observed to bind the identical region of the *hTERT* promoter in the differentiated but not undifferentiated cells. Similarly, upon the shutting-off of c-Myc expression in p493-6 cell line, the occupancy of c-Myc on the E-box was replaced by Mad1 at the *hTERT* promoter region, which was accompanied by the presence of RBP2. Taken together, the co-existence of Mad1 and RBP2 on the *hTERT* promoter suggests that Mad1 is responsible for the recruitment of RBP2.

As mentioned above, RBP2 is a HDM specific for tri- and dimethylated H3-K4. We found that the di- and tri- methylation of histone H3-K4 was abundant on the *hTERT* promoter in the control HL60 cells but became diminished upon the cellular differentiation following their exposure to DMSO. These results are well consistent with a temporary up-regulation of RBP2 and its presence at the *hTERT* promoter. In addition, consistent with the RBP2 recruitment to the *hTERT* promoter upon the shutting-off of c-Myc expression in p493-6 cell line, histone H3-K4 di- and tri-methylation decreased significantly. Furthermore, our ChIP results showed the disappearance of RBP2 from the *hTERT* promoter accompanied by the enhanced H3-K4 di- and tri-methylation in the RBP2-depleted cells.

In summary, the Myc/Max/Mad network proteins regulate *hTERT* gene transcription via recruiting the HDM RBP2, thereby affecting H3-K4 methylation status, resulting in subsequent altered chromatin structure.

7. Human normal T lymphocytes and lymphoid cell lines express alternative splicing variants of *hTERT* mRNA (paper V)

Alternative splicing of *hTERT* mRNA, as a post-transcriptional modification mechanism, plays a part role in regulating telomerase activity in many types of human cells and tissues.

An earlier observation indicated that normal human T and B lymphocytes only expressed the full-length *hTERT* mRNA [111]. It raised a question: Are there the fundamental differences in regulating telomerase activity between normal

lymphocytes and other types of human cells? To answer this question, we first detected the splicing profile of *hTERT* transcripts in various T and B cell-derived tumor cell lines. *hTERT* mRNA variants including full-length, α- and β-deletion were observed in all these malignant cells. Driven by this, we re-examined *hTERT* mRNA variants in normal T cells. In contrast with the earlier finding, we observed that T cells treated with the mitogenic stimuli including CD3/CD28 antibodies, ConA and PHA exhibited an identical *hTERT* splicing pattern as seen in lymphoid cell lines, consistent with increased telomerase activity. Whereas resting T cells expressed only low levels of the β-form of *hTERT* mRNA variants without detectable full-length *hTERT* mRNA, in accordance with minimal telomerase activity detected in these cells. Our results thus demonstrate that alternatively spliced *hTERT* mRNA is present in both normal and malignant lymphoid cells.

8. Requirement of c-Myc for induction of full-length *hTERT* mRNA (paper V)

Having shown the presence of splicing variants of *hTERT* mRNA in both activated normal T cells and lymphoid cell lines, we wanted to further explore the potential mechanisms underlying the expression of splicing variants and full-length *hTERT* transcripts.

Several lines of evidence indicate a close association between c-Myc and expression of the full-length *hTERT* mRNA [44,45]. In addition, it is also known that telomerase activity is linked to growth factor-stimulated cell proliferation [103]. Therefore, we were interested in determining whether c-Myc, or growth factors, or both of them are required for expression of the full-length *hTERT* mRNA. For this purpose, a B cell line p493-6 with an inducible c-Myc system was firstly incubated with tetracycline in serum-free medium for 72h to turn off c-Myc expression, and then manipulated through different treatments for 24h: c-Myc+/FCS+, c-Myc+/FCS−, c-Myc−/FCS−, and c-Myc−/FCS+. Our results clearly show that without c-Myc, FCS up-regulated the alternatively spliced *hTERT* mRNA while it had minimal effects on the full-length *hTERT* mRNA expression in p493-6 cells. c-Myc could induce expression of both full-length and other variants of *hTERT* mRNA, regardless of the presence or absence of FCS, consistent with the highest level of telomerase activity in these cells.

These results suggest that c-Myc may be the key factor that contributes to expression of the full-length *hTERT* mRNA.

9. Expression and distribution of *hTERT* protein in normal human resting and activated T cells (paper V)

One earlier study [112] indicated that telomerase activation in human T lymphocytes does not require up-regulation of *hTERT* protein expression but is associated with *hTERT* phosphorylation and nuclear translocation. However, a later study showed that many of commercially available *hTERT* antibodies widely used in earlier publications recognize non-specific proteins rather than *hTERT* [113], and we noticed that one of such antibodies was applied in the study above. Therefore, we want to re-examine *hTERT* protein expression in both resting and activated T cells by using an established *hTERT* antibody. The immunohistochemical analyses show that the majority of resting T cells expressed no *hTERT* protein in neither cytoplasm nor nucleus compartments. In contrast, *hTERT* protein staining was positive in most T cells stimulated with ConA. Moreover, although the *hTERT* staining signal was present in both the cytoplasm and nucleus of the ConA-treated T cells, we observed a significant amount of *hTERT* protein in cytoplasmic compartments.

CONCLUSIONS

1. MAPK cascade-mediated histone H3 phosphorylation at Ser10 is required for transcriptional activation of the *hTERT* gene induced by proliferation stimulation, and the cooperative interplay between histone H3 phosphorylation and acetylation contributes to constitutive expression of *hTERT*/telomerase in human cells.

2. LSD1 and RBP2, the HDMs that specifically demethylate histone H3-K4 mono-, di- or tri-methylation, target the *hTERT* promoter and participate in the establishment of a stable repression state of the *hTERT* gene in human normal or differentiated malignant cells.

3. HMT SMYD3 occupies its binding motifs on the *hTERT* promoter, activates *hTERT* transcription through enhancing histone H3-K4 trimethylation, and thereby induces *hTERT* expression/telomerase activity in normal and malignant human cells.

4. Alternatively spliced *hTERT* mRNA is present in both normal and malignant lymphoid cells, and involved in the post-transcriptional regulation of the *hTERT* gene and control of telomerase activity in these cells. c-Myc may be the key factor that stimulates the expression of the full-length *hTERT* mRNA.

Overall, the present study reveals a significant role of histone H3 modifying-enzymes and alternative splicing in regulating *hTERT*/telomerase expression in human cells. These findings may be implicated in manipulation of telomerase activity for anti-aging and anti-cancer therapeutic purposes.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to everyone who has contributed to this thesis in various ways, thank you for all your help and support.

I would like to begin by expressing my deepest gratitude and appreciation to my supervisor, Dawei Xu. Thank you for giving me the opportunity to be your PhD student and for teaching me from the beginning towards the dissertation. I am always grateful for your brilliant mind and your endless scientific enthusiasm. I greatly acknowledge your endless encouragement, constant supports, scientific guidance and discussions provided to me. Thank you so much for your constant help and guidance with my thesis. Thank you very much, my supervisor, I do appreciate all you have done for me!

Dear Astrid Gruber, my co-supervisor, you are such an excellent person, always so kind and helpful. Thank you for your constant concern about my life and your smile always made me feel like at home. Thank you very much for your kind support and guidance. I do appreciate your encouragement during my study here. Thank you very much for finding time out of your busy schedule to check my progress. I greatly acknowledge your careful reading of my thesis and very helpful suggestions.

Magnus Björkholm, my dear professor, you are such a kind and wonderful professor. I am very much impressed by your spirit and enthusiasm on science, by your vast knowledge, and by your sense of humor. I greatly acknowledge your constant support and guidance. Thank you for all your wise scientific advices. I do appreciate your time allotments for me in your busy schedule. I am so grateful to you for reading my thesis carefully and for the valuable suggestions and discussions.

Dear Jan Sjöberg, any words seem to be too pale to express my gratitude to you. Thanks for all your help!! I will never forget those help you gave me when I was in the difficulties. I feel very lucky to know you here. You are so sincere, warm-hearted, and helpful, and you are so active and optimistic! I am extremely impressed by your love for nature and life. Do you remember that fishing rod? I am thinking how to bring it back to China, so I can catch another big fish there…

Dear Margareta Andersson (Meta), I will never forget the apples you put on my writing desk when I was sick…You are always so kind, letting me feel I am at home instead of a place far away from my home country. You always consider

other people more than yourself, letting me feel so lucky to be in this country where people are so polite and elegant. And thank you very much for helping me with flow cytometry. Dear Ingrid Arvidsson, you are so kind and helpful. Whenever I need some help, you always tried your best to help me. I still kept those maps you drew for me when I was confusing to find some places. Whenever I saw them, I felt so warm in my heart… And thank you very much for helping me with immunofluorescence. Dear Ann-Marie Andreasson, you are always so active and energetic. Thank you for your help! When I put the order sheet on your bench, I always feel hearty thanks for your kind help to support my lab work…And thank you for arranging many things to make our working environment lovely. Dear Anne Sundblad, you are very kind. Thank you for a lot of interesting talking. Thank you very much for your encouragement and help. Dear Selina Parvin, I feel very happy to have met you here. You are very friendly and helpful. Sometimes I feel you are like my sister not only one of my colleagues. Thank you so much for your invitation to have Christmas Eve dinner with you. I enjoyed a lot of happiness: nice food, interesting talking and lovely Christmas environment… Thank you for giving me so many good memories! I also do appreciate all your help in the lab. Dear Margareta Söderquist, thank you very much for invitation to your lovely summer house and for your baked cakes. Dear Ninni Petersen, you are so nice and helpful. Thank you for your kindness and many help.

I also would like to thank Qingjun Zhu, my former colleague, although you stayed in our lab only for one year, I feel very lucky to have such a great colleague and many thanks come from my heart. You are such a sincere, friendly and helpful person! Thank you very much for sharing your precious lab experiences with me and for teaching me many things in the lab, which are so helpful for me. I extremely thank you for sharing your experiences on lab techniques and giving me a lot of wise advices. Dear Frida, you are such a confident, brave and sweet girl! You can always manage very well with both your work in the lab and your life out of the lab. Thank you for a lot of fun in the lab, for your encouragement and for your help to translate Swedish for me. Good luck with your thesis! Cheng Liu, thank you to let me know many things in this field and thank you very much for sharing your tourism experiences with me. Good luck with your thesis! Marit, my dear former colleague, you are a lovely and happy girl. Thank you for your “Holland Cookies”! Wenjuan Li, the new member in our lab, thank you for your Chinese TV play series.

My special thanks to Priya, my good friend, I feel very happy to have such a great friend during my stay here. We went through this long journey together,

sharing happy and difficult time, supporting and encouraging each other. My dear friend, thank you so much for all your help! Good luck with your future life! Dear Elaine, you like as my older sister, giving me so many support and consideration. Thank you for your talking and teaching. My dear friend Dexiu, you are so confident and brave girl. You always know what you should do and always do good job! Thank you for your help and encouragement. I am extremely impressed by your language talent, thank you for your interesting and beautiful letters…Good luck with your career in USA. Dear Dara, you are a wonderful friend, always so polite and friendly. Thank you for all your help and a lot of happy time. Dear Uli, I feel very lucky to be your roommate. Although we stayed only three months, I have so many good memories. I will never forget the happy time together with you and your friends. Liang Yu, you are very kind and helpful person. Thank you for your friendship. Also thank you very much for a lot of help and advices.

Xiongbiao Wang, you are so warm-hearted and helpful. Thank you very much for all your help, especially for helping me with moving, buying and setting new furniture. I do appreciate your encouragement when I was in difficulties. I hope that you have wonderful life in China. Mi Hou, you are such nice person and very helpful. I admire your scientific spirit. Thank you for a lot of help and wise advices. Chengyun Zheng, thank you very much for to be my external mentor. Thank you for your kindness and many help. Dear Monika Grudzinska, you are such an active, confident and optimistic girl! I am amazed by your capability of doing many different things at once, by your mastering a new language so quickly…You are so friendly and helpful. Thank you for a lot of help, especially for helping me with many language problems. Also thank you for a lot of interesting talking. Daniel Ketelhuth, you are so kind and friendly. I am impressed by your broad knowledge of science. Thank you so much for your discussions of protein issue and your wise advices. Also thank you very much for your encouragement. Yuri, thank for the jokes in the writing room and for your help in the lab. Thank you for all your advices and encouragement when I was in difficulties. Mohsen Karimi, you are very nice person and excellent scientist. Thank you for all your kindness. Jiajing, thank for your friendship and encouragement. Hong Xu, good to know you at CMM and thank you for your help in the lab and discussions. Rainier de Klark, you are very nice person, always helpful and friendly. Thank you very much for trying your best to find the reagent for my urgent use on certain weekend, and thank you for a lot of interesting talking during the lunch time. Klas and Nina, thanks so much for helping me taking pictures of my experimental results. Yu Ming, Yin-Choy

Chuan and Ming Gu, I am very happy to know all of you here and thank you for a lot of help.

I would like to thank all my colleagues at CMM. Especially, all the members in cardiovascular group, CMV group and HIV group on the 3rd floor. I worked with all of you for more than 3 years, enjoying a lot of wonderful time and lovely working environment. Thank you for all the help! Also, I would like to thank all the members of neurogenetics group on the ground floor. All of you are so friendly and helpful.

I express my thanks to Department of Medicine: Camilla Berg. You are so kind and helpful. Thank you very much for all your help with the administration process. My hearty thanks to Karolinska Institutet's library, thanks for providing me with such excellent study environment.

My dear Papa and Mama, no any words are enough to express my deepest thanks to both of you. You brought me to this world, making me grow up in a safe and pleasure sky. You always tried your best to make me happy and to give me the best you have. In my life tour, you always accompany me, take care of me, encourage me and support me! You are the best parents for me! If I was asked what the greatest happiness in my life is, the answer is only one: "I have the best parents in the world! Thanks God!"

Xi, you are one of the most important persons in my life. Without you, probably my life would go to another direction and I would not be here as well. Thank you for a lot of encouragement and support. Thank you for your care in your special way. Thank you for always remembering those special days and for those presents from you…

My thanks go to my dear aunts in China, Jianing and Jiazeng. Thank you very much for all you had done for me and for my parents! My hearty thanks to Professor Ao and Luhua in China, thank you so much for all your constant support and encouragement. My closest friend in China, Ninghong Pang, I feel very happy to have such a great friend, thank you very much for your friendship and a lot of help.

I am sure I forgot someone here but I hope you will understand that you were not less important than those who are listed above…

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