HBZ-induced functional deregulation of menin - new insights into the mechanism of telomerase activation during HTLV-1-mediated leukemogenesis

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N° national de thèse: 2013ENSL0828

THESE

En vue d'obtenir le grade de

Docteur de l'Université de Lyon, délivré par l'École Normale Supérieure de Lyon

Spécialité: Sciences de la vie

Laboratoire de Biologie Moléculaire de la Cellule CNRS UMR5239

Ecole Doctorale de Biologie Moléculaire, Intégrative et Cellulaire

Soutenue publiquement le 16 juillet 2013

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Acknowledgements

I am grateful to the members of the jury for accepting to judge my work. I thank the reviewers, Françoise Bex and Isabelle Jariel-Encontre, for careful reading of the manuscript, all the comments and remarks, which allowed me to improve it; as well as the examiners, Patrick Gaudray and Jean-Marie Peloponese for their eager acceptance of the invitation to Lyon.

I would like to express my gratitude to the members of my PhD committee, Franck Mortreux and Jean-Michel Mesnard for following the advancement of my work throughout my PhD and valuable discussions helping to orient the work in a good direction.

I thank Olivier Hermine from Necker Hospital in Paris and Naoki Mori from the University of the Ryukyus in Japan for providing the blood samples from ATL patients. I am also grateful to Benoit Barbeau and Emilie Larocque from the Université de Montréal for the collaboration on the APH-3 and APH-4.

Finally it is my pleasure to thank my PhD director, Madeleine Duc Dodon, for entrusting me this project, which strengthen my passion for science. I am very grateful for all these years of guidance and trust, which allowed me to do mature as an independent scientist.

I would like to thank Anne-Sophie Kuhlmann and Sophie Girard whose experiments gave the background to herein PhD project. I am particularly grateful to Anne-Sophie for passing me the baton in this research relay.

I would like also to express my gratitude to Pierre Jalinot and Renaud Mahieux for their reception in CEGOV and OR teams, work in which was for me a fantastic experience both on scientific and personal level. I thus thank all the members of both teams for their warm attitude, which created a great atmosphere to share the passion for science. "Merci" to Louis, for organizing the everyday work in the lab and his advices always coming in the good moment. I am especially indebted also to Chloé, Julien and Vincent for very stimulating discussions and giving me the opportunity to benefit from their knowledge. Warmest thanks to Morgan, Jean-Philippe, Armelle, Christelle, Sandrine and Estelle for

everyday bench support and sharing their technical secrets; Jocelyn, Julien, Anne, Sébastien, Morgan, Maname, Eléonore, Céline and Khanh for their always eager help with translations and various challenges of French bureaucracy, as well as creating this very friendly atmosphere during everyday work on neighbouring benches, providing me with such an enjoyable workplace during these several years.

I would like to thank also the stuff of the ENS platforms, whose trainings and advices also contributed to the advancement of this work. To Bariza Blanquier for the qPCR training; the PLATIM platform - especially great thanks to Olivier Hamant for helping me to discover the confocal microscopy and to Christophe Chamot and David Cluet for their helpful advices on the analysis of the images; Adélaïde Saint-Leger who performed with me the telomere length analysis; and all very friendly people that I met on the corridors of the ENS, always eager for scientific exchange.

Last but not least, I would like also to thank Morgan, Maname, and the Polish team Monika, Julia and Armel, for all the moments after work, which are adding this very warm memorable supplement to my French adventure. A special place in my memory is also reserved for the "couch surfers" Anna and Julien and the great trio Monica, Fabrice and Maxence, without whom the Lyon experience wouldn't be the same – muchas gracias! Great thanks also to the very international group of people with whom I shared the life of the PhD student during this several years, Michel, Robie, Emel, Kaja, Cristi and Adrian - thanks for simply being around.

Also great thanks to the "Le Chimistes", Jacek, Ollie, Guillaume, Jinping, Monica, Anna, Menaf, who accepted me as if I was a part of their team as well. Special thanks to Ollie for being a man "do tańca i do różańca", for scientific exchange, for sharing all the good and bad times of the PhD period, and much, much more.

Finally warm "dziękuję" to Jacek for sharing with me this adventure abroad and being the invaluable support during those four years. Dziękuję również mojej Rodzinie i Rodzinie Kolanowskich za nieocenione wsparcie i polskie paczki pozwalające przetrwać na ciężkiej żabiej diecie – jesteście kochani!

Mikrokosmos, Wisława Szymborska

Kiedy zaczęto patrzeć przez mikroskop,
powiało grozą i do dzisiaj wieje.
Życie było dotychczas wystarczająco szalone
w swoich rozmiarach i kształtach.
Wytwarzało więc także istoty maleńkie,
jakieś muszki, robaczki,
ale przynajmniej gołym ludzkim okiem
dające się zobaczyć.

A tu nagle, pod szkiełkiem,
inne aż do przesady
i tak już znikome,
że to co sobą zajmują w przestrzeni,
tylko przez litość można nazwać miejscem.

Szkiełko ich nawet wcale nie uciska,
bez przeszkód dwoją się pod nim i troją
na pełnym luzie i na chybił trafił.

Powiedzieć, że ich dużo - to mało powiedzieć.
Im silniejszy mikroskop,
tym pilniej i dokładniej wielokrotne.

Nie mają nawet porządnych wnętrzności.
Nie wiedzą, co to płeć, dzieciństwo, starość.
Może nawet nie wiedzą czy są - czy ich nie ma.
A jednak decydują o naszym życiu i śmierci.

Niektóre zastygają w chwilowym bezruchu,
choć nie wiadomo, czym dla nich jest chwila.
Skoro są takie drobne,
to może i trwanie
jest dla nich odpowiednio rozdrobnione.

Pyłek znoszony wiatrem to przy nich meteor
z głębokiego kosmosu,
a odcisk palca - rozległy labirynt,
gdzie mogą się gromadzić
na swoje głuche parady,
swoje ślepe iliady i upaniszady.

Od dawna chciałam już o nich napisać,
ale to trudny temat,
wciąż odkładany na potem
i chyba godny lepszego poety,
jeszcze bardziej ode mnie zdumionego światem.
Ale czas nagli. Piszę.

wszystkim tym
zdziwionym światem
choć odrobinę

Microcosmos, Wisława Szymborska

When we first started looking through microscopes
a cold fear blew and it's still blowing.
Life hitherto had been frantic enough
in all its shapes and dimensions.
Which is why it created small-scale creatures,
assorted tiny worms and flies,
but at least the naked human eye
could see them.

But then suddenly beneath the glass,
foreign to a fault
and so petite,
that what they occupy in space
can only charitably be called a spot.

The glass doesn't even touch them,
they double and triple unobstructed,
with room to spare, willy-nilly.

To say they're many isn't saying much.
The stronger the microscope
the more exactly, avidly they're multiplied.

They don't even have decent innards.
They don't know gender, childhood, age.
They may not even know they are—or aren't.
Still they decide our life and death.

Some freeze in momentary stasis,
although we don't know what their moment is.
Since they're so minuscule themselves,
their duration may be
pulverized accordingly.

A windborne speck of dust is a meteor
from deepest space,
a fingerprint is a farflung labyrinth
where they may gather
for their mute parades,
their blind iliads and upanishads.

I've wanted to write about them for a long while,
but it's a tricky subject,
always put off for later
and perhaps worthy of a better poet,
even more stunned by the world than I.
But time is short. I write.

to all astonished with the world
at least just a bit

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Abstract

Adult T-cell leukemia (ATL) is an aggressive lymphoproliferative disorder associated with human T-cell leukemia virus type 1 (HTLV-1) infection. Reactivation of telomerase, a critical event in tumor progression observed in the late phases of ATL development, has been shown to be caused by HBZ (HTLV-1 bZIP factor), a regulatory protein encoded by the negative strand of the HTLV-1 genome. The HBZ-mediated up-regulation of the telomerase catalytic subunit is dependent on JunD, which in the cellular context occurs in the complex with menin, the product of the *MEN-1* tumor suppressor gene. Interaction with menin represses JunD-dependent transcription and converts JunD into a growth suppressor, whereas it acts as a growth promoter in the absence of menin.

My results demonstrate that the viral protein HBZ abrogates the tumor suppressor function of menin, resulting in the activation of JunD transcriptional activity and finally in the up-regulation of its target gene, the human telomerase reverse transcriptase (*hTERT*). I showed that HBZ, JunD and menin can coexist in the same protein complex and that HBZ and menin exert opposite effects on JunD transcriptional activity. Moreover menin inhibits the JunD-mediated activation of the *hTERT* proximal promoter and HBZ is able to counteract this effect. Finally, I proposed that HBZ, by recruiting p300 histone acetyltransferase, reverses the histone deacetylation conducted by menin-recruited HDACs and therefore up-regulates the expression of the *hTERT* gene.

Altogether, my work led to the identification of the molecular mechanism leading to the functional impairment of the menin tumor suppressor, which results in the deregulation of AP-1 signaling in HTLV-1 infected cells. Finally this work gave new insights into the mechanism of the transcriptional up-regulation of the *hTERT* gene upon HTLV-1 infection, being a key event during the development of Adult T-cell leukemia and a necessary step towards the progression into more aggressive courses.

Key words: HTLV-1, HBZ, menin, tumor suppressors, AP-1 signaling, telomerase, *hTERT*, leukemogenesis

Résumé

La leucémie T de l'adulte (ATL) est une pathologie lympho-prolifératives aiguë associée à l'infection par le virus HTLV-1 (human T-cell leukemia virus type 1). La réactivation de la télomérase observée lors de la phase tardive du développement de l'ATL est un évènement crucial dans la progression tumorale. Elle est induite au niveau transcriptionnel par la protéine HBZ (HTLV-1 bZIP factor), une protéine régulatrice encodée par le brin antisens du génome viral. L'activation du gène codant pour la sous-unité catalytique de la télomérase par HBZ est dépendante du facteur de transcription JunD. Ce dernier est normalement associé en complexe avec le produit du gène suppresseur de tumeur *MEN-1*, la ménine, dont l'interaction avec JunD réprime la transcription JunD-dépendante et convertit JunD en inhibiteur de croissance, alors qu'il joue le rôle d'activateur de croissance en absence de ménine.

Mes résultats démontrent que la protéine virale HBZ inhibe la fonction suppresseur de tumeur de la ménine, induisant l'activité transcriptionnelle de JunD et donc l'activation de la transcription de son gène cible : la transcriptase inverse télomérase humaine (*hTERT*). J'ai démontré que HBZ, JunD et la ménine peuvent coexister dans un même complexe protéique, et que HBZ et la ménine ont des effets opposés sur l'activité transcriptionnelle de JunD. En effet la ménine inhibe l'activation du promoteur proximal de *hTERT* par JunD, alors que HBZ est capable de contre balancer cet effet. Finalement, je propose qu'en recrutant l'histone acétyltransférase p300, HBZ réverse la déacétylation des histones induite par le recrutement des HDACs par la ménine, et par conséquent active le promoteur d'*hTERT*.

L'ensemble de ces résultats a permis d'identifier les mécanismes moléculaires aboutissant à l'inhibition fonctionnelle de la protéine suppresseur de tumeur ménine, résultant en la dérégulation de la voie AP-1 dans les cellules infectées par HTLV-1. Finalement, ce travail apporte de nouvelles précisions sur le mécanisme de la surexpression transcriptionnelle de la télomérase lors de l'infection par HTLV-1, une étape importante de la mise en place et du développement de la leucémie T de l'adulte vers des stades plus agressifs.

Mots clés : HTLV-1, HBZ, ménine, suppresseur de tumeur, voie AP-1, télomérase, *hTERT*, leucémie

List of abbreviations

AP-1	*activator protein 1*
APH	*antisens protein of HTLV*
ASK	*activator of S-phase kinase*
ATF	*activating transcription factor*
ATL	*adult T-cell leukemia*
bHLH	*basic helix loop helix*
bZIP	*basic leucine zipper*
CDKI	*cyclin dependent kinase inhibitor*
CHES1	*checkpoint suppressor 1*
CRE	*cyclic adenosine monophosphate-response element*
CREB	*CRE binding protein*
ERE	*estrogen responsive element*
ERK	*extracellular-signal-regulated kinases*
ERα	*estrogen receptor-alpha*
FANCD2	*Fanconi anemia, complementation group D2*
Foxo1	*forkhead box O1*
FoxP3	*forkhead box P3*
GABP	*GA binding protein*
GFAP	*glial fibrillary acidic protein*
HAT	*histone acetyltransferase*
HBV	*hepatitis B virus*
HBZ	*HTLV-1 bZIP factor*
HDAC	*histone deacetylase complex*
HMT	*histone methyltransferase*
hTERT	*human telomerase reverse transcriptase*
HTLV	*human T-cell leukemia virus*
hTR	*human telomerase RNA*
IGFBP-2	*insulin-like growth factor binding protein 2*

INK	*inhibitor of cyclin-dependent kinase*
JNK	*Jun N-terminal kinase*
LEDGF	*lens epithelium derived growth factor*
LTR	*long terminal repeat*
MAPK	*mitogen-activated protein kinase*
MEF	*mouse embryonic fibroblasts*
MEN-1	*multiple endocrine neoplasia type 1*
MI	*muscle initiator*
MLL	*mixed lineage leukemia*
NF-κB	*nuclear Factor kappa B*
NFY	*nuclear factor Y*
NLS	*nuclear localization signal*
Nm23	*nonmetastatic gene 23*
NMHC II-A	*nonmuscle myosin II-A heavy chain*
NRSE	*neuron-restrictive silencer element*
PAX5	*paired box protein 5*
PBMC	*peripheral blood mononuclear cells*
Pem	*placenta and embryonic expression*
PKC	*protein kinase C*
pRb	*retinoblastoma protein*
PTLV	*primate T-cell leukemia virus*
RPA2	*subunit of replication protein A*
Rq-Trap	*real time quantitative telomeric repeat amplification protocol*
Runx2	*runt-related transcription factor 2*
shRNA	*short hairpin RNA*
Sin3A	*SIN3 homolog A*
SINE	*short interspersed nuclear element*
Smad	*mothers against decapentaplegic homolog*
Sp1	*Specificity protein 1*
TAL1	*T-cell acute lymphoblastic leukemia 1*
Tax	*transcriptional activator of pX region*
TFIID	*transcription factor II D*
TGF-β	*transforming growth factor-β*
TRE	*TPA responsive element*
TSA	*trichostatin A*
TSP/HAM	*tropical spastic paraparesis / HTLV-1-associated myelopathy*
TSS	*transcription start site*
TxRE	*Tax responsive element*
UTR	*untranslated region*

Part 1

Bibliographical studies

General introduction

Human T-cell leukemia virus type 1 (HTLV-1) is the first human retrovirus associated with cancer. After a long latent period a subset of infected individuals develop adult T-cell leukemia (ATL) (Yoshida 2005). A key step in HTLV-1 induced leukemogenesis is the induction of abnormal T-cell growth, and the transformation of target lymphocytes. To achieve clonal expansion, HTLV-1 infected cells must acquire an increased capacity to replicate their DNA and telomeric ends (Bellon and Nicot 2007). This is achieved by the activation of the telomerase, which is in fact a common mechanism during cellular transformation and carcinogenesis provoked by multiple human DNA and RNA tumor viruses (Bellon and Nicot 2008). Consistent with this, HTLV-1 transformed cell lines and the leukemic cells from ATL patients are characterized by increased telomerase activity. Moreover patients with acute and chronic ATL exhibit higher level of telomerase expression than asymptomatic carriers or those with smoldering ATL, thus there is a direct correlation between telomerase activity and the stage of ATL progression (Uchida, Otsuka et al. 1999; Kubuki, Suzuki et al. 2005). Kuhlmann *et al.* previously reported that HTLV-1-induced activation of telomerase occurs *via* up-regulation of the catalytic subunit of telomerase (*hTERT*) dependent on the HBZ viral protein (Kuhlmann, Villaudy et al. 2007). The HBZ-mediated transcriptional activation of *hTERT* rely on cellular AP-1 transcription factor, JunD, allowing for HBZ recruitment to the proximal *hTERT* promoter (Kuhlmann, Villaudy et al. 2007).

In normal cells *hTERT* expression is a subject of multiple levels of control by tumor suppressors like p53 and retinoblastoma (pRB) as well as Mad1/c-Myc and TGF-β

pathways, and direct *hTERT* repressors including menin (Cong, Wright et al. 2002; Ducrest, Szutorisz et al. 2002; Lin and Elledge 2003).

Traditionally cancer is understood as a disorder of abnormal cell proliferation controlled by a series of mutations, typically affecting proto-oncogenes or tumor suppressor genes, which confer the growth advantage. Indeed compelling evidence supports an important role for altered expression of tumor suppressors in the development of ATL as well as the progression of the disease from the chronic/smoldering to the acute/lymphoma type. The main genetic events have been reported to center around the cyclin dependent kinase inhibitors (CDKIs), as well as p53 and pRb proteins (Hatta and Koeffler 2002). In addition to genetic mutations multiple tumor suppressors were also reported to undergo a functional inactivation in the course of ATL development. As such *TP53* gene, encoding p53 tumor suppressor, is mutated only in a small subset of leukemic cells, however additional mechanisms render this protein inactive in most of the ATL cases. The function of p53 is inhibited by Tax *via* diverse mechanisms including constitutive phosphorylation of p53 at specific serine residues, sequestration of p300/CBP coactivators by Tax and also multiple indirect effects involving NF-κB and CREB/ATF pathways (Tabakin-Fix, Azran et al. 2006). Also tumor suppressor pRb, is inactivated in HTLV-1-infected cells, involving mechanisms conferring its proteasomal degradation (Kehn, Fuente Cde et al. 2005).

The interference with the functions of tumor suppressors, playing a role in the regulation of cell-cycle progression, apoptosis and DNA repair; strongly contributes to the leukemogenic process mediated by HTLV-1 and is the driving force fueling the development and the progression of the adult T-cell leukemia.

Herein PhD work addresses the role of menin tumor suppressor in the development of ATL in order to better understand the HTLV-1-mediated pathogenesis and reveal the molecular mechanisms leading to leukemogenic process. I investigated menin expression and function upon HTLV-1 infection, and the significance of their deregulation in the leukemogenesis.

Menin, encoded by Multiple Endocrine Neoplasia type 1 (*MEN-1*) gene, plays a pivotal role in the regulation of gene expression, cell proliferation, apoptosis and genome stability, which are often malfunctioning in cancer cells (Balogh, Racz et al. 2006; Yang and Hua 2007). Among the known functions of menin, I focused on its role in the

transcriptional regulation of telomerase reverse transcriptase (*hTERT*) (Lin and Elledge 2003), highly implicated in the development and progression of ATL (Uchida, Otsuka et al. 1999; Lin and Elledge 2003).

Before presenting the experimental data, I am going to introduce the domain of research by discussing the state of the art. First I am going to summarize the knowledge on HTLV-1 and associated pathologies and the role of viral proteins in the development of the adult T-cell leukemia, focusing on the HBZ antisense protein. Subsequently I will describe the function of the telomerase in tumorigenesis and the transcriptional regulation of its catalytic subunit (*hTERT*) in normal cells. I will also present the knowledge on the deregulation of *hTERT* gene expression during the infection with HTLV-1, which gave a background to herein PhD work. In the last part of the bibliographical introduction I will introduce menin, including the information on the regulation of its expression, structure of the protein and its function as a tumor suppressor, focusing on the relationship with JunD transcription factor.

Chapter 1

Human T-cell leukemia virus type 1 and associated pathologies

Human T-cell leukemia virus type 1 (HTLV-1) is the first human retrovirus identified 30 years ago (Poiesz, Ruscetti et al. 1980; Yoshida, Miyoshi et al. 1982). HTLV-1 belongs to *Deltaretrovirus* genus and together with HTLV-2, HTLV-3, HTLV-4 and their simian counterparts has been classified to the group of Primate T lymphotropic viruses (PTLV), sharing a number of epidemiological and molecular characteristics.

HTLV-1 is the causative agent of adult T-cell leukemia (ATL) (Uchiyama, Yodoi et al. 1977) and neurological disorder TSP/HAM (tropical spastic paraperesis/HTLV-1-associated myelopathy) (Jacobson, Raine et al. 1988). HTLV-1 infection was also associated with a variety of other hematological and nonhematological disorders including: uveitis, dermatitis, ocular lesions, inflammatory arthropathy and polymyositis (Lairmore, Haines et al. 2012).

ATL is an aggressive tumor malignancy characterized by monoclonal or oligoclonal proliferation of $CD4^+$ $CD25^+$ T-cells (Wattel, Cavrois et al. 1996). This lymphoproliferative disorder develops after a long latent period in a subset of 6% (males) to 2% (females) of HTLV-1 infected individuals (Taylor and Matsuoka 2005). ATL is a heterogeneous disease clinically divided into four subtypes: chronic, smoldering, acute and lymphoma. The acute and lymphoma subtypes are the most aggressive, and their prognosis is poor, with less than a year survival following the onset of the first symptoms (Goncalves, Proietti et al.

2010). ATL manifests in the adult life and its characterized by presentation of skin lesions, lymphadenopathy, hepatosplenomegaly, elevated counts of white blood cells and the presence of abnormal lymphoid cells with T-helper phenotype and deeply indented or multilobulated nuclei called flower cells (Takatsuki 2005).

Currently, the estimated number of people infected with HTLV-1 worldwide is between 15 and 25 millions. The areas of the highest prevalence of HTLV-1 include Japan, Africa, the Caribbean islands, and Central and South America (Proietti, Carneiro-Proietti et al. 2005) (Figure 1). There are three major routes of HTLV-1 transmission: (1) mother-to-infant transmission (mainly through breastfeeding), (2) sexual intercourse, and (3) blood contact (Proietti, Carneiro-Proietti et al. 2005).

The second member of HTLV serotype, the HTLV-2, has been for the first time identified in 1982 in the cell line established from leukemic cells isolated from a patient suffering from atypical hairy T-cell leukemia, however an association of this pathology

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Figure 1. Estimated geographic distribution of HTLV-1-infected cases. The HTLV-1 prevalence rates have been stratified into high with more than 5% prevalence in the population (emphasized with asterisk), middle (5% to 1%), and low (less than 1%). From (Goncalves, Proietti et al. 2010).

with HTLV-2 infection has not been clearly demonstrated so far (Kalyanaraman, Sarngadharan et al. 1982). HTLV-2 is prevalent in Central and West Africa, in native Amerindian populations in America and among cohorts of intravenous drug users in the United States and Europe (Manns and Blattner 1991). HTLV-2 carriers were shown to develop a neurological disorder clinically similar to TSP/HAM, however exhibiting a slower outcome than the TSP/HAM induced by HTLV-1 (Araujo and Hall 2004). HTLV-2 shares approximately 70% nucleotide sequence homology with HTLV-1 and exhibit similar overall genomic organization (Shaw, Gonda et al. 1984). Similarly to HTLV-1, this virus is also targeting T-lymphocytes and it induces their proliferation. Therefore HTLV-2 infected individuals exhibit persistently higher lymphocyte and platelets counts however no cancer syndrome has been identified so far among HTLV-2 carriers (Kwaan, Lee et al. 2006; Bartman, Kaidarova et al. 2008).

Two more strains of HTLV were recently discovered, the HTLV-3 and HTLV-4. The analysis of a complete sequence of HTLV-4 provirus published in 2009 revealed that this strain is the closest to HTLV-2 (Switzer, Salemi et al. 2009), whereas HTLV-3 was shown to be more closely related to its simian counterpart, the STLV-3 (Calattini, Chevalier et al. 2005; Wolfe, Heneine et al. 2005). For the moment no pathology has been associated with the infection by HTLV-3 and HTLV-4 (Thomas, Perzova et al. ; Duong, Jia et al. 2008), however until now only 4 individuals infected with HTLV-3 were identified (Calattini, Chevalier et al. 2005; Wolfe, Heneine et al. 2005; Calattini, Betsem et al. 2009; Zheng, Wolfe et al. 2010), while the HTLV-4 type consists only of a unique human strain, whose provirus was found in the PBMCs obtained from a hunter living in Cameroon (Wolfe, Heneine et al. 2005).

1.1. Viral oncogenes and mechanisms of HTLV-1-mediated leukemogenesis

1.1.1. HTLV-1 encoded proteins

HTLV-1 genome is composed of a single-strand diploid RNA and have a similar structure to genomes of other retroviruses, with *gag*, *pro-pol* and *env* genes, flanked by long terminal repeats (LTRs) (Figure 2). The whole sequence of HTLV-1 proviral genome, of approximately 9kb, was reported for the first time in 1983 (Seiki, Hattori et al. 1983). It

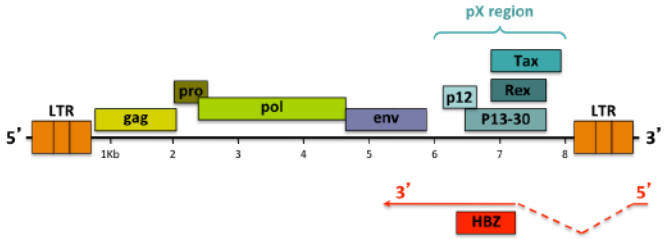

Figure 2. Genomic organization of the HTLV-1 provirus. The *gag*, *pro-pol* and *env* structural genes are flanked by 5' and 3' LTRs. The open reading frames of the pX region, encoding Tax, Rex, p12, p13 and p30 regulatory and accessory proteins, as well as the ORF of HBZ antisense protein are shown.

encodes structural and regulatory proteins and enzymes required for the virus replicative cycle. Key structural proteins include nucleocapsid (p15/NC), capsid (p24/CA) and matrix (p19/MA) all encoded from the *gag* (group-specific antigen) gene (Figure 3). The *env* open reading frame encodes proteins that mediate binding to the surface receptors and subsequent fusion with the cellular membrane of the target cell, enabling viral entry, namely the surface gp46 and transmembrane gp21 glycoproteins. The *pro-pol* gene encodes enzymatic components necessary for viral replication and integration to the host genome, including: integrase (IN), reverse transcriptase (RT) and protease (Pro) (Lairmore, Haines et al. 2012).

A unique sequence in the HTLV-1 genome was found between *env* and 3'LTR and is known as the pX region. This part of HTLV-1 genome contains several open reading frames that encode regulatory and nonstructural proteins implicated in viral infectivity and proliferation of infected cells, including: Tax (p40), Rex (p27), p12, p13, p30 and p21 (Nicot, Harrod et al. 2005). The mRNAs transcribed from the pX region are alternatively spliced and generally less expressed comparing to transcripts of the structural proteins. More recently, also the complementary minus strand RNA of the pX region has been found to encode an antisense protein, the HTLV-1 basic leucine zipper factor (HBZ) (Gaudray, Gachon et al. 2002).

Among viral proteins, the key oncogenic property was attributed to 40kDa Tax (transcriptional activator of pX region) and 25kDa HBZ translated from the antisense HTLV-1 transcript (Bex, Murphy et al. 1999; Matsuoka and Green 2009; Lodewick, Lamsoul et al. 2011).

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Figure 3. Structure of the HTLV-1 viral particle. The virus core components, including nucleocapsid (p15/NC), capsid (p24/CA) and matrix (p19/MA) proteins, are surrounding the single-stranded diploid RNA genome and enzymatic proteins encoded by *pro-pol*. Transmembrane glycoprotein gp21 and surface gp46 embedded in lipid bilayer are forming viral envelope, critical for viral infectivity. Taken from (Lairmore, Haines et al. 2012).

1.1.2. Tax and HBZ - main players in HTLV-1-mediated oncogenesis

Development of ATL is a multistage process involving several factors. Therefore a long clinically latent period, lasting about 60 years, is needed before the onset of ATL. The mechanisms behind the HTLV-1-induced leukemogenesis are not yet fully understood, however the main transforming entity has been attributed to Tax and HBZ regulatory

proteins. Interestingly, Tax and HBZ have distinct, often even opposite, effects on the regulation of viral and host transcription and the cellular signaling pathways (Table 1). Tax activates 5'LTR transcription; enhances AP-1 and classical NF-κB signaling, and suppresses TGF-β pathway (Bex and Gaynor 1998). In the contrary, HBZ suppresses Tax-mediated transcription form the 5'LTR and activates its own expression from the 3'LTR; inhibits activated AP-1 and classical NF-κB signaling, at the same time enhancing TGF-β pathway (Matsuoka and Green 2009). Moreover both Tax and HBZ are promoting cell proliferation, however in the context of HBZ, rather RNA is implicated in this process than HBZ protein, indicating, distinct regulatory mechanisms of cell cycle control used by Tax and HBZ. HTLV-1 may take an advantage of this distinct regulatory mechanisms employed by Tax and HBZ, and by fine modulation of cellular signaling pathways, precisely induce the transformation and allow for better survival of HTLV-1-infected cells and escape from the immune surveillance (Lairmore, Haines et al. 2012).

Property	Tax	HBZ
Expression in ATL cells	~40%	100%
Role in the form of RNA transcript	ND	Yes
HTLV-1 5'LTR transcription	Activation	Inhibition
HTLV-1 3'LTR transcription	Activation	Activation
Target of CTL response	Yes	No
Effect on host immune response	Enhancement	Suppression
Immortalization of T cells	Yes	No
Induction of T cell proliferation	Yes	Yes
Classical NF-κB pathway	Activation	Inhibition
Alternative NF-κB pathway	Activation	No effect
AP-1 signaling	Activation	Inhibition
JunD transcription	Activation	Activation
TGF-β signaling	Inhibition	Activation

Table 1. Properties exhibited by Tax and HBZ viral oncogenes. Tax and HBZ show distinct expression profiles throughout the ATL development process, exert also distinct, often opposite, biological functions. Tax and HBZ are implicated in the regulation of viral replication, modulation of host immune response, deregulation of cell cycle progression and cellular signaling pathways, therefore leading to cellular transformation while assuring viral persistence. Adapted from (Zhao and Matsuoka 2012).

Tax is recognized as an oncoprotein, since it immortalizes primary T-cells and confers proliferative properties to HTLV-1-infected cells. In the contrary to Tax, HBZ seems to be dispensable for viral-induced immortalization process, as disruption of HBZ protein in an infectious proviral clone had no effect on the ability of the virus to immortalize T-lymphocytes in tissue culture (Arnold, Yamamoto et al. 2006). However HBZ promotes cell proliferation and enhance viral infectivity and persistence (Arnold, Zimmerman et al. 2008). The loss of HBZ resulted in a significant reduction of proviral load in a rabbit model of infection, even very early following the infection, suggesting an important role of HBZ in establishing the chronic infection and maintaining viral persistence, from the very early stages post-infection (Arnold, Yamamoto et al. 2006).

Taken together, the multiple functions of Tax and HBZ render these proteins predominant in the leukemogenic process leading to the ATL development. However, despite its pleiotropic functions during HTLV-1 mediated leukemogenesis, Tax expression is frequently lost in ATL cells, while the expression of HBZ is consistently detected in all ATL cells (Satou, Yasunaga et al. 2006). This property is due to the unique location of the *HBZ* gene on the negative strand of the provirus (Gaudray, Gachon et al. 2002). Therefore, HBZ transcription is regulated by a promoter within the 3' long terminal repeat (LTR) rather than by the 5'LTR promoter that is responsible for transcription of all other HTLV-1 genes (Yoshida, Satou et al. 2008).

While 3'LTR remains intact, the 5'LTR is often deleted (Tamiya, Matsuoka et al. 1996) or methylated in ATL cells (Koiwa, Hamano-Usami et al. 2002; Takeda, Maeda et al. 2004; Taniguchi, Nosaka et al. 2005), thereby eliminating the expression of all viral proteins encoded by the sense strand of the provirus, including Tax. Additionally Tax expression in ATL cells is disrupted by several complementary mechanisms, including genetic changes within the *tax* gene - deletions and insertions as well as nonsense mutations, e.g. caused by APOBEC3A (Takeda, Maeda et al. 2004; Fan, Ma et al. 2010). It was also recently shown, that PDLIM2 ubiquitin E3 ligase, is promoting degradation of Tax, mediating its polyubiquitination and recruiting Tax from its functional sites into the nuclear matrix where the polyubiquitinated Tax is degraded by the proteasome (Fan, Ma et al. 2010).

Therefore, due to above-mentioned genetic and epigenetic changes in the HTLV-1 provirus, supported by proteasomal Tax degradation mechanisms, the Tax expression is

not detectable in approximately 60% of ATL cases (Tamiya, Matsuoka et al. 1996; Takeda, Maeda et al. 2004). In contrast, repressive modifications are not known to occur within HBZ coding sequence, and analyses of HTLV-1 provirus showed that the 3' LTR is not deleted and remains unmethylated in ATL cells (Saito, Matsuzaki et al. 2009; Fan, Ma et al. 2010). Thus HBZ expression has been consistently detected in all ATL cell lines and cells freshly isolated from ATL patients (Gaudray, Gachon et al. 2002; Murata, Hayashibara et al. 2006; Satou, Yasunaga et al. 2006). In addition, expression of the *HBZ* gene is detected also in HTLV-1-infected asymptomatic carriers.

These observations stay in accordance with the studies on kinetics of viral gene expression in PBMCs from newly infected rabbits. They revealed that *tax/rex* and *gag/pol* mRNAs were expressed at the highest levels immediately after infection and then progressively declined over time, eventually stabilizing at low levels (Li, Kesic et al. 2009). Conversely, *HBZ* was expressed at a low level early after infection, and increased to reach a plateau, having the highest expression level of all mRNA measured (an average of nine fold more represented than *tax* mRNA) (Li, Kesic et al. 2009).

Furthermore, it has been reported that HBZ expression level is well correlated with the amount of integrated provirus in HTLV-1 infected individuals, while Tax expression does not correlate with the proviral load (Usui, Yanagihara et al. 2008; Saito, Matsuzaki et al. 2009). Importantly the *HBZ* transcription levels were also shown to correlate with the severity of HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Saito, Matsuzaki et al. 2009).

Taken together, unlike other viral proteins encoded by the sense strand of the HTLV-1 provirus, the *HBZ* gene is constantly present in ATL cells from all patients and in many ATL cases, *HBZ* is the only viral gene that is expressed. Therefore HBZ is believed to be indispensable both during the initial stages of ATL development as well as during the maintenance of transformed phenotype.

1.1.3. Leukemogenic process provoked by HTLV-1

The current view on the leukemogenic process provoked by HTLV-1, assumes that ATL arises in four stages, which are first acute viral infection, followed by polyclonal

proliferation of infected cells, then clinical latency and finally tumorigenesis (Figure 4). Tax is believed to be important in the first two stages towards development of ATL, as it activates viral transcription and stimulates cell proliferation by triggering changes in a variety of intracellular signal transduction pathways, deregulates mitotic checkpoints and inactivates tumor suppressors (Peloponese, Kinjo et al. 2007; Matsuoka and Jeang 2011). However, Tax appears to be expendable during the late stages of disease. Moreover since Tax is a major target of cytotoxic T lymphocytes its extended expression is highly unfavorable for viral persistence (Kannagi, Harada et al. 1991).

On the other hand *HBZ* gene, which is constantly expressed seems to be important throughout the whole process of ATL development, contributing to the oncogenesis *via*

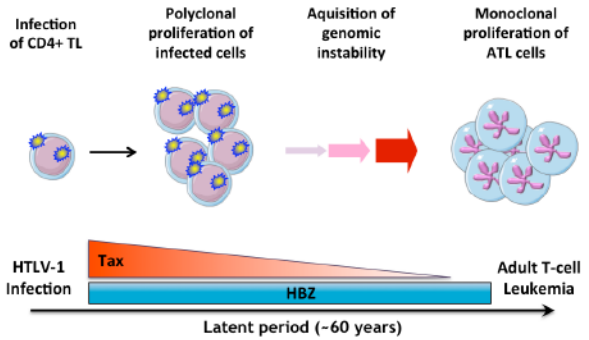

Figure 4. Schematic model of adult T-cell leukemia development. HTLV-1 infects $CD4^+$ T cells *via* direct cell-to-cell contact. Infected cells express viral oncogenes: Tax and HBZ, which are promoting clonal proliferation of HTLV-1 positive T cells. During the latency period, the genetic and epigenetic alterations accumulate in the host genomes. The late phase of ATL is characterized by monoclonal/oligoclonal proliferation of transformed cells. Throughout the multistage process of ATL development HBZ viral protein is constitutively expressed, while Tax expression is inactivated and Tax expressing cells are eliminated by host immune system. This suggests that Tax is only important in the initial stages of leukemogenesis, while HBZ plays important roles in all phases. Figure modified after (Yasunaga and Matsuoka 2007).

promoting cell growth and destabilizing cellular pathways (Basbous, Arpin et al. 2003; Arnold, Zimmerman et al. 2008; Zhao, Yasunaga et al. 2009). The increasing data on HBZ role in HTLV-1 mediated pathogenicity imply that HBZ can be considered as a key player in HTLV-1 mediated leukemogenesis, being important both in the initial stages after viral infection (possibly also in the carrier state) as well as after establishing leukemogenic phenotype, with the cumulation of its function in the late stages of ATL development. HBZ seems to be a necessary prerequisite for the malignant transformation of infected cells and is essential for the maintenance of oncogenesis. Therefore, despite the fact that the reports on the role of Tax in HTLV- mediated leukemogenesis are more numerous, as it was discovered more than 20 years before we knew about the existence of HBZ, we can not neglect the importance of HBZ and further studies are necessary to fully understand the HTLV-1 oncogenic process.

1.2. HTLV-1 antisense protein

HBZ (HTLV-1 bZIP factor) is an antisense protein encoded by the negative strand of HTLV-1 provirus. The phenomenon of antisense transcription was first detected in HTLV-1 infected cell lines in 1989 (Larocca, Chao et al. 1989). While the presence of a protein was confirmed in HTLV-1-transformed cell lines a decade after the first reports on the discovery of HTLV-1 antisense mRNA. HBZ was then described as a binding protein to CREB-2 (cAMP-response element binding protein-2) (Gaudray, Gachon et al. 2002).

1.2.1. Transcription of *HBZ* gene

The HBZ-coding region is located between Tax exon 3 and Env exon 2 in the antisense strand (Gaudray, Gachon et al. 2002). Two HBZ transcripts are produced, the spliced and unspliced one giving rise to sHBZ and usHBZ of 206 and 209 amino acids respectively (Cavanagh, Landry et al. 2006; Murata, Hayashibara et al. 2006; Satou, Yasunaga et al. 2006). Consequently, these isoforms share >95% amino acid sequence identity, and differ only in 4-7aa at their N terminus (Murata, Hayashibara et al. 2006).

sHBZ has multiple transcriptional initiation sites in the U5 and R regions of the 3' LTR, whereas the us*HBZ* gene initiates within the *tax* gene (Cavanagh, Landry et al. 2006;

Yoshida, Satou et al. 2008). Detailed sequence analysis revealed that sHBZ antisense transcript is spliced at positions 367 of the antisense strand and joined to an internal region of the HBZ ORF at position 1767 (Figure 5), leading to an intronic region of 1400 nt (Cavanagh, Landry et al. 2006). Translation initiates in the exon 1, the PolyA was positioned at a distance of 1450nt from the codon stop (Cavanagh, Landry et al. 2006). Promoters for both the sHBZ and usHBZ genes are TATA-less (Yoshida, Satou et al. 2008). Detailed studies showed that the transcription of sHBZ is dependent on the constitutively expressed transcription factor Sp1, binding to U5 region. Moreover the activities of both sHBZ and usHBZ gene promoters were upregulated by Tax through Tax responsible elements (TREs), in the U3 region of 3' LTR. However since ATL cells frequently lose the expression of Tax due to genetic or epigenetic changes in the HTLV-1 provirus, the influence of Tax on the transcription of HBZ is likely limited in ATL cells (Yoshida, Satou et al. 2008). In agreement with this hypothesis Yoshida et al. reported that the promoter activity of the sHBZ gene was much higher than that of the usHBZ gene, presumably due to additional role of Sp1 factors in the expression of sHBZ isoform (Yoshida, Satou et al. 2008).

Importantly the expression of HBZ is a subject of auto-stimulatory loop, as recently it was demonstrated that HBZ up-regulates its own promoter (Gazon, Lemasson et al. 2012). This HBZ mediated stimulation of 3'LTR was dependent on heterodimerization with JunD and additionally required Sp1 factors. Suggesting that HBZ is recruited to its own promoter thanks to JunD, which in turn, associates with cellular Sp1 factors at 3' LTR promoter (Gazon, Lemasson et al. 2012).

The two isoforms of the *HBZ* gene are not equally expressed in ATL cells. The expression level of the s*HBZ* gene was found to be four times higher than that of the us*HBZ* gene in both ATL patients and HTLV-1 carriers (Usui, Yanagihara et al. 2008), which may be a direct result of a stronger promoter activity of the *sHBZ* gene reported by Yoshida. In addition, the half-life of the sHBZ protein isoform is longer than that of the usHBZ isoform (Yoshida, Satou et al. 2008). Therefore in ATL cells, only sHBZ protein could be detected on the protein level (Arnold, Zimmerman et al. 2008; Usui, Yanagihara et al. 2008), indicating significantly higher prevalence of spliced HBZ isoform. Additionally the proteins derived from the spliced and the unspliced HBZ exhibit some differences on the functional level, despite the fact that they differ only by seven amino acids on their

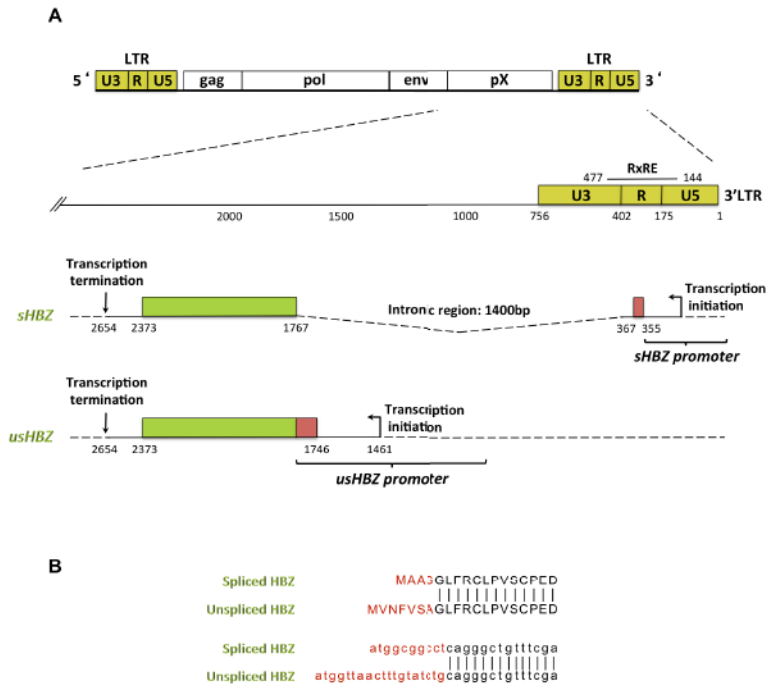

Figure 5. A. Transcription map of *HBZ*. Two alternatively spliced *HBZ* transcripts are shown. The U5 and a part of R region of 3'LTR compose the promoter for the *HBZ* gene. The first exon of the spliced *HBZ* gene corresponds to the region that encodes the Rex responsive element (RxRE). HBZ: HTLV-1 bZIP factor, sHBZ: spliced HBZ, usHBZ: unspliced HBZ , proteins encoded by each mRNA are represented as green boxes. Figure adapted from: (Journo, Douceron et al. 2009; Matsuoka and Green 2009). **B. Amino acid and nucleotide sequence** at the N-terminus of two HBZ isoforms. The differences in the proteins derived from the spliced and the unspliced HBZ are 4 amino acids in the spliced HBZ and 7 amino acids in the unspliced HBZ (Cavanagh, Landry et al. 2006).

N-terminus. Namely sHBZ show a stronger suppression of Tax-mediated transcriptional activation through the 5' LTR than usHBZ (Yoshida, Satou et al. 2008). Additionally sHBZ RNA promotes T-cell growth, whereas usHBZ RNA does not have growth-promoting activity (Yoshida, Satou et al. 2008). Therefore it appears that a spliced HBZ plays more important role in HTLV-1 related pathogenicity, and in my PhD work I focused on this HBZ isoform.

1.2.2. Localization and structure of HBZ

The spliced and unspliced *HBZ* transcripts are translated into polypeptides of 206 and 209 amino acids, respectively. The HBZ protein is localized in the nucleus and accumulates there within characteristic nuclear speckles, different from Cajal bodies, splicing factor compartments, or promyelocytic leukemia oncoprotein bodies (Gaudray, Gachon et al. 2002; Hivin, Frederic et al. 2005). Nuclear targeting of HBZ is mediated by three distinct nuclear localization signals (NLSs), which were all mapped to the region in the central domain of HBZ. NLS-1 and NLS-2 corresponds to two regions rich in basic amino acids (BR1 and BR2, Figure 6), and NLS-3 corresponds to the DNA-binding domain (DBD). At first glance, NLS-1 and NLS-2 contain the required stretch of 3-5 positively charged residues sufficient to provide the nuclear localization. However, none of the NLS motifs alone is sufficient for HBZ to be retained exclusively in the nucleus and at least two NLS motifs are required to work together to promote nuclear translocation. Moreover, the integrity of HBZ protein sequence is necessary for the speckled distribution in the nucleus (Hivin, Frederic et al. 2005). Interestingly, despite 95% amino acid sequence identity, the sHBZ and usHBZ show distinct subnuclear distribution profiles (Murata, Hayashibara et al. 2006). Namely sHBZ was also found in the nucleolus, while usHBZ was not. Since both isoforms differ only in the N-terminal part, it is believed that this region prevents the transport of usHBZ into the nucleoli, probably by interacting with nucleoplasmic proteins (Murata, Hayashibara et al. 2006). Both isoforms of HBZ contain three domains: N-terminal activation domain (AD), central domain (CD), and basic ZIP domain (bZIP) in the C-terminus (Hivin, Frederic et al. 2005; Yoshida, Satou et al. 2008).

The activation domain is composed of two LXXLL-like motifs, which are responsible for mediating the interaction with the KIX domain of p300/CBP transcriptional

coactivators (Lemasson, Lewis et al. 2007; Clerc, Polakowski et al. 2008). The interaction with HBZ leads to the sequestration of p300/CBP in the nuclear bodies, and finally the repression of Tax-dependent transactivation of viral LTRs and possibly to the downregulation of other genes that are regulated by p300/CBP.

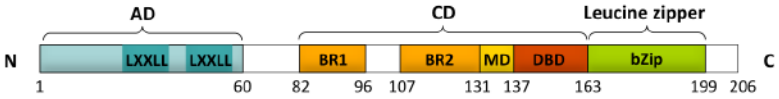

Figure 6. Molecular structure of HBZ. N-terminal activation domain (AD) containing two LXXLL-like motifs, two basic regions (BR1 and BR2), a DNA-binding domain (DBD) and a C-terminal leucine zipper (bZIP) are shown. The numbers below indicate the first and last residues of the domains (Adapted from Hivin *et al*. 2005).

Domain	Function	Reference
AD	Interact with p300/CBP	(Clerc, Polakowski et al. 2008)
	Activate TGF-b signaling	(Zhao, Satou et al. 2011)
	Bind to 26S proteasome	(Isono, Ohshima et al. 2008)
CD	Responsible for nuclear localization	(Hivin, Frederic et al. 2005)
bZIP	Interact and inhibit c-Jun, JunB	(Basbous, Arpin et al. 2003)
	Interact abd inhibit CREB and CREB2	(Gaudray, Gachon et al. 2002; Lemasson, Lewis et al. 2007)
	Inhibit 5'LTR activation	(Lemasson, Lewis et al. 2007)
AD + bZIP	Bind to p65 and inhibit canonical NF-kB pathway	(Zhao, Yasunaga et al. 2009)
	Stimulate transcriptional activity of JunD	(Thebault, Basbous et al. 2004; Gazon, Lemasson et al. 2012)

Table 2. Molecular functions of HBZ domains. Adapted from (Matsuoka 2010; Zhao and Matsuoka 2012).

The central domain contains basic regions and DNA binding domain, however the detailed analysis of the amino acid sequence of those basic regions argues against direct DNA binding (Reinke, Grigoryan et al.). Effectively the direct recruitment of HBZ to the DNA was not yet shown.

The bZIP domain located on the C-terminus, consists of a basic region followed by heptad repeats of hydrophobic residues forming an α-helical leucine zipper. This domain allows for dimerization required for efficient interaction with the DNA. HBZ is unable to form stable homodimers, however eagerly heterodimerizes with cellular bZIP proteins (Basbous, Arpin et al. 2003). *Via* bZIP domain HBZ interacts with transcriptional activators that are necessary for Tax to transactivate the 5'LTR, such as CREB or CREB-2, playing also important role in regulation of cellular genes *via* cyclic AMP response elements (CRE) (Gaudray, Gachon et al. 2002; Lemasson, Lewis et al. 2007). Therefore by sequestration of those factors in nuclear bodies, HBZ prevents their recruitment to TRE sites within viral LTR and leads to decrease of Tax-mediated HTLV-1 transcription. HBZ can also bind to transcription factors from AP-1 family, including c-Jun, JunB (Basbous, Arpin et al. 2003) and JunD (Thebault, Basbous et al. 2004; Gazon, Lemasson et al. 2012), leading to the deregulation of their transcriptional activity and finally to the expression of their target genes.

In addition, HBZ can also bind to the p65 subunit of NF-κB and both the AD and bZIP domains are important in establishing this interaction (Zhao, Yasunaga et al. 2009).

1.2.3. Role of HBZ in HTLV-1-mediated pathogenicity

1.2.3.1. Activation of cellular proliferation

Increasing number of studies indicate that HBZ has a growth promoting activity and can stimulate the proliferation of T lymphocytes *in vitro* and *in vivo*. Namely the suppression of *HBZ* gene transcription by specific shRNA inhibits proliferation rate of ATL cells (Satou, Yasunaga et al. 2006), as well as HTLV-1 transformed T-cell lines and newly immortalized T-lymphocytes (Arnold, Zimmerman et al. 2008). Moreover stable expression of *HBZ* maintains the growth of Kit-225 and increases Jurkat cell proliferative capacity (Satou, Yasunaga et al. 2006). Additionally as shown by Arnold *et al.* the tumor

formation and tumor infiltration are significantly reduced in NOG mice engrafted with HBZ-knocked-down HTLV-1 transduced cells, in comparison to animals challenged with control HTLV-1 transduced cells (Arnold, Yamamoto et al. 2006). This indicates that *HBZ* expression enhances the proliferative capacity of HTLV-1– infected cells and plays a critical role in cell survival and, ultimately, HTLV-1 tumorigenesis in infected host (Arnold, Yamamoto et al. 2006).

Detailed studies on HBZ-mediated T-cell proliferation revealed, that presumably the *HBZ* RNA is implicated in this process (Satou, Yasunaga et al. 2006). Interestingly the growth-promoting activity was observed only in a T-cell line expressing sHBZ, but not in usHBZ-expressing T-cells (Yoshida, Satou et al. 2008). The difference between sHBZ and usHBZ lies with the presence of the first exon. This region corresponds to the Rex-responsive element (RxRE) within the 3'LTR, which forms a stem-loop secondary structure (Matsuoka and Green 2009). Indeed mutation analyses of the *HBZ* gene showed that this stem-loop structure promoted T-cell proliferation (Satou, Yasunaga et al. 2006), however the exact molecular mechanism implicated in this process remains unknown. Although it is speculated that the stem-loop structure, known to interact with Rex viral protein, might interact with host factors to induce the proliferation of ATL cells (Matsuoka and Green 2009).

HBZ RNA was shown to up-regulate the transcription of *E2F1* and its downstream targets (Satou, Yasunaga et al. 2006). E2F1 is considered as a critical regulator of cell cycle progression, as it plays a pivotal role in the G1-to-S-phase transition by transactivating specific target genes (Nevins 2001), and its overexpression is associated with oncogenesis (Pierce, Gimenez-Conti et al. 1998). Therefore the HBZ-induced T-cell proliferation is likely to employ the mechanism involving this transcription factor.

Interestingly as shown by Gazon *et al.* JunD may play a role in HBZ-mediated stimulation of cellular proliferation, as knockdown of JunD negated HBZ proliferative effect in cells expressing HTLV-1 molecular clone and the HTLV-1 infected cell lines (Gazon, Lemasson et al. 2012). However, further studies are required to confirm those observations.

Another study indicated that HBZ growth promoting activity seems to depend also on the ATF3 factor, which is known to bind HBZ. Suppressing ATF3 expression inhibited proliferation and strongly reduced the viability of ATL cells (Hagiya, Yasunaga et al. 2011). In normal cells ATF3 expression has both positive and negative effects on cellular

proliferation and survival. Namely ATF3 up-regulates the transcription of factors that control the G1-to-S phase transition - cell division cycle 2 (CDC2) and cyclin E2, and at the same time enhances the p53 transcriptional activity. HBZ was shown to impair the ATF3 negative effect on cell cycle progression *via* mechanism involving p53, at the same time leaving ATF3 to promote proliferation of ATL cells *via* mechanisms including up-regulation of CDC2 and cyclin E2 (Hagiya, Yasunaga et al. 2011).

Summarizing the HBZ-mediated induction of cellular proliferation seems to be a very complex process employing different mechanisms, possibly implicating two different molecular forms of HBZ - both the RNA and the protein.

The stimulation of cellular proliferation is especially important in HTLV-1 mediated pathogenicity as HTLV-1 infects cells *via* virological synapses requiring direct cell-to-cell contact (Yoshida 2005). Therefore HTLV-1 to facilitate the transmission, increases the number of infected T cells by clonal expansion (Wattel, Cavrois et al. 1996). The growth promoting activity was also reported for Tax, however its expression is frequently lost in ATL cells. Therefore HBZ that is expressed constitutively seems to play a critical role in oncogenesis mediated by HTLV-1, even in late stages of oncogenesis when Tax is not expressed (Matsuoka and Jeang 2007). HBZ mediated induction of cellular proliferation ensures viral persistence, supports infected cell survival and, ultimately, maintain the leukemic state of affected cells (Satou, Yasunaga et al. 2006).

1.2.3.2. Modification of cellular signaling pathways

1.2.3.2.1. Modification of AP-1 signaling

One of the very important axes of HBZ mediated oncogenesis depends on the deregulation of cellular transcription pathways, including the activator protein 1 (AP1). The AP1 factors are important for regulating many biological processes including proliferation, differentiation, apoptosis and transformation (Shaulian and Karin 2001). Interestingly HTLV-1 infected cells exhibit high expression level of AP1 factors and their enhanced activity (Hall and Fujii 2005). Therefore this family of transcription factors is believed to contribute to the transformation process during ATL development.

The common structural feature of these regulatory proteins is the presence of a bZIP domain that consists of a basic region followed by heptad repeats of hydrophobic residues forming a leucine zipper. This domain allows for heterodimerization with C-terminal leucine zipper of HBZ, however not all of the AP1 proteins containing this domain can interact with HBZ. Thus HBZ is binding to c-Jun (Basbous, Arpin et al. 2003; Matsumoto, Ohshima et al. 2005), JunB (Basbous, Arpin et al. 2003), and JunD (Thebault, Basbous et al. 2004), but not c-Fos (Matsumoto, Ohshima et al. 2005). As a result, HBZ, affects the cellular localization of bound transcription factors, modulates their transcriptional activity and deregulates the expression of target genes. Interestingly HBZ can both act as an activator and repressor of AP1. In particular it was shown that JunB and c-Jun were repressed by HBZ, while JunD was strongly activated, both on the transcriptional and functional level.

The HBZ-mediated inhibition of transcriptional activity of JunB and c-Jun occurs on different levels and involve distinct molecular mechanisms. First the heterodimerization of HBZ with JunB and c-Jun results in diminishing their affinity of DNA binding, thereby attenuating their transcriptional activity (Basbous, Arpin et al. 2003; Matsumoto, Ohshima et al. 2005; Hivin, Arpin-Andre et al. 2006). It was shown that the DNA-binding domain of HBZ plays an important role in in this process (Hivin, Arpin-Andre et al. 2006). Indeed the noncanonical amino acid sequence of HBZ basic region argues against direct DNA binding (Reinke, Grigoryan et al.), therefore it is believed that dimerization with HBZ might decrease the DNA affinity of interacting proteins.

Secondly HBZ was shown to sequester JunB and c-Jun to the nuclear bodies (Hivin, Basbous et al. 2007; Clerc, Hivin et al. 2009). Those structures where shown not to colocalize with RNA polymerase II and considered as transcriptionally inactive nuclear sites (Hivin, Basbous et al. 2007). Paradoxically the speckled localization of HBZ is altered by the deletion of its leucine zipper domain, suggesting that the heterodimerization partners are involved in controlling their own nuclear trafficking (Hivin, Basbous et al. 2007). Importantly as shown by Clerc *et al.* in the context of c-Jun inhibition of transcriptional activity, the sequestration to the nuclear bodies have a greater importance *in vivo*, than blocking the binding to the DNA (Clerc, Hivin et al. 2009).

Moreover HBZ was shown to suppress c-Jun transcriptional activity by an additional mechanism. Namely HBZ promotes degradation of c-Jun, which occurs through

a proteasome dependent pathway, as the HBZ-mediated decrease of steady-state c-Jun expression levels were restored by treatment with the proteasome inhibitor (Matsumoto, Ohshima et al. 2005; Isono, Ohshima et al. 2008). Although most proteasomal substrates are targeted for degradation *via* conjugation of polyubiquitin chains, Isono *et al.* showed that ubiquitination is not required for HBZ-mediated proteasomal degradation of c-Jun. HBZ directly interacts with both the 26S proteasome and c-Jun and facilitates the delivery of c-Jun to the proteasome without ubiquitination. HBZ acts therefore as a tethering factor between the 26S proteasome and its substrate, thereby bypassing the targeting function of ubiquitination (Isono, Ohshima et al. 2008).

In conclusion the interaction of HBZ with c-Jun and JunB results in the decrease of their transcriptional activity *via* various mechanisms and prevents those factors from activating the transcription of AP1-regulated genes. Additionally as c-Jun is implicated in the up-regulation of basal activity of HTLV-1 promoter, in the presence of HBZ the viral expression is also diminished (Basbous, Arpin et al. 2003).

Conversely JunD, which was shown by yeast two-hybrid assay to also interact with HBZ *via* the bZIP domain (Thebault, Basbous et al. 2004), oppositely to other AP1 factors is transcriptionally activated by HBZ. Luciferase assays using a construct encoding luciferase under the control of a collagenase promoter that contains a canonical AP1 element, reveled that in the presence of HBZ, the JunD transcriptional activity substantially increased (Thebault, Basbous et al. 2004). Moreover the effect of HBZ on JunD did not depend on affecting its DNA-binding activity (Thebault, Basbous et al. 2004). Additionally JunD, opposite to other Jun proteins, in the presence of HBZ did not change its localization into transcriptionally inactive nuclear bodies. In the contrary, in COS cells ectopically expressing JunD, HBZ did not show at all granular localization pattern and was rather diffused throughout the nucleoplasm colocalizing with JunD (Hivin, Basbous et al. 2007), suggesting that JunD participated in determining HBZ localization, and not the other way round.

The stimulatory effect of HBZ and resulting activation of JunD-dependent transcription required the N-terminal activation domain of HBZ (Thebault, Basbous et al. 2004). As this domain contains two LXXLL motifs responsible for binding to p300/CBP transcriptional coactivators (Clerc, Polakowski et al. 2008), one may speculate that those factors are important in this process. Moreover HBZ ΔAD in the luciferase assays repressed the

transcriptional activity of JunD, suggesting not only that the amino-terminus of HBZ is necessary for HBZ to stimulate JunD-dependent transcription but also that HBZ lacking the AD could antagonize JunD action (Thebault, Basbous et al. 2004). Additionally it was shown that six charged amino acids immediately adjacent to the DNA binding domain of HBZ, were particularly important in regulation of Jun transcriptional potency. It is speculated that this subdomain could be involved in efficient presentation of the AP1 complex to the transcriptional machinery (Hivin, Arpin-Andre et al. 2006). However, further studies are required to fully understand the role of AD and the basic region of HBZ in activation of JunD-directed transcription.

As a result of the processes described herein, the HBZ/JunD heterodimers were reported to increase the expression of both cellular and viral genes. For example the up-regulation of *hTERT* gene expression was observed (Kuhlmann, Villaudy et al. 2007), as well as stimulation of the antisense transcription from the viral 3'LTR (Gazon, Lemasson et al. 2012). In both cases the transcriptional activation was dependent on the Sp1 factors. As JunD homodimers exhibit very weak DNA binding *in vitro* (Thebault, Basbous et al. 2004), and so far there are no reports on direct interaction of HBZ with the DNA, we may therefore speculate that to participate in the regulation of gene expression, HBZ/JunD heterodimers require additional factors allowing for their recruitment to the gene promoters.

In addition to the stimulation of JunD transcriptional activity HBZ increases the protein expression levels of this transcription factor (Gazon, Lemasson et al. 2012). This observation is in accordance with previous reports indicating an increase in the abundance and activity of JunD in freshly isolated ATL cells (Fujii, Iwai et al. 2000; Mori, Fujii et al. 2000). The exact mechanism of this process still remains unknown, however the growing amount of data on the pathogenic effect of JunD encourages the development of this axis of the research. Previous studies suggest that JunD plays a critical role in leukemic-cell survival (Mori, Fujii et al. 2000) and the increase of JunD abundance correlates with emergence of the features of transformed cells in immortalized fibroblasts (Gazon, Lemasson et al. 2012). Moreover the knockdown of JunD inhibits HBZ induced cell proliferation, indicating the importance of this factor in HTLV-1 mediated pathogenicity (Gazon, Lemasson et al. 2012).

Summarizing the studies on the regulation of AP1 factors by HBZ, presented

herein, reveal distinct mechanisms of HBZ action, leading to repression of JunB and c-Jun and the activation of JunD-dependent transcription. Despite high sequence similarities, the Jun family members exhibit distinct biological roles in the cellular context. For example c-Jun is a positive regulator, while JunD acts as a negative regulator of the cell cycle (Mechta-Grigoriou, Gerald et al. 2001). Therefore distinct regulation of the AP1 factors by HBZ contributes in a very elaborated way to the development of ATL.

1.2.3.2.2. Inhibition of the classical NF-κB pathway

Tax can activate both the classical and alternative pathways of nuclear factor-κB (NF-κB), playing therefore an important role in the proliferation of ATL cells, inhibition of apoptosis and finally in the transforming activity of HTLV-1 (Yamaoka, Inoue et al. 1996; Sun and Yamaoka 2005). However at the same time activation of NF-κB pathway is thought to play an important role in the protective response of the host during viral infection, resulting in viral clearance.

In contrast to Tax, HBZ inhibits the classical NF-κB pathway, leaving intact the alternative one (Zhao, Yasunaga et al. 2009). To do so HBZ employs two distinct mechanisms. First, HBZ interacts with the NF-κB subunit, p65, and prevents it from the DNA binding. Secondly, HBZ induces p65 degradation through increasing the expression of the *PDLIM2* gene, which encodes an ubiquitin E3 ligase. Therefore HBZ is promoting p65 ubiquitination and its proteosomal degradation resulting in decreased expression of target genes. Importantly both the activation and the bZIP domains are responsible for the interaction of HBZ with the Rel homology domain of p65 (Zhao, Yasunaga et al. 2009).

Both the classical and alternative NF-κB pathways are involved in the regulation of different biological processes and exhibit distinct functions. Recent studies suggest that the classical NF-κB pathway is mostly involved in innate immunity and inflammatory responses. The inhibition of this NF-κB signaling is common among different viruses, suggesting that it may be a common mechanism to escape viral clearance by the immune system. Effectively by suppressing the classical NF-κB pathway, HBZ inhibits the transcription of the *IFNg* and *IRF4* genes, facilitating the HTLV-1 escape from the host immune response (Zhao and Matsuoka 2012).

On one hand, HBZ by suppressing selectively the classical NF-κB pathway, renders the alternative pathway to be activated by Tax. Such a function of HBZ in cooperation with Tax activity may be beneficial for proliferation of infected cells and oncogenesis. On the other hand *via* inhibition of the classical NF-κB pathway, HBZ allows for sneaking through the innate and adaptive antiviral response, ensuring the virus survival and successful spread of infection (Zhao and Matsuoka 2012).

1.2.3.2.3. Enhancement of TGF-β signaling

TGF-β controls a variety of biological processes, including cell growth, differentiation, apoptosis, development, and immune homeostasis. The signaling pathway involve Smad mediators, transducing the signal from the activated TGF-β cell surface receptors to the nucleus, to regulate the transcription of target genes (Massague, Blain et al. 2000). Smads can regulate gene expression positively by recruiting coactivators, such as CBP/p300, or negatively by direct recruitment of histone deacetylases or corepressors, such as c-Ski and SnoN (Feng and Derynck 2005).

HBZ activates TGF-β signaling and its N-terminal LXXLL-like motifs are required for this function (Zhao, Satou et al. 2011). Effectively, HBZ was shown to interact with Smad2/3 and recruit p300 coactivator to enhance TGF-β/Smad transcriptional responses. HBZ enhance the association between Smad3 and p300, forming a ternary complex together with Smad3 and p300. By this mean HBZ can overcome the suppressive effect of Tax on TGF-β pathway and activates the expression of TGF-β target genes even in the presence of Tax. Therefore the HBZ-mediated enhancement of TGF-β leads to the up-regulation of FoxP3 target gene expression in T cells (Zhao, Satou et al. 2011). Effectively the expression of FoxP3 was detected in two-thirds of examined ATL cases (Karube, Ohshima et al. 2004). Consistent with the known role of FoxP3 in the development and function of regulatory T cells (Tregs), a higher proportion of $FoxP3^{+}$ Tregs was found among the HTLV-1–infected cells rather than among the HTLV-1–negative CD4+ cells (Toulza, Heaps et al. 2008). Such a Treg phenotype of ATL cells is considered to suppress the immune response and may be implicated in immunodeficiency.

Summarizing, HBZ, by enhancing TGF-β signaling and Foxp3 expression, enables HTLV-1 to convert infected T cells into regulatory T cells, which is thought to be a critical strategy for virus persistence. Moreover HBZ seems to selectively modulate the actions of TGF-β/Smad signaling pathway, as it does not influence the expression of genes associated with inhibition of cell growth and therefore is able to maintain the cell proliferation and contribute to HTLV-1 mediated oncogenesis (Zhao and Matsuoka 2012).

1.3. Other antisense proteins

HTLV-1 is not unique in its capacity to produce the negative-strand RNA transcripts. Similarly to HTLV-1, the other members of HTLV strain encode for antisense proteins. The phenomenon of antisense transcription enables viruses to greatly enhance their coding capacity. It appears to be therefore common among different families of viruses regardless of the type of genetic material and the replication strategy used. Bidirectional transcription was found for example among dsDNA viruses like Varicella zoster human herpes virus, and reverse transcribing dsDNA viruses including woodchuck hepatitis virus and the hepatitis B virus (Meier, Luo et al. 1994; Velhagen, Hilger et al. 1995; Shimoda, Sugata et al. 1998). Also the human hepatitis D virus, which posses a circular single-stranded RNA genome, uses a complementary antigenomic RNA intermediate as a template for transcription (Lai 1995). Among retroviruses, despite HTLV, the antisense transcription was also reported for lentiviruses, HIV-1 and FIV (human and feline immunodeficiency viruses) (Michael, Vahey et al. 1994; Briquet, Richardson et al. 2001) (Ludwig, Ambrus et al. 2006). However since the phenomenon of antisense transcription has been discovered relatively recently, the antisense proteins are still very poorly studied.

The antisense transcription initiating in 3'Long Terminal Repeat (LTR) of the HTLV-2 was reported for the first time in 2009 (Halin, Douceron et al. 2009). The protein encoded by the complementary strand of HTLV-2 provirus was named APH-2 for antisense protein of HTLV-2, as in the contrary to HBZ of HTLV-1 it does not contain the consensus basic leucine zipper motif (bZIP). Nevertheless this protein shares certain molecular characteristics and functional properties with HBZ.

The APH-2 transcript is structured similarly to the transcript encoding HBZ - it is spliced, initiates in the 3'-LTR at multiple sites, and is polyadenylated. The length of the intron and of the 5' and 3' untranslated regions is also similar to that of the HBZ transcript. Interestingly all APH-2 transcripts undergo splicing, as the unspliced APH-2 transcript was not detected, in contrast to HBZ, which produces a substantial amount of unspliced transcript encoding for a different HBZ isoforms (Halin, Douceron et al. 2009). APH-2 is constitutively expressed in infected cells, and was detected in vivo in most HTLV-2 carriers. Its expression correlates with PVL (Douceron, Kaidarova et al. 2012), reminiscent to HBZ. However APH-2 does not promote cell proliferation and does not cause lymphocytosis *in vivo* (Douceron, Kaidarova et al. 2012).

The APH-2 protein is composed of 183 amino acids. It localizes in the cell nucleus, but unlike HBZ, was not found in the nucleolus (Halin, Douceron et al. 2009). Similarly to HBZ, the APH-2 protein contains an LXXLL domain and an LXXLL-like (ILKLL) motif, however their localization is different. The LXXLL motif of APH-2 is located at its C-terminus, while ILKLL motif within the central domain of APH-2. In contrary to HBZ, those motifs exhibit only a very weak interaction with p300 (Halin, Douceron et al. 2009), however it remains unknown whether it is caused by different localization of the interaction domains.

Despite the lack of a classic bZIP domain, APH-2 retains the capacity to inhibit Tax2-dependent LTR activation and to interact with the CREB transcription factor (Halin, Douceron et al. 2009). According to a recent study, the LXXLL motif of APH-2 is involved in its interaction with CREB and in the repression of Tax function on viral genes (Yin, Kannian et al. 2012).

The central domain of APH-2 contains so called non-conventional bZIP motif, as the intervals between leucine residues are not regular, modifying therefore the secondary structure of an α-helix. Nevertheless, this non-conventional bZIP domain allows for the interaction with c-Jun and JunB transcription factors (Marban, McCabe et al. 2012). However, unlike HBZ, APH-2 acts as an activator of AP-1 basal activity and stimulates c-Jun and JunB-mediated transactivation. Interestingly also JunD transcriptional activity is stimulated by APH-2, despite the fact that APH-2 and JunD are not able to form a stable complex in vivo. Therefore APH-2 stimulates JunD transcriptional activity indirectly, and employs different mechanism than in the case of c-Jun and JunB. As APH-2ΔbZIP fails to stimulate JunD-mediated transcriptional activity, we can conclude that the leucine zipper

is required for APH-2 to mediate JunD transactivation, however further studies are required to elucidate precise molecular mechanism and protein partners implicated in this process. Moreover, HTLV-2 Tax was also shown to enhance the AP-1 transcription. Surprisingly co-expression of both Tax2 and APH-2 results in a suppression of Tax2-mediated transactivation by APH-2, whereas APH-2 or Tax2 expressed individually highly enhance AP-1 transcription (Marban, McCabe et al. 2012). As the AP-1 pathway is involved in numerous cellular functions susceptible to affect the fate of the cell, these distinct biological properties between HBZ and APH-2 may contribute to the differential pathogenic potential of HTLV-1 and HTLV-2 (Marban, McCabe et al. 2012).

The newly discovered HTLV-3 and HTLV-4 are equally capable of producing antisense proteins (Larocque, Halin et al. 2011). The antisense transcripts of HTLV-3 and HTLV-4 are spliced and polyadenylated, the splicing pattern is simillar to that of HBZ with a similar intronic size (Larocque, Halin et al. 2011). The encoded proteins, were named in analogy to APH-2, the antisense proteins of HTLV-3 and HTLV-4 respectively. These proteins do share certain similarities with HBZ and APH-2 although their cellular localization and amino acid sequence do indicate functional differences. The APH-4 viral protein exhibits a nuclear-restricted pattern while APH-3 localizes both in the cytoplasm and in the nucleus. Both proteins also show partial co-localization with nucleoli and HBZ-associated structures. In addition, alike APH-2, both APH-3 and APH-4 are devoid of a typical bZIP domain but contain basic amino acid-rich regions, which are highly conserved and predicted to form a leucine zipper-like coiled-coil secondary structures (Larocque, Halin et al. 2011). Also LXXLL and LXXLL-like motifs (known to be responsible for binding p300/CBP) were also observed but their positions were different from that observed for HBZ (Larocque, Halin et al. 2011). Finally, both proteins inhibited Tax1- and Tax3-mediated HTLV-1 and HTLV-3 LTR activation (Larocque, Halin et al. 2011).

The oncogenic property of all members of HTLV strain is different. Unlike HTLV-1, the human HTLV-2 has been linked to HAM-like pathologies, but not to leukemia (Roucoux and Murphy 2004), while HTLV-3 and HTLV-4 infection for the moment is not associated with any pathology (Duong, Jia et al. 2008; Thomas, Perzova et al. 2010). The distinct clinical manifestations can be attributed to distinct biological functions of viral proteins, including HBZ/APH proteins. As presented above, the antisense proteins encoded by different members of HTLV strain, exhibit structural and functional

differences, suggesting potential dissimilarities in their capacity to modulate cellular and/or viral expression. These functional differences could eventually condition their different oncogenic property and account for the distinctive outcomes in infected patients. Further comparative studies of these viral proteins should provide important information on the pathogenic mechanisms and lead to further understanding of leukemogenesis induced by HTLV-1.

Chapter 2

Telomerase and its role in carcinogenesis

2.1. Structure of telomerase, function and role in cancer development

For the first time, in 1961 Hayflick and Moorhead noticed that normal somatic cells have a limited capacity to divide, after which they become senescent (Hayflick and Moorhead 1961). Later on Olovnikov *et al.* hypothesized that this effect may result from the incomplete replication of the extremities of the linear DNA molecules (Olovnikov 1973). In the mid-1970s, the first telomere sequence from ciliate *Tetrahymena* was cloned (Blackburn and Gall 1978), and ten years later the complete human telomere was sequenced (Moyzis, Buckingham et al. 1988). At that time also the telomerase holoenzyme was discovered and described as an enzyme that adds the telomeric repeats necessary for the replication of the chromosome ends in eukaryotes (Greider and Blackburn 1985). Due to the extensive research efforts, we now know that the up-regulation or reexpression of this telomere-synthesizing ribonucleoprotein is a critical event responsible for continuous tumor cell growth. Therefore nowadays the telomerase activity is often used as a parameter in the cancer diagnostics and the targeting of the telomerase is one of the major goals in the cancer therapy.

Telomerase is a ribonucleoprotein complex, that has an activity of an RNA-dependent DNA polymerase (Morin 1989). The telomerase holoenzyme consists of two essential components: the RNA strand (in humans called hTR or hTERC) and a catalytic

subunit (hTERT) with reverse transcriptase activity (Figure 7). In contrast to the hTR component, which is ubiquitously expressed, the catalytic component hTERT is repressed in telomerase negative cells and therefore is the limiting factor and the primary determinant for the enzyme activity (Avilion, Piatyszek et al. 1996). The structural RNA component is 451-nucleotide long and near the 5'end of the molecule contains an 11-bp motif (CUAAUCCCAAC), which is complementary to the telomeric single-stranded overhang and functions as a template for the synthesis of telomeric DNA (Feng, Funk et al. 1995). Telomerase adds the tandem TTAGGG telomeric repeats to the 3'end of the G rich strand. Subsequently the complementary strand of chromosomes is elongated at its 5' end by conventional DNA polymerases (Greider and Blackburn 1985).

Telomerase is not capable of extending a blunt-ended DNA molecule in vitro. Therefore, following conventional DNA replication, the blunt-ended chromosome end that is created as a consequence of leading strand synthesis must be processed to create a single-strand extension prior to next round of telomerase action. However the mechanism of creating such a structure is not yet known (Lingner and Cech 1996).

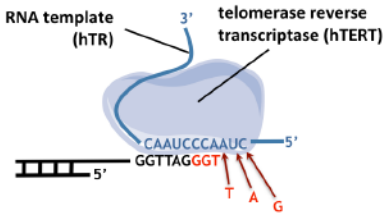

Figure 7. The schematic structure of telomerase holoenzyme. Telomerase is composed of the RNA strand (hTR) and the catalytic subunit, the telomerase reverse transcriptase (hTERT). The hTR is complementary to the telomeric single-stranded overhang and serves as a template for the synthesis of the telomeric repeats. The extension of the G rich strand is catalyzed by the hTERT. Subsequently the complementary strand of the DNA is elongated by conventional DNA polymerases.

Telomerase activity can be reconstituted in rabbit reticulocyte lysates from in vitro-transcribed *hTR* and in vitro-transcribed and -translated *hTERT*, suggesting that those components determine the minimal requirement for telomerase activity (Weinrich, Pruzan et al. 1997). However in addition to the main telomerase components several accessory factors have been identified, which assist in telomerase assembly, maturation, recruitment and activation (Cong, Wright et al. 2002). The chaperon complexes p23 and hsp90 (heat shock protein 90) interact with human telomerase to assist in proper ribonucleoprotein assembly and the formation of active telomerase enzyme. Proteins from the 14-3-3 family interact with the C-terminal region of hTERT and promote its nuclear localization. The snoRNA (small nucleolar ribonucleoprotein) binding proteins, including hGAR1, dyskerin/NAP57, hNOP10, are involved in the stability, accumulation, maturation, and localization of hTR. Other RNA binding proteins, like hStau and L22, may play a role in hTR processing, localization and telomerase assembly. Moreover proteins like TEP1 (telomerase associated protein 1) and many nuclear ribonucleoproteins (hnRNPs) C1 and C2 and A1 and UP1 have been shown to associate with hTR, however their role has to be confirmed (Cong, Wright et al. 2002). Despite the fact that non of the above-mentioned accessory proteins seems to be essential for the telomerase activity *per se*, they seem to support the full activity and biological function of the enzyme.

The telomerase activity is repressed in majority of the cell types and only some highly regenerative tissues such as hematopoietic progenitor, intestinal crypt, skin basal layer, cervical keratinocyte, endometrium, and germ line cells as well as lymphocytes subjected to mitogenic stimuli, are able to maintain and/or extend their telomere lengths using telomerase (Hiyama, Hirai et al. 1995; Harle-Bachor and Boukamp 1996; Yasumoto, Kunimura et al. 1996).

Human telomeres typically consists of tandem GT-rich repeats, (TTAGGG)n, with a single-stranded 3'-end overhang, which invades the double stranded telomeric DNA repeat array to form a T-loop structure stabilized by the shelterin complex (Blackburn 2001). Telomeres are involved in several essential biological functions. Thanks to the T-loop capping they protect chromosomes from recombination, end-to-end fusion, and recognition as damaged DNA. Additionally they serve as a molecular clock that controls the replicative capacity of human cells and their entry into senescence (Cong, Wright et al. 2002). In normal cells the length of telomere is between 5–15 kilobase pairs and in the

absence of telomerase, the telomeres shorten by about 50nt per cell population doubling (Figure 8) (Ducrest, Szutorisz et al. 2002). This results from the fact that conventional DNA polymerases are unable to completely replicate the 3'end of the lagging strand at the chromosome ends, due to so called "end replication problem". This progressive shortening of telomeres occurring with each cell division eventually results in structural changes at the telomeres and the induction of cellular senescence. The cells with the critically shortened telomeres enters a permanent growth arrest known as replicative senescence or mortality stage 1 (M1), induced by p53- and p16/retinoblastoma protein (p16/pRB) (Harley, Vaziri et al. 1992). Telomerase, by extending telomeres, resets this molecular clock, and leads to cellular immortality. Therefore, the expression of telomerase is tightly controlled in normal human cells.

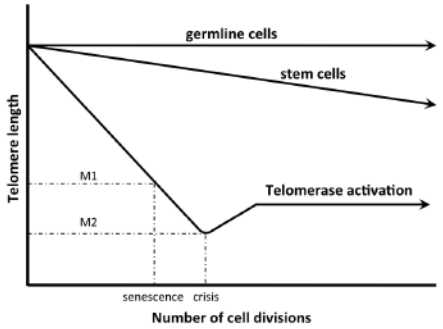

Figure 8. Schematic representation of the relationship between the cell division and the telomere length in different cell types. Normal human somatic cells are negative for telomerase and therefore undergo the telomere shortening with each cell division. Critically shortened telomeres may signal cells to enter senescence at M1 phase. Such cells continue to suffer telomere erosion and ultimately enter crisis at M2 phase, characterized by widespread cell death. Rare surviving cells acquire the telomerase activity and unlimited proliferative potential and stabilization of telomere length, almost universally by activation the telomerase. In the contrary germ cells exhibit unlimited proliferation potential, as their telomere length is maintained by telomerase. Pluripotent stem cells exhibiting moderate telomerase activity and their telomere length shortens in stem cells at rates slower than that of telomerase-negative somatic cells (From (Cong, Wright et al. 2002).

In the contrary in approximately 90% of human cancers, telomerase is up-regulated. The activation of the telomerase allows for balancing the fast telomere shortening occurring in those actively replicating cells, therefore allows for cell immortalization.

Although the reexpression of the telomerase is not sufficient to induce a tumorigenic state *per se,* but it can contribute to the tumorigenesis in the presence of additional oncogenes and/or the disruption of tumor suppressors (Bellon and Nicot 2008). The acquisition of this "replicative immortality" is a critical step in the malignant transformation, therefore 10% of cancers which has inactive telomerase, use the alternative telomere lengthening (ATL) to maintain their telomeres and provide the unlimited proliferative potential of tumor cells (Bellon and Nicot 2007).

As the RNA component of the telomerase is expressed ubiquitously, the critical element determining the telomerase activity is the catalytic subunit, the human telomerase reverse transcriptase (hTERT). Therefore the expression of the *hTERT* is up-regulated in majority of the cancerous lesions. Increase in transcription of the telomerase reverse transcriptase is also a common mechanism during cellular transformation and carcinogenesis provoked by multiple human DNA and RNA tumor viruses including Epstein- Barr virus, Kaposi sarcoma–associated herpesvirus, human papillomavirus, hepatitis B virus, hepatitis C virus, and human T-cell leukemia virus-1 (Bellon and Nicot 2008).

2.2. Regulation of telomerase activity in normal and cancer cells

In normal human cells, the telomere length and telomerase activity are tightly regulated to meet the proliferative demand of specific cellular functions while at the same time preserving proliferative barriers (senescence) against tumorigenesis (Cong, Wright et al. 2002). The regulation of telomerase activity occurs at various levels, including transcription, mRNA splicing, maturation and modifications of hTR and hTERT subunits, transport and subcellular localization of each component, assembly of active telomerase ribonucleoprotein, and accessibility of the telomeres for their elongation (Cong, Wright et al. 2002).

Importantly, the rate-limiting factor for assembly of an active telomerase complex

seems to be the catalytic component hTERT, as the second core component, hTR is usually expressed ubiquitously. Additionally substantial experimental data demonstrate that hTERT is mainly regulated on the transcriptional level and the transcriptional activation of the *hTERT* gene is the primary event in the reactivation of telomerase (Cong, Wen et al. 1999; Horikawa, Cable et al. 1999; Takakura, Kyo et al. 1999). In agreement with this, a striking correlation between the *hTERT* mRNA and the telomerase activity has been proved. At the same time the transcriptional activation of *hTERT* is believed to be a key factor in the process of cancer initiation and progression (Ducrest, Szutorisz et al. 2002). Concerning those facts the following chapter of this manuscript will primarily focus on the regulation of the hTERT catalytic subunit of the telomerase, with the emphasis on its transcription.

2.2.1. Transcriptional regulation of *hTERT* gene expression

2.2.1.1. Structure of the *hTERT* promoter

The *hTERT* gene is located on the short arm of the chromosome 5 (5p15.33), and is composed of 16 exons and 15 introns (Cong, Wen et al. 1999; Cukusic, Skrobot Vidacek et al. 2008). The promoter of *hTERT* gene was cloned and analyzed independently by 3 groups in 1999 (Cong, Wen et al. 1999; Horikawa, Cable et al. 1999; Takakura, Kyo et al. 1999). Although rich in CpG dinucleotides, the *hTERT* promoter lacks both TATA and CAAT boxes (Horikawa, Cable et al. 1999; Takakura, Kyo et al. 1999). The transcription initiation sites map 55 to 120 bp upstream of the codon ATG, depending on the methods and cell lines used in different laboratories (Horikawa, Cable et al. 1999; Takakura, Kyo et al. 1999; Wu, Grandori et al. 1999). The core *hTERT* promoter (Figure 9) contains two E-boxes (CACGTG) and five GC-rich elements (GGGCGG), which are believed to be the main regulatory sequences implicated in the basal *hTERT* expression (Horikawa, Cable et al. 1999).

The regulation of the *hTERT* gene expression is a subject of a complex transcriptional control occurring on the multiple levels and involving many cellular signaling pathways. A number of factors have been identified to directly or indirectly regulate the *hTERT* promoter, including cellular transcriptional activators (c-Myc, Max,

Sp1, Smad3, HIF-1, AP2, ER, Ets, etc.) as well as the repressors, most of which comprise tumor suppressor gene products, such as p53, WT1, Mad1 and menin (summarized in Table 3) (Kyo, Takakura et al. 2008; Daniel, Peek et al. 2012). It is believed that the 180bp proximal sequence of the *hTERT* promoter is important for maintaining the basal transcriptional activity and that c-Myc/Max and Sp-1 are the main activators of the *hTERT* core promoter (Takakura, Kyo et al. 2005).

In the following chapters the findings on the transcriptional regulation of the *hTERT* gene expression will be summarized. However due to the complexity of the *hTERT* gene regulation, the emphasis will be given only to the key transcription factors, playing the most significant role in this process.

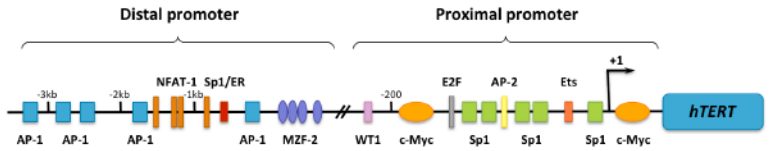

Figure 9. Schematic representation of the *hTERT* promoter. Scheme represents the main transcription factor binding sites and the transcription start site marked as (+1).

Signaling pathway	Transcription factor	Primary role	Transcription factor	Primary role
Cell cycle regulation	CDK2	Activator	p27	Mostly repressor
	CDK4	Activator	Rb	Mostly repressor
	Cyclin D	Activator	E2F1	Mostly repressor
	Cyclin E	Activator	p14 (p19)	Repressor
	p21	Repressor	MDM2	Activator
	p53	Repressor	p16	Repressor
TGF-β	TGF-β	Repressor	SMAD4	Repressor
	TGF-βR1	Repressor	E2F4	Repressor
	TGF-βR2	Repressor	E2F5	Repressor
	SMAD3	Repressor		
PI3K/Akt	PI3K	Activator	mTOR	Activator
	Akt	Activator	IKK	Activator
	PTEN	Repressor	hTERT	Activator
NF-κB (canonical)	NF-κB (p50)	Activator	IKK	Activator
	NF-κB (p65)	Activator	IκB	Repressor
NF-κB (non-canonical)	NIK	Activator	NF-κB (p100)	Activator
	IKK	Activator	NF-κB (RelB)	Activator
MAPK	RAS	Activator	ERK	Activator
	MEK	Activator		
ErbB	EGF	Activator	HER2/neu	Activator
	EGFR	Activator	PI3K	Activator
TGF-β and cell cycle	p107	Repressor		
TGF-β and PI3K/Akt	Jab1	Activator		
cell cycle, TGF-β, PI3K/Akt and MAPK	c-MYC	Activator		
	MAD1	Repressor		
Other	AP2	Activator	MAD3	Repressor
	AP4	Activator	MAZ	Activator
	BRCA1	Repressor	Menin	Repressor
	CCAC	Activator	MNT	Repressor
	c-Ets-2	Activator	MyoD	Mixed
	c-IAPI	Activator	MZF-2	Repressor
	c-Myb	Activator	NF1	Activator
	CREB/ATF	Activator	NFAT	Activator
	CTCF	Mostly repressor	NF-E2	Activator
	AP1	Mixed	PITX1	Repressor
	ERβ	Mostly activator	Sp1	Mostly activator
	IK2	Activator	USF1/USF2	Mixed
	INHBC	Activator	WT1	Mostly repressor

Table 3. Signaling pathways and transcription factors implicated in the regulation of *hTERT* gene expression (After (Daniel, Peek et al. 2012).

2.2.1.2. E-box-mediated regulation of *hTERT* promoter

The members of the c-Myc/Max/Mad network regulates a variety of genes implicated in diverse processes such as cell growth, differentiation, apoptosis and metabolism (Grandori, Cowley et al. 2000). This family of transcription factors regulates the *hTERT* expression *via* E boxes present in the proximal part of the promoter (Wang, Xie et al. 1998; Greenberg, O'Hagan et al. 1999; Wu, Grandori et al. 1999). They predominantly act as dimers and are capable to play a role of either activator or inhibitor in the regulation of gene expression. Both c-Myc and Mad can dimerize with the ubiquitously expressed Max. Heterodimers c-Myc/Max bound to E boxes activate gene expression (Blackwood and Eisenman 1991), while Mad/Max heterodimers competing with them for binding sites, repress the transcription (Gunes, Lichtsteiner et al. 2000; Oh, Song et al. 2000),

Chromatin immunoprecipitation experiments suggested that the differential regulation of the *hTERT* promoter by activator Myc/Max and inhibitor Mad/Max complexes, result from their differential modulation of the histone acetylation and therefore opposite effects on the chromatin structure (Xu, Popov et al. 2001). Namely c-Myc is a part of the histone acetyltransferase SAGA (Spt-Ada-Gcn5-acetyltransferase) complex containing the histone acetyltransferase hGCN5 (McMahon, Wood et al. 2000), and activates *hTERT* transcription by recruiting the histone acetyltransferases to the *hTERT* promoter. In contrast, Mad1 has been shown to interact with mammalian Sin3, which associates with HDAC1, HDAC2, and a nuclear hormone transcriptional corepressor called NcoR (nuclear corepressor) (Cress and Seto 2000). Therefore the Mad1 replacement of c-Myc on the E-boxes of the *hTERT* promoter is accompanied with the reduction of the histone acetylation (Xu, Popov et al. 2001).

The Myc/Max/Mad network is regulated by many signaling and regulatory pathways, which therefore indirectly influence the *hTERT* expression. In particular, the TGF-β pathway has been shown to down-regulate *hTERT* expression by increasing Mad1 and suppressing c-Myc (Zhu, Blenis et al. 2008; Wu, Hultquist et al. 2009). Additionally several investigators demonstrated that the P13K/Akt and MAP kinase pathways up-regulate *hTERT* transcription *via* the phosphorylation of Mad1, leading to its ubiquitin-mediated proteolysis (Zhu, Blenis et al. 2008).

Consistent with the opposite role of c-Myc and Mad1 in the regulation of *hTERT* gene expression, in most normal human somatic cells and in differentiated cells, in which the *hTERT* gene is repressed, the Mad1 is often up-regulated, while c-Myc expression is usually low. Inverse situation occurs in telomerase-positive cells, such as immortal cell lines and primary tumor cells, which express high levels of c-Myc (Cong, Wright et al. 2002). Consistent with this Xu *et al*. reported that endogenous c-Myc expression was well correlated with the induction of *hTERT* in proliferating leukemic cells (Xu, Popov et al. 2001).

Summarizing, the rapidly proliferating cells often contain high levels of c-Myc and the up-regulation of c-Myc is implicated in a wide variety of cancers being a key driving force behind sustained *hTERT* expression (Nesbit, Tersak et al. 1999).

2.2.1.3. GC box-mediated regulation of *hTERT* promoter

Among main transactivators, responsible for basic *hTERT* expression, despite c-Myc, an important player is the Sp1 transcription factor. It was shown to cooperate with c-Myc and strongly activate the *hTERT* expression (Kyo, Takakura et al. 2000).

Sp1 is a zinc finger transcription factor and it is binding to the *hTERT* promoter *via* GC-boxes (Xu, Popov et al. 2001). There are five such elements within the core promoter of *hTERT*, which are able to bind Sp1, as proved by the electrophoretic mobility shift assay (EMSA) (Xu, Popov et al. 2001). Those GC rich elements are located between two E boxes responsible for c-Myc binding, at approximately 110 bp upstream of the transcriptional start site (Kyo, Takakura et al. 2000).

Overexpression of Sp1 in cells that contain relatively low levels of endogenous Sp1 enhances the *hTERT* promoter activity, while any mutations in the Sp1 binding sites drastically reduces it, as shown by the luciferase reporter assays performed by Cong *et al*. (Cong, Wen et al. 1999). This suggests the critical role of Sp1 factors in the regulation of *hTERT* transcription. However, it should be noted that because Sp1 is often universally expressed in normal cells, the Sp1 alone can not account for the cancer-specific *hTERT* activation (Kyo, Takakura et al. 2008). Sp1 has been therefore shown to activate the *hTERT* expression *via* cooperation with MBD1-containing chromatin-associated factor 1

(MCAF1) (Liu, Ishihara et al. 2009; Li, Lin et al. 2011) and the high-mobility group A2 (HMGA2) impeding the recruitment of histone deacetylase 2 (HDAC2) to the *hTERT* proximal promoter; as well as *via* the cooperation with the nuclear factor of activated T cells (NFAT) as demonstrated by Chebel *et al.* (Chebel, Rouault et al. 2009).

2.2.1.5. Tumor suppressors implicated in the negative regulation of *hTERT*

Multiple tumor suppressor/oncogene pathways were shown to coordinately repress *hTERT* expression. Among the most recognized tumor suppressors involved in *hTERT* regulation we can list the p53 factor acting in a Sp1-dependent manner (Xu, Wang et al. 2000), and the retinoblastoma protein Rb, presumably inhibiting *hTERT* by influencing the chromatin structure *via* post-translational modifications of histones like methylation and deacetylation (Xu, Zhou et al. 1997). Several transcriptional repressors, including Wilms' tumor 1 (WT1) (Oh, Song et al. 1999) and myeloid specific zinc finger protein-2 (MZF-2) (Fujimoto, Kyo et al. 2000) are also known to repress *hTERT* transcription, although the exact molecular mechanisms standing behind this repression remains unclear. Moreover by the means of gene screening that used enhanced retroviral mutagenesis (ERM) Lin *et al.* identified several additional negative regulatory factors of *hTERT* (Lin and Elledge 2003). One of them is the Mad1/c-Myc pathway, which I have extensively described in the paragraph 3.2.1.2, concerning the E-box mediated regulation of *hTERT*. The second factor identified by Lin *et al.*, SIP1, is a transcriptional target of the TGF-β pathway, and mediates the TGF-β regulated repression of *hTERT*. Two other less studied genes, BRIT1 and RAK, encoding a protein kinase, were also identified as negative regulators of *hTERT*, however further studies are required to fully understand the molecular mechanism of their action (Lin and Elledge 2003).

The screening performed by Lin *et al.* reveled the menin tumor suppressor, as a direct inhibitor of *hTERT*. Menin physically associates with the *hTERT* promoter, as shown by the chromatin immunoprecipitation assay, however the molecular mechanism of menin action has not yet been elucidated. Nevertheless menin is known to act as a negative regulator of gene expression through its ability to inhibit the transcriptional activity of several positively acting transcription factors. Therefore menin-mediated

inhibition of *hTERT* could potentially involve its target factors like JunD and/or NF-κB. Above all menin seems to be a very important factor implicated in the *hTERT* regulation, as depleting menin results in telomerase reactivation, leads to immortalization of primary human fibroblasts and causes a transformation phenotype when coupled with expression of SV40 Large and Small T antigen and oncogenic ras (Lin and Elledge 2003).

Presented above tumor suppressors provide an endogenous mechanism for *hTERT* transcriptional repression, which are often inactivated during carcinogenesis. Consistent with this, many tumor disorders exhibit mutations within the coding sequence of those factors or are characterized by their inactivation on the post-translational level, including increased degradation or the functional impairment.

2.2.2. Epigenetic regulation of *hTERT* expression

The *hTERT* gene expression, in addition to a complex network of transcription factors, is also regulated by the epigenetic modifications, which influence the chromatin structure. This involve the covalent modifications of core histones, including acetylation, methylation and phosphorylation; as well as the DNA methylation by the DNA methyltransferases (DNMTs). Those modifications are affecting the local chromatin structure, and therefore its accessibility for transcription factors, thus directly influencing the transcriptional potential of affected sequences.

The most studied epigenetic regulation mechanism in the context of *hTERT* expression concerns the histone acetylation. Transcriptionally inactive genes tend to be associated with hypoacetylated histones, whereas transcriptionally active genes tend to be associated with hyperacetylated ones. Consistent with this hypothesis it has been shown that, trichostatin A, a potent inhibitor of classes I and II histone deacetylases (HDACs), induced *hTERT* transcription in normal cells and telomerase-negative immortal cell lines (Cong and Bacchetti 2000; Takakura, Kyo et al. 2001; Hou, Wang et al. 2002; Wang and Zhu 2003). In addition, *hTERT* transcription was also associated with hyperacetylation of core histones at the *hTERT* promoter in several types of cells, as revealed by chromatin immunoprecipitation (ChIP) (Xu, Popov et al. 2001; Wang, Hu et al. 2007).

The enzymes modifying histones acetylation, including histone acetyltransferases (HATs) and histone deacetylases (HDACs), are often recruited to the promoter by numerous transcription factors regulating the *hTERT* gene expression. Both Mad1 and Sp1 have been shown to be able to recruit repressor complexes containing the histone deacetylase presumably by their respective association with E-box and Sp1 sites within the *hTERT* promoter (Cong and Bacchetti 2000; Won, Yim et al. 2002). In agreement with this, Takakura *et al.* showed that the proximal 181bp promoter, which contains two E boxes and five GC sites, is the responsive TSA sequence (Takakura, Kyo et al. 2001). However, mutational analysis of the *hTERT* promoter demonstrated that the activation of *hTERT* transcription induced by TSA is dependent rather on GC sites, than on E-boxes, implying that Sp1 may play more important role in the inhibition of *hTERT via* HDACs recruitment (Takakura, Kyo et al. 2001; Won, Yim et al. 2002). Moreover the E2F–pocket protein–histone deacetylase (HDAC) complex have been also reported to recruit the HDACs to the *hTERT* promoter, resulting in its transcriptional repression (Won, Chang et al. 2004). Similarly the tumor suppressor menin, which was shown by Lin *et al.* to inhibit *hTERT* expression is known to recruit the HDAC1 and HDAC2 *via* associated mSin3A corepressor (Kim, Lee et al. 2003; Lin and Elledge 2003), however their role in *hTERT* inhibition still has to be confirmed.

Another post-translational histone modifications involved in the regulation of *hTERT* gene expression concern methylation and phosphorylation. Namely the SET and MYND domain-containing protein 3 (SMYD3), a histone methyltransferase was shown to directly trans-activate the *hTERT* expression *via* histone H3-K4 trimethylation (Liu, Fang et al. 2007). Similarly as shown by Ge *et al.* the phosphorylation of Ser10 on Histone H3 induced by the MAPK signaling, results in the transcriptional activation of the telomerase (Ge, Liu et al. 2006). However both types of histone modifications are for the moment very poorly studied in the context of the *hTERT* gene expression. Therefore further studies are required to draw more conclusions on this type of regulation.

Another widely studied epigenetic modification in the context of *hTERT* gene expression is the DNA methylation. As the *hTERT* promoter contains a high density of CpG dinucleotides, it is therefore a potential target for this modification (Dessain, Yu et al. 2000).

It is believed that the protection from DNA methylation, however not sufficient, it is

necessary for active gene transcription. Therefore it might be expected that telomerase positive cells will be free from DNA methylation within the *hTERT* promoter region. However despite many data acquired on this topic, the general pattern of the DNA methylation in normal and cancerous cells has not been revealed until now. Initial studies failed to reveal a correlation between gene expression and methylation of the *hTERT* promoter in a variety of cancerous and immortalized cell lines (Devereux, Horikawa et al. 1999; Dessain, Yu et al. 2000). Nevertheless, it has been shown that the treatment with the inhibitor 5-azacytidine induced *hTERT* transcription and the telomerase activity in certain telomerase-negative cell lines (Devereux, Horikawa et al. 1999; Dessain, Yu et al. 2000). Additionally the detailed analysis of *hTERT* promoter methylation in a variety of telomerase-positive cancer cell lines revealed that the region immediately before the transcription start site has very little or no methylation (Zinn, Pruitt et al. 2007). Also, a reverse correlation between the degree of methylation in the hTERT promoter and telomerase activity has been observed in B-cell lymphocytic leukemia (Bechter, Eisterer et al. 2002). This indicates that DNA methylation plays an important role in the epigenetic regulation of *hTERT* gene expression, however further studies are required to fully elucidate this phenomenon.

2.2.3. Post-transcriptional regulation of telomerase activity

The transcriptional regulation of *hTERT* is undoubtedly the primary mechanism in controlling telomerase activity. However, substantial evidence suggests that the post-transcriptional regulation might provide an additional layer of the control of telomerase activity. This involve the alternative splicing, protein folding, post-translational modification of hTERT, the assembly of the functional ribonucleoprotein holoenzyme, transport to functionally relevant nuclear compartments, interaction with protein partners influencing the enzymatic activity, and the access of the enzyme to the telomeres (Cong, Wright et al. 2002).

I will now briefly summarize the most interesting findings on the post-transcriptional regulation of the telomerase, in order to outline the complexity of this process.

Alternative splicing represents one of the major axes in the post-transcriptional regulation of telomerase activity. *hTERT* gene is composed of 16 exons, which may be differentially spliced, and more than 10 different alternative splice variants have been identified so far (Kilian, Bowtell et al. 1997). However only the full-length *hTERT* transcript, giving rise to the 127kDa protein, is associated with telomerase activity (Ulaner, Hu et al. 1998; Ulaner, Hu et al. 2001). Interestingly, this full length *hTERT* transcript (often referred as α+ β+) commonly accounts for only ~5% of total *hTERT* mRNA, with inactive variants comprising the rest (Yi, Shay et al. 2001). As ectopic expression of *hTERT*α-β+ splice variant results in dominant-negative inhibition of telomerase activity, the *hTERT* deletion variants likely play a role in the negative regulation of telomerase (Colgin, Wilkinson et al. 2000).

Post-translational control of the telomerase activity involves the modifications of hTERT, like phosphorylation and ubiquitination (Kang, Kwon et al. 1999; Kim, Park et al. 2005; Oh, Lee et al. 2010). As treatment with phosphatase 2A was shown to inhibit telomerase, it suggests that the phosphorylation is a necessary modification to maintain the catalytic properties of telomerase (Li, Zhao et al. 1997). Indeed phosphorylation of hTERT by the serine/threonine kinases, protein kinase C (PKCα and PKCζ) has been shown to stabilize the telomerase holoenzyme complex and enhance telomerase enzymatic activity in breast and prostate cancer cell lines (Li, Zhao et al. 1998; Yu, Lo et al. 2001; Chang, Lu et al. 2006). Moreover Akt protein kinase (also called protein kinase B), a downstream kinase of the phosphoinositide-3 kinase pathway, is also a positive regulator of telomerase activity (Kang, Kwon et al. 1999; Breitschopf, Zeiher et al. 2001). Activated Akt is able to phosphorylates two sites within hTERT (protein sequences 220-GARRRGGSAS-229 and 817-AVRIRGKSYV-826) and activate telomerase activity (Kang, Kwon et al. 1999; Breitschopf, Zeiher et al. 2001).

One interesting mechanism by which phosphorylation of hTERT may regulate telomerase activity was proposed by Liu *et al.*, who showed that hTERT upon its phosphorylation was translocated from the cytoplasm to the nucleus during $CD4^+$ T cell activation (Liu, Hodes et al. 2001). Therefore indicating that this post-translational modification affects the hTERT localization and by allowing its translocation to its functional nuclear compartment may play a role in the regulation of telomerase activity. These results were repeated by another group, showing a phosphorylation-dependent translocation of hTERT and

associated enzymatic activation in rat vascular smooth muscle cells (Minamino, Mitsialis et al. 2001). However it is worth to notice that in the contrary to PKC and Akt kinases, the phosphorylation of hTERT by another tyrosine kinase, c-Abl, results in the inhibition of the telomerase activity (Kharbanda, Kumar et al. 2000). Therefore hTERT phosphorylation may potentially exhibit a bimodal function, depending on the participating enzyme and the phosphorylation site.

The hTERT localization can be also affected *via* interaction with its protein partners. Akiyama *et al.* showed that hTERT interacts directly with NF-κB p65 and that TNFα –mediated activation of NF-κB pathway results in modulation of telomerase activity by inducing nuclear translocation of hTERT bound to NF-κB p65 (Akiyama, Hideshima et al. 2003). Similarly the members of the 14.3.3. protein family were also shown to promote the nuclear localization of hTERT. However the mechanism seems to be different. Namely the 14.3.3 protein are blocking the interaction of hTERT with CRM1/exportin 1, a receptor for the nuclear export machinery, allowing for efficient accumulation of hTERT in the nucleus (Seimiya, Sawada et al. 2000). Furthermore, PML-IV has been shown to negatively regulate telomerase activity in vivo by direct binding and recruitment of hTERT to PML bodies (Oh, Ghim et al. 2009).

Another post-translational regulatory mechanism involves chaperone proteins p23 and hsp90, which are involved in correct folding of hTERT (Akalin, Elmore et al. 2001; Forsythe, Jarvis et al. 2001), therefore participating in the effective assembly of the telomerase holoenzyme (Holt, Aisner et al. 1999).

Additionally several protein partners were shown to modulate the telomerase recruitment to the telomeres. For example the Ku70/80 protein, or the shelterin component TPP1, which directly interacts with TEN domain of hTERT (Chai, Ford et al. 2002; Xin, Liu et al. 2007; Zaug, Podell et al. 2010).

However shelterin components, normally are recognized as negative regulators of telomere elongation, as they participate in the formation of "closed" structure at the chromosomal ends, blocking them therefore from the access of the enzyme (Evans and Lundblad 2000). Consistent with this view, it was shown that telomeres exist in two states: uncapped, accessible by telomerase and capped where telomere elongation does not occur (Teixeira, Arneric et al. 2004). This effect of telomere capping is connected to the fact that G-rich single-stranded overhang at the chromosome terminus invades the

telomeric DNA duplex to form a t-loop, the structure that is subsequently stabilized by shelterin proteins (Greider 1999; Griffith, Comeau et al. 1999). Therefore the telomere-associated proteins, like TRF1 and TRF2 (TTAGGG repeat binding factors 1 and 2), which are involved in t-loop formation, are acting as negative regulators of telomere length (van Steensel and de Lange 1997; Smogorzewska, van Steensel et al. 2000). In addition to the t-loop, telomeric G rich overhang have also the potential to form G-quadruplexes, and this structures also prevent the elongation of telomeres by telomerase (Zahler, Williamson et al. 1991). Collectively, the formation of the secondary structures at the chromosome termini, which provide an architectural solution to protect telomere integrity, have the potential also to monitor the telomere homeostasis by modifying the access of telomerase to its substrate. This provides additional axes to the already complex transcriptional regulation of *hTERT* gene expression, adding further layers of the control of telomere length, both on the post-transcriptional and post-translational level.

2.3. Deregulation of telomerase upon HTLV-1 infection

Adult T-cell leukemia, like many other tumor disorders, has been associated with the activation of telomerase, as the leukemic cells isolated form blood samples of ATL patients exhibit higher telomerase activity compared to non-infected peripheral blood mononuclear cells (PBMC) from healthy volunteers and asymptomatic HTLV-1 carriers (Kubuki, Suzuki et al. 2005). Moreover a close correlation between telomerase activity and the clinical stage of the disease was observed, with higher telomerase activity in acute type relative to the less aggressive subtypes of ATL and asymptomatic carriers. This suggests that the reactivation of telomerase is a crucial event in the progression of ATL (Uchida, Otsuka et al. 1999). Interestingly leukemic HTLV-1$^+$ cells are also characterized by short telomere length (Kubuki, Suzuki et al. 2005), presumably connected to increased proliferation rate of ATL cells. Both high telomerase activity and shortened telomeres were associated with poor prognosis of ATL patients and are predicted to be good clinical markers for monitoring disease development, assessment of its prognosis and efficacy of the treatment (Kubuki, Suzuki et al. 2005).

Telomerase activity, strongly depends on the expression of its catalytic subunit,

the human telomerase reverse transcriptase (*hTERT*). Among HTLV-1 encoded proteins, Tax and HBZ were both reported to interfere with the transcription of the *hTERT* gene. Tax plays a bimodal role in *hTERT* gene regulation, being both the activator and repressor of its expression depending on the cell state. In resting lymphoid cells the ectopic expression of Tax increases *hTERT* expression whereas Tax represses this gene after T-cell activation (Gabet, Mortreux et al. 2003; Sinha-Datta, Horikawa et al. 2004). In the contrary to Tax, HBZ was found to always activate the *hTERT* expression, and its function is mediated *via* JunD (Kuhlmann, Villaudy et al. 2007).

Taking together, the regulatory mechanism of *hTERT* gene expression upon HTLV-1 infection seems to be very complex, involving both the inhibition and activation mediated by different viral oncogenes. Taking into account that Tax and HBZ exhibit different expression profiles throughout the ATL development, this differential regulation of *hTERT* may play a precise role in the establishment and the progression of ATL.

2.3.1. Bimodal function of Tax in the regulation of *hTERT* expression

Tax effect on the *hTERT* expression is still poorly understood, as the regulation of *hTERT* by Tax seems to be a very complex issue. Both an activatory and inhibitory functions were attributed to this viral protein.

Gabet *et al.* showed that Tax transactivator inhibits the expression of *hTERT* gene. Namely the analysis of different HTLV-1 cell lines, showed a decreased telomerase activity in the cell lines expressing Tax, the same effect was observed in HeLa cells upon ectopic expression of Tax. Detailed analysis of the promoter revealed that the inhibitory effect of Tax was dependent on the CANNTG consensus sequence located within the proximal promoter of *hTERT* (Gabet, Mortreux et al. 2003). This consensus sequence, referred as E box, functions as a binding site for the basic helix–loop–helix (bHLH) proteins, and mediates the activation of *hTERT* promoter *via* c-Myc. *hTERT* promoter contains two E boxes, but only the proximal one, located between positions 21 and 26 downstream of the *hTERT* transcription initiation site, was shown to be involved in Tax- mediated inhibition. It is proposed that Tax competes with c-Myc for the recruitment to the *hTERT* promoter, as c-Myc overexpression rescued the Tax-mediated transrepression of *hTERT*.

The decreased telomerase activity upon Tax-expression was observed both in HeLa and Jurkat cell lines as well as in primary $CD4^+$ and $CD8^+$ peripheral blood lymphocytes stimulated by PHA and subsequently transduced by Tax (Gabet, Mortreux et al. 2003).

Additionally Terme *et al.* showed that the negative effect of Tax on *hTERT* expression is reinforced by TAL1 (T-cell acute lymphoblastic leukemia 1) in transfected HeLa cells. As ATL cells exhibit higher TAL1 expression levels, both proteins may cooperate in the inhibition of *hTERT* expression upon HTLV-1 infection (Terme, Mocquet et al. 2009).

Another study showed that transduction of Tax in resting primary lymphocytes resulted in the activation of telomerase expression and increase of telomere length when cells were cultured in the absence of any exogenous stimulation (Sinha-Datta, Horikawa et al. 2004). While Tax-mediated inhibition of *hTERT* transcription was observed in response to mitogenic stimulation, confirming previous results. It was proposed that Tax stimulates the *hTERT* promoter in quiescent cells through the NF-κB pathway. This hypothesis was based on the observation that Tax mutants M22 and G148V, deficient for NF-κB activation, were neither able to activate the *hTERT* promoter nor sustain telomere length in transduced primary lymphocytes. Similarly in the presence of I-κBα, the specific NF-κB inhibitor, Tax was unable to activate the *hTERT* proximal promoter, thereby confirming that Tax-mediated NF-κB activation is required for the transcriptional activation of the *hTERT* gene in quiescent cells. On one hand, the proximal *hTERT* promoter does not contain any NF-κB consensus binding sites and the p65 was not detected on the *hTERT* promoter. On the other hand, analysis of the *hTERT* promoter occupancy in vivo using chromatin immunoprecipitation assays suggested that Tax-mediated activation of the *hTERT* promoter is connected to the increased binding of c-Myc and Sp1. Therefore the effect of NF-κB seems be indirect, and occur *via* c-Myc and Sp1, which may cooperate in activating the *hTERT* promoter (Sinha-Datta, Horikawa et al. 2004).

Subsequently Hara *et al.* confirmed that Tax can play both a role of an activator and an inhibitor of *hTERT* expression, and their findings suggested that the nature of Tax-mediated regulation of *hTERT* is dependent on the state of the cell cycle (Hara, Matsumura-Arioka et al. 2008). Reporter assays revealed that Tax activated the *hTERT*

promoter in quiescent Kit 225 cells, while the promoter activity was repressed by Tax in proliferating Jurkat cells. Additionally Tax induced endogenous *hTERT* gene expression in normal resting PBL, while this effect was not observed in IL-2-induced cells. Furthermore, in resting T-cells, Tax up-regulated the *hTERT* promoter but in activated growing T-cells the down-regulation of the *hTERT* promoter was observed. Both the up-regulation and down-regulation by Tax were mediated through the 43-bp sequences within the *hTERT* promoter, containing the proximal E-box (Hara, Matsumura-Arioka et al. 2008).

One of the known features of Tax is the inhibition of cell proliferation, resulting in cell-cycle arrest, presumably due to Tax-dependent stabilization of cdk inhibitors p21CIP1/WAF1 (Sieburg, Tripp et al. 2004). Therefore Hara *et al.* proposed that Tax-mediated down-regulation of the *hTERT* promoter in Jurkat cells may be attributed to this function of Tax (Hara, Matsumura-Arioka et al. 2008).

Moreover previous studies have shown that antigen-specific induction of telomerase occurs in vitro through CD3 and CD28 costimulatory signals and that telomerase is highly inducible in peripheral lymphocytes following activation through the CD3 or after stimulation with phorbol myristate acetate (PMA)/ionomycin. It is therefore important to note that HTLV-1–infected T cells have profound dysfunctions in CD3 responses, mediated in part by Tax. Thus, through its interference with the CD3 signaling pathway, Tax may lower the effect of PHA stimulation on the *hTERT* gene activation, thereby leading to a reduced activation of *hTERT* in PHA stimulated T cells (Sinha-Datta, Horikawa et al. 2004).

Summarizing the effect of Tax on the expression of *hTERT* gene seems to be very complex issue, involving many cellular pathways. Depending on the cell cycle state Tax seems to play a bimodal function in the regulation of the *hTERT* gene expression. Namely Tax activates the *hTERT* promoter in quiescent T-cells, but in growing T-cells mediates its repression (Hara, Matsumura-Arioka et al. 2008). This bimodal function of Tax could directly contribute to the development and the progression of Adult T-cell leukemia. Following model was proposed - during active T-cell proliferation, as observed in response to antigen or PHA stimulation, interference of Tax with the full *hTERT* induction may result in a transient genetic instability state. Once the mitogenic effect is passed, Tax-mediated activation of *hTERT* gene expression offers a long-term proliferative advantage to these cells that have acquired chromosomal abnormalities (Sinha-Datta,

Horikawa et al. 2004). Therefore, in one hand, Tax-mediated repression of telomerase activity may contribute to the increase genomic instability, resulting in the transformation of T-cells in the early stages of HTLV-1 infection (Gabet, Mortreux et al. 2003). On the other hand, Tax-mediated induction of the *hTERT* gene may contribute to extending the division capacity of HTLV-1 infected cells in the later stages following the transformation (Hara, Matsumura-Arioka et al. 2008).

Further studies are required to fully understand the role of Tax in the expression of *hTERT* gene, and confirm the raised hypotheses and proposed molecular mechanisms. Moreover comparison of different HTLV-1–positive leukemia cell lines showed no quantitative relationship between the *hTERT* and Tax expression levels, indicating that Tax may not be the major regulator of *hTERT* expression (Sinha-Datta, Horikawa et al. 2004).

2.3.2. Mechanism of HBZ-mediated activation of *hTERT*

HBZ viral protein was shown to exert a positive effect on the *hTERT* gene transcription in transfected HeLa cells, and it required heterodimerization with JunD AP-1 transcription factor to exhibit this function (Kuhlmann, Villaudy et al. 2007). Luciferase reporter assays revealed that the responsive sequence is located within the -378/+1 proximal promoter of *hTERT*, which is devoid of any AP-1 sites, however it contains two E boxes and five GC boxes. Chromatin immunoprecipitation revealed that the recruitment of HBZ/JunD heterodimer is mediated *via* Sp1 factors, therefore suggesting that the recruitment occurs *via* GC boxes. Kuhlmann *et al.* also showed that both the bZIP domain responsible for the interaction with JunD, and the activation domain of HBZ were critical for the HBZ-mediated activation of the *hTERT* expression. Additionally the luciferase reporter assays revealed that the positive effect of HBZ on the *hTERT* promoter was reversed by overexpression of Tax, confirming that both proteins have an inverse effect on the transcription of the *hTERT* gene (Kuhlmann, Villaudy et al. 2007).

Another study revealed a supplementary mechanism of HBZ-mediated activation of *hTERT* expression. Namely HBZ was found to directly interact and promote the degradation of TAL1, a negative regulator of *hTERT* expression (Terme, Mocquet et al.

2009). As the proteasome inhibitor, MG132, was able to block this effect, HBZ-mediated degradation of TAL1 presumably depends on the proteasome, similarly to previously reported degradation of c-Jun (Matsumoto, Ohshima et al. 2005). Therefore *via* inactivation of the endogenous TAL1, HBZ is reinforcing its transactivator effect on *hTERT* expression, mediated *via* mechanism involving JunD. Thus in the presence of HBZ, due to above mentioned double mode of action, the strong *hTERT* gene activation is observed.

2.3.3. Deregulation of *hTERT* in adult T-cell leukemia

Taking into account the acquired knowledge on the differential regulation of the *hTERT* gene by Tax and HBZ and on their expression profiles in ATL cells, the following model of the development of ATL was proposed. Tax, important in the early stages of the ATL development, inhibits telomerase expression and at the same time promotes proliferation of infected T cells (Gabet, Mortreux et al. 2003). This results in progressive shortening of telomeres, as terminal sequences of linear DNA molecules, due to so called end replication problem, are not amplified. Short telomeres are recombinogenic and are prone to promote chromosome fusions. Due to simultaneous inactivation of DNA damage mitotic checkpoints, such as p53 and MAD1 (Jin, Spencer et al. 1998; Tabakin-Fix, Azran et al. 2006), the cells with acquired chromosomal aberrations can evade the cellular senescence. This extended cell survival accompanied by continued telomere shortening leads to the accumulation of chromosomal rearrangements. Once the mitogenic effect is passed, Tax switches from telomerase inhibitor to telomerase activator, at the same time promoting cell proliferation (Sinha-Datta, Horikawa et al. 2004). At the later stage, when Tax expression is repressed, the high telomerase activity is maintained by HBZ, which is constantly expressed in the ATL cells (Kuhlmann, Villaudy et al. 2007). This allows for stabilization of the earlier established pro-cancer genotype and endows the cells with immortal growth potential, finally leading to the malignant transformation, the clonal expansion of HTLV-1-infected cells and as a result development of ATL.

Chapter 3

Menin tumor suppressor

Menin is a ubiquitously expressed tumor suppressor encoded by *MEN-1* gene (Wautot, Khodaei et al. 2000). Mutations in its coding sequence predispose to multiple endocrine neoplasia (MEN-1 syndrome) (Gaudray and Weber 2009). This autosomal dominant disorder has a prevalence ranging from 1 in 10,000 to 1 in 100,000. Affected individuals develop hyperplastic and neoplastic disorders of endocrine organs such as parathyroid, anterior pituitary and duodenopancreatic endocrine tissues (Agarwal, Kester et al. 1997; Marx, Agarwal et al. 1998).

Consistent with its proposed function as a tumor suppressor, menin overexpression in the human endocrine pancreatic tumor cell line, results in an inhibition of cell growth (Stalberg, Grimfjard et al. 2004). Moreover the ectopic expression of menin in Ras transformed fibroblasts, results in a decreased proliferation *in vitro*, and suppresses Ras-induced tumor growth in nude mice (Kim, Burns et al. 1999). Additionally, menin expression in *MEN-1*-deficient tumor cell lines induces cell cycle arrest and apoptosis (Hussein, Casse et al. 2007). At the same time, depletion of menin in human fibroblasts, results in their immortalization (Lin and Elledge 2003), further supporting the role of menin as a tumor suppressor.

On the cellular level menin has been shown to play a role in maintaining the genome stability and ensuring appropriate control of cell proliferation and apoptosis, which are among the hallmarks deregulated in cancer cells (Yang and Hua 2007). Growing evidence indicate that menin exerts its function by the transcriptional regulation of genes

involved in cell cycle regulation and DNA repair or *via* direct contact with those factors and modulations of their functions on the protein level (Thakker 2010).

3.1. Regulation of *MEN-1* gene expression

Regulation of *MEN-1* expression is for the moment very poorly understood and only few reports are available describing *MEN-1* promoter. I will summarize herein the knowledge on the structure of *MEN-1* gene and regulation of its expression acquired until now.

The *MEN-1* gene was mapped to the long arm of the chromosome 11 (11q13) in 1988 by the researchers from the Uppsala University and Karolinska Institute in Stockholm (Larsson, Skogseid et al. 1988; Bystrom, Larsson et al. 1990). The entire gene was cloned and described 10 years later by the scientists form NIH in Bethesda (Chandrasekharappa, Guru et al. 1997). The *MEN-1* gene spans 9kb of the genome and contains 10 exons, from which entire exon 1 and the 3832bp of exon 10 are untranslated (Guru, Manickam et al. 1998; Chandrasekharappa and Teh 2003).

In humans, a single transcript of 2.8kb that encodes menin is detected in most tissues. However, in a few tissues like pancreas and thymus, a second transcript of 4.2kb occurs, which structure and function remains undetermined (Lemmens, Van de Ven et al. 1997). The *MEN-1* 2.8kb transcripts are heterogeneous, as several alternatively spliced transcripts have been identified, which differ in their 5' untranslated region (5'UTR) and encode the same protein. Six distinct exons 1 (e1A–e1F) were isolated, however the e1B splice-variant, with transcription start sites (TSS) at positions: 1338 and 1832, seems to be the major *MEN-1* transcript in humans. The ATG indicating the initiation of translation is located within the exon 2 (position +457) (Fromaget, Vercherat et al. 2003).

The level of *MEN-1* expression is regulated alongside the cell-cycle, and reaches the highest level when cells enter the S-phase, similarly to other well known tumor suppressors such as BRCA1, BRCA2 and p53 (Kaji, Canaff et al. 1999). Additionally a cell type specific regulation of *MEN-1* expression has been suggested, as not all of the tissues express menin. It was detected in variety of organs including brain cortex, kidney, pituitary, testis and thymus as well as thyroid, adrenal glands and heart muscle, however

was undetectable in liver, lungs, pancreas and skin (Wautot, Khodaei et al. 2000). Interestingly the highest menin expression level was detected in proliferating tissues (Ikeo, Sakurai et al. 2000), suggesting a compensatory mechanism as a reaction for increased proliferation. Indeed menin expression was shown to be up-regulated in case of DNA damage and increased cell proliferation (Wrocklage, Gold et al. 2002).

The minimal *MEN-1* promoter was mapped to the region between **-**135 and **-**36, lying closely upstream the most commonly expressed first exon, and the region further upstream modifies its activity (Fromaget, Vercherat et al. 2003; Zablewska, Bylund et al. 2003). *MEN-1* promoter is TATA-less, and it contains instead Inr (initiator) elements (Fromaget, Vercherat et al. 2003).

Sequence analysis of the *MEN-1* promoter revealed existence of several putative transcription factors binding sites. The distal promoter contains the estrogen responsive element (ERE) and the forkhead box O1 (Foxo1, from -980 to -806) and a putative nuclear factor kappa B (NF-κB) binding site (Fromaget, Vercherat et al. 2003; Zablewska, Bylund et al. 2003; Zhang, Li et al. 2012). The proximal *MEN-1* promoter contains putative E box (-232/-227), neuron-restrictive silencer element (NRSE), as well as binding sites for paired box protein (PAX5), GA binding protein (GABP), NF-κB (-182/-171), nuclear factor Y (NFY), muscle initiator (MI), and Sp1 (schematically shown on Figure 10).

Sp1 sites are often present in TATA-less promoters, where they are demonstrated to activate transcription through direct interaction with basic transcriptional machinery factors, like TFIID. *MEN-1* promoter contains 3 putative binding sites for Sp1 and one is located within the minimal promoter, thus Sp1 may be responsible for its basic activation. Also a CCAAT box was identified within the proximal *MEN-1* promoter (-178/-174), however there are two contradictory reports, one stating that mutation CCAAT site reduces 30% of global promoter activity, while according to another group site-directed mutagenesis of this box had no effect on the expression from the *MEN-1* promoter (Fromaget, Vercherat et al. 2003; Zablewska, Bylund et al. 2003). Therefore additional studies are required to confirm whether identified CCAAT box is functional and play a role in the regulation of *MEN-1* expression.

MEN-1 promoter contains as well CpG islands, one encompasses the minimal promoter (from -194 to +73). Methylation of this region was documented in clinical samples of pancreatic ductal adenocarcinoma (Cavallari, Silic-Benussi et al. 2009). The second stretch

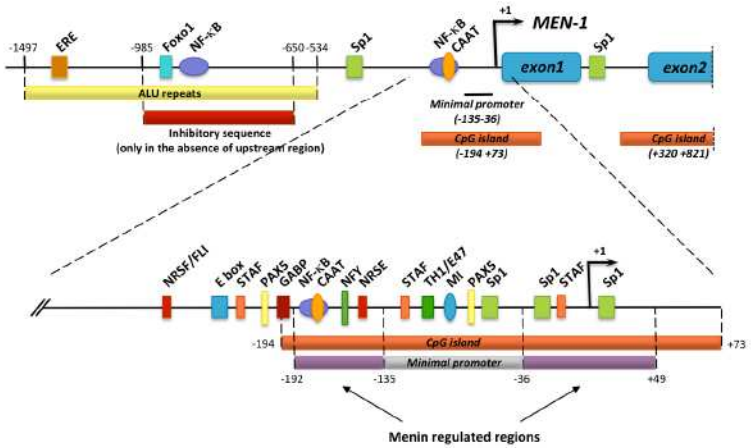

Figure 10. Schematic representation of *MEN-1* promoter. First two exons, minimal promoter, CpG islands and menin responsive sequences are indicated. Putative estrogen responsive element (ERE), neuron-restrictive silencer element (NRSE), CAAT and E boxes as well as putative binding sites for transcription factors are shown, including the nuclear factor kappa B (NF-κB), paired box protein (PAX5), forkhead box O1 (Foxo1), GA binding protein (GABP), nuclear factor Y (NFY), muscle initiator (MI) and Sp1. Black arrow indicates the most common transcription start site (Based on (Fromaget, Vercherat et al. 2003; Zablewska, Bylund et al. 2003; Zhang, Li et al. 2012).

of CpGs is located within the untranslated region downstream the transcription start site, including the first intron and exon2 (from +320 to +821), however this region was found to be entirely unmethylated both in endocrine tumors and normal tissue (Chan, Kim et al. 2003).

Additionally an inhibitory sequence was described encompassing the region between -985 and -651, however no specific factors have been associated with its activity. Moreover this inhibitory element was effective only in the absence of the sequence further upstream, even though this upstream region didn't exert any promoter activity alone. Whole region spanning from -1497 to -534 is entirely covered with short interspersed (SINE)/Alu repetitive elements interrupted by two simple repeats, a polyA

repeat (-813/-786) and a TATATG one (-785/-709). As the SINE/Alu repeats are able to work both as silencers and activators of transcription, they may play a role in the regulation of above-mentioned region (Zablewska, Bylund et al. 2003).

3.2. Localization, structure and function of menin

Menin is highly conserved, from fruit flies, zebrafish, mice, to humans, and it is ubiquitously expressed in the majority of the tissues (Trump, Farren et al. 1996; Hendy, Kaji et al. 2009).

Menin contains 610 amino acids and is predominantly nuclear (Guru, Goldsmith et al. 1998). Moreover during and immediately after cell division menin is also found in the cytoplasm and at that time of the cell cycle it can associate with cytoskeletal elements (Huang, Zhuang et al. 1999; Wautot, Khodaei et al. 2000). However menin functions in the cytoplasm are still poorly understood.

The nuclear localization of menin is determined by two NLSs (nuclear localization signals, Figure 11) at the C-terminal part of the protein (NLS1 - amino acids: 479–497, NLS2 - amino acids: 588–608) (Guru, Goldsmith et al. 1998). Both NLSs are functionally independent and a single NLS is sufficient for the nuclear targeting of menin. Additionally an accessory signal (NLSa - amino acids: 546–572) has also been found, which plays an auxiliary role (La, Desmond et al. 2006).

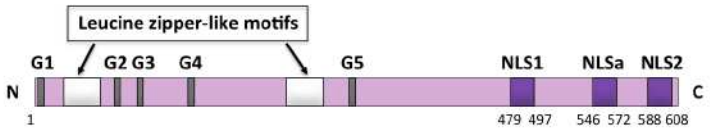

Figure 11. Schematic representation of the functional domains of menin. Menin has two main nuclear localization signals at its C-terminus, NLS1 (spanning amino acids from 479 to 497), NLS2 (aa 588–608) and an alternative NLSa (aa 546– 572). N terminal part of menin contains five putative guanosine triphosphatase (GTPase) sites (G1-G5) and two leucine zipper-like motifs. After (Balogh, Racz et al. 2006; Thakker 2010).

The N-terminal part of menin contains five consensus sequences, so called G motifs. Those sequences are characteristic of GTPases or GTP-binding proteins. Effectively menin is able to interact with GTP or GDP, however, the affinity of this binding is low, and menin alone has no detectable GTPase activity. Nevertheless it was shown that it can exhibit those properties upon interaction with a tumor metastasis suppressor nm23H1/nucleoside diphosphate kinase (nm23) (Yaguchi, Ohkura et al. 2002).

Additionally leucine zipper-like motifs were found in the N-terminal part of menin. Their exact roles have not been confirmed, however it was suggested that they could potentially play a role in direct binding to the double stranded DNA in a sequence specific manner (Balogh, Racz et al. 2006).

In studies addressing the function of menin, several protein partners have been identified (Poisson, Zablewska et al. 2003). Many described tumor suppressor properties of menin are steaming from the modulation of the functions of its interacting proteins. Such direct contact play a role, for example, in menin-mediated maintenance of genome stability. Namely menin was shown to interact and modulate the function of proteins involved in DNA replication, recombination and repair, like FANCD2 protein and the subunit of replication protein (RPA2) (Jin, Mao et al. 2003; Sukhodolets, Hickman et al. 2003). Menin also has a role in direct regulation of cell division as it interacts with the cytoskeletal elements, including nonmuscle myosin heavy chain IIA (NMHC II-A), glial fibrillary acidic protein (GFAP) and vimentin, which are involved in the intermediate filament network (Lopez-Egido, Cunningham et al. 2002). Additionally a function in cell cycle control is exerted *via* binding and suppression of the activator of the S-phase kinase (ASK), which is a component of the Cdc7/ASK kinase complex, that is crucial for promoting cell proliferation (Schnepp, Hou et al. 2004).

Moreover menin has been found to interact with a variety of transcription factors including NF-κB and Smad family members, JunD, Pem, ERα, β-catenin, and MLL complex, which play a role in transcriptional regulation of genes implicated in the control of cell growth, survival, apoptosis, signal transduction, DNA replication and repair (Yang and Hua 2007).

Menin binding proteins are summarized in Table 4, and the amino acids in the sequence of menin implicated in the interaction are indicated. Furthermore following chapters will

also describe more in details the relationship of menin with different signaling pathways and its role in the regulation of gene expression.

3.3. Menin-mediated regulation of gene expression

The tumor suppressor functions of menin are gathered around the regulation of gene expression at the level of transcription. Chromatin immunoprecipitation studies revealed hundreds of menin occupied chromatin regions, indicating that it can be implicated in the regulation of great number of genes (Scacheri, Davis et al. 2006; Agarwal, Impey et al. 2007). Interestingly menin exerts a dual role in the regulation of

Function	Interacting partner	Binding domain
Transcriptional regulation	JunD	codons 1–40, 139–242, 323–428
	NF-κB (p50, p52, p65)	codons 305–381
	Pem	codons 278–476
	Sin3A	codons 371–387
	HDAC1	codons 145–450
	Smad1	ND
	Smad3	codons 40–278, 477–610
	Smad5	ND
	Runx2	ND
	MLL	aa 241, 278, 281
	ERα	ND
	CHES1	codons 428–610
Genome stability	RPA2	codons 1–40, 286–448
	FANCD2	codons 219–395
Cell division	NMHC II-A	codons 154–306
	GFAP	ND
	Vimentin	ND
Cell cycle control	Nm23	codons 1–486
	ASK	codons 558–610

Table 4. Protein partners of menin. The binding domains and the cellular function steaming from the identified interaction are indicated (excluding those marked as ND-non determined). After (Huang, Gurung et al. ; Thakker 2010).

transcription, as it can act both as an activator and repressor of gene expression. Thus menin was found to inhibit the tumorigenic target genes, such as the *hTERT* (human telomerase reverse transcriptase) and *IGFBP-2* (insulin-like growth factor binding protein 2) (Lin and Elledge 2003; La, Schnepp et al. 2004), and stimulate the expression of tumor-suppressors such as cyclin-dependent-kinase inhibitors p18INK4c and p27Kip1, as well as the homeobox Hoxc8 and the apoptosis effector caspase 8 (Hughes, Rozenblatt-Rosen et al. 2004; Milne, Hughes et al. 2005; La, Yang et al. 2007). These diverse effects on transcription can be reconciled by the fact of differential regulation of distinct signaling pathways by menin. It has been shown to interact with the AP-1-family transcription factor JunD, changing it from an oncoprotein into a tumor suppressor (Agarwal, Guru et al. 1999). At the same time menin maintains TGF-β-mediated signal transduction and inhibit the NF-κB signaling (Heppner, Bilimoria et al. 2001; Kaji, Canaff et al. 2001). Additionally menin is also known to recruit various chromatin-modifying protein complexes like mSin3A-histone deacetylase (HDAC) complex as well as mixed lineage leukemia (MLL1 and MLL2) and associated histone methyltransferase (HMT) complexes (Kim, Lee et al. 2003; Hughes, Rozenblatt-Rosen et al. 2004; Yokoyama, Wang et al. 2004). Those chromatin-remodeling complexes mediate both positive and negative effects on the regulation of gene expression.

Interestingly menin does not contain known DNA-binding motifs and therefore it relies on DNA-sequence-specific transcription factors for its targeting to the promoters. Additionally it is believed that menin functions as a scaffold protein to regulate transcription of its target genes by concomitant association with transcription factors and chromatin modifying complexes.

The following chapters will aim on summarizing the knowledge on menin-mediated regulation of gene expression.

3.3.1. Association with chromatin remodeling complexes

Menin is involved in epigenetic regulation of transcription by associating with nuclear proteins involved in chromatin remodeling. This involves the protein complexes implicated in histone deacetylase and histone methyltransferase activity.

The theory that the transcription function of menin is tied to chromatin regulation was raised after the finding that menin is an integral component of human histone methyltransferase complexes (Hughes, Rozenblatt-Rosen et al. 2004; Yokoyama, Wang et al. 2004). These complexes contain members from the mixed lineage leukemia and trithorax protein family (MLL1/MLL2, Rbbp5, WDR5 and hAsh2), which *via* their SET domains can direct the trimethylation of the fourth lysine residue of the histone H3 (H3K4-me3). Unlike other methylation marks (for example H3K9 and H3K27), H3K4 trimethylation is strongly associated with transcriptional activation (Dreijerink, Hoppener et al. 2006). Effectively the H3K4-me3 was shown to coincide with the presence of RNA pol II and the basal transcription factors on the promoter of target genes (Dreijerink, Hoppener et al. 2006).

Consistent with its identified function, in pancreatic tumors of heterozygous *MEN-1* knock-out mice the global levels of H3K4-me3 were decreased (Karnik, Hughes et al. 2005), supporting the role of menin as a scaffold protein recruiting histone methyltransferases to the promoters of target genes. Being a molecular adaptor physically linking MLL histone methyltransferase with transcription factors, menin acts as a coactivator up-regulating the transcriptional activity of activated estrogen receptor, vitamin D receptor and the lens epithelium derived growth factor (LEDGF) (Dreijerink, Mulder et al. 2006; Yokoyama and Cleary 2008).

In addition to histone methyltransferases, menin associates also with histone deacetylase complexes (HDACs) (Kim, Lee et al. 2003; Mould, Duncan et al. 2007). Removal of the acetyl groups on histones by deacetylases results in chromatin remodeling into more packed structure and therefore this property of menin links it to the inhibition of gene transcription (Jenuwein and Allis 2001). Menin was shown to recruit HDACs through association with mSin3A, a general transcriptional corepressor. mSin3A is recruited *via* SID domain located in the central part of menin, which is composed of a cluster of hydrophobic residues forming an amphipathic α-helix (Kim, Lee et al. 2003).

Thanks to the recruitment of HDACs menin was shown to indirectly down-regulate the expression of certain genes. One of the examples is the *Ccnb2* encoding the cyclin B2, implicated in the G2/M transition during the cell cycle progression. Menin was shown to associate with the promoter of *Ccnb2* gene and recruit the HDAC3. Resulting reduction of

histone H3 acetylation at the *Ccnb2* locus, repressed the expression of cyclin B2 (Wu, Zhang et al. 2010).

The recruitment of HDACs by menin was also shown to result in the inhibition of transcriptional activity of JunD, and therefore indirectly implicated in the down-regulation of JunD regulated genes (Kim, Lee et al. 2003). Detailed analysis of menin-mediated inhibition of JunD transcriptional activity will be presented in the next chapter.

3.3.2. Inhibition of JunD transcriptional activity

JunD was the first menin-interacting protein described, and was identified as such through yeast two-hybrid screening (Agarwal, Guru et al. 1999). JunD is a basic leucine zipper (bZIP) DNA-binding protein and a member of the Jun family of transcription factors (Ryder, Lanahan et al. 1989; Berger and Shaul 1991). Jun proteins can homo- and heterodimerize with other bZIP proteins of Jun (c-Jun, JunB, or JunD), Fos (c-Fos, FosB, Fra-1, or Fra-2), Maf and ATF/CREB (activating transcription factor / cAMP-response element-binding protein) protein families to form the activator protein 1 (AP-1) transcription factor (Nakabeppu, Ryder et al. 1988). The common feature of all AP-1 family members is the presence of above mentioned basic leucine zipper (bZIP) motif, which consists of a DNA binding domain rich in basic amino acids adjacent to a leucine zipper structure required for protein-protein dimerization (Landschulz, Johnson et al. 1988). AP-1 dimers thanks to this domain can directly bind dsDNA. This group of transcription factors can recognize either TPA response elements (TRE - TGACTCA) or cAMP response elements (CRE - TGACGTCA) which are present in the promoter region of many cellular genes involved in a large spectrum of biological processes including cell proliferation, apoptosis and oncogenic transformation (Shaulian and Karin 2001).

Two isoforms of JunD, resulting from alternative translation start codons within the JunD mRNA are produced: a 39 kDa full-length JunD protein (JunD-FL, Figure 12) by initiation at the first AUG codon downstream of the mRNA 5'cap and a shorter, 34 kDa JunD protein (ΔJunD) by initiation at a second in-frame AUG codon (Okazaki, Ito et al. 1998). These two isoforms are ubiquitously expressed at approximately the same stoichiometry and are identical except for 48 N-terminal amino acids in the longer

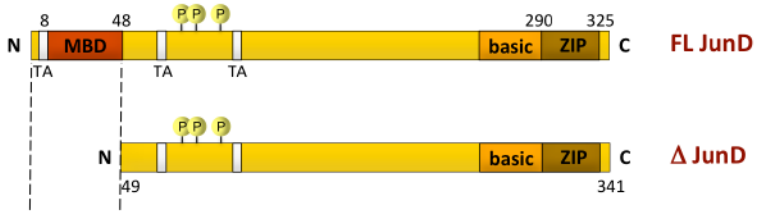

Figure 12. Schematic representation of ΔJunD and full-length JunD (FL). Menin binding domain (MBD) is located within the first 48aa of the N-terminal part of FL JunD, truncated in ΔJunD. TA- transactivation domain, ZIP- leucine zipper, basic - region responsible for direct interaction with DNA. Phosphoacceptor sites for mitogen-activated protein kinase (MAPK) are indicated (serine 90, serine 100, and threonine 117). Scheme modified after (Yazgan and Pfarr 2001).

full-length JunD (Yazgan and Pfarr 2001). Interestingly, the JunD-FL isoform has higher basal transcriptional activity than the ΔJunD isoform, which suggests that JunD-FL activity must be more tightly regulated (Yazgan and Pfarr 2001).

An important regulatory mechanism of JunD function involves its phosphorylation through the activation of MAPK (mitogen-activated protein kinase) cascades, including JNK (Jun N-terminal kinase) and ERK (extracellular-signal-regulated kinases) (Karin 1996). Both JunD isoforms undergo this regulation, as the three MAPK phospho-acceptor sites (serine 90, serine 100, and threonine 117) are all located within the N-terminal transactivation domains, present in both ΔJunD and JunD-FL (Vinciguerra, Vivacqua et al. 2004). MAPK phosphorylation is mediated by the presence of docking sites, including N-terminal D domain and the C-terminal DEF motif (Vinciguerra, Vivacqua et al. 2004). While the D domain is sufficient to induce JunD activation by JNK signaling, both the D and DEF motifs are required for efficient phosphorylation and activation of JunD in response to transient activation of ERK (Vinciguerra, Vivacqua et al. 2004).

JunD was shown to interact with menin, which represses its transcriptional activity (Agarwal, Guru et al. 1999). The interaction occurs between N-terminal 8–48 residues of JunD-FL and the C-terminal part of menin (Agarwal, Guru et al. 1999; Yazgan and Pfarr

2001). As the menin binding domain (MBD) is contained within the first 48 amino acids, precisely the same region that is truncated in ΔJunD, menin has the ability to interact only with the full-length isoform. Therefore menin strongly suppresses the transcriptional activity of JunD-FL, whereas it does not affect the activity of the shorter ΔJunD isoform (Yazgan and Pfarr 2001).

Menin-induced inhibition of JunD involves two separate mechanisms occurring on the level of post-translational modification of JunD or on the level of JunD-mediated transcription on the promoters of target genes (Figure 13).

One of the mechanisms employed by menin to inhibit JunD transcriptional activity involves the MAPK pathway. It has been shown that menin interplay with PKC (protein kinase C), an up-stream regulator of MAPK cascade, and exerts an inverse role in regulating JunD transcriptional activity (Kim, Lee et al. 2005). Namely menin was shown to down-regulate the phosphorylation state of the JunD-N-terminal domain by uncoupling JunD phosphorylation from both JNK and ERK activation (Gallo, Cuozzo et al. 2002).

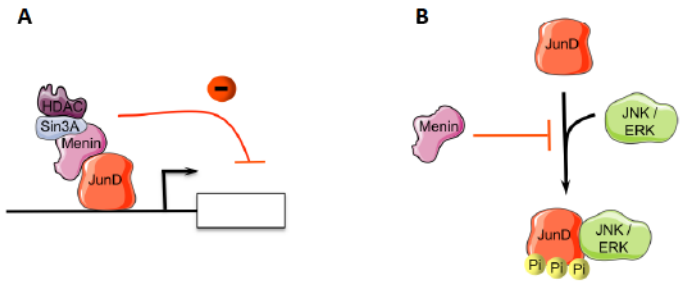

Figure 13. Double mode of menin-mediated inhibition of JunD transcriptional activity. A. Menin recruits HDAC/mSin3A histone deacetylase complex to the promoters of JunD regulated genes. B. Menin inhibits JunD transcriptional activity preventing its phosphorylation by JNK/ERK kinases.

It was recently shown that JNK-mediated phosphorylation is inhibited by menin through blocking the JNK-docking on JunD, as the MBD (menin binding domain) and JNK docking motif are immediately adjacent (Huang, Gurung et al.). The exact mechanism by which menin perturb the ERC-mediated phosphorylation is not known so far. However, it was shown that the N-terminal deletion mutant of menin, which lacks JunD binding activity, retained the ability to efficiently inhibit ERK-dependent phosphorylation of JunD, while as expected was not able to inhibit JunD phosphorylation by JNK (Gallo, Cuozzo et al. 2002). These findings indicate that the physical interaction between JunD and menin is not required to inhibit ERK, and distinct mechanisms account for the inhibition of signal-induced phosphorylation of JunD by both ERK and JNK. However, both mechanisms act at the levels that are downstream of MAPK activation, since menin overexpression does not alter signal-dependent activation of either ERK or JNK (Gallo, Cuozzo et al. 2002). Thus menin functions as suppressor of ERK-induced phosphorylation without directly altering its activity, however the exact mechanism remains to be elucidated.

The second mechanism employed by menin to inhibit transcriptional activity of JunD is histone deacetylase (HDAC)-dependent, as trichostatin A (TSA), an inhibitor of HDAC reverses menin-mediated inhibition (Gobl, Berg et al. 1999; Kim, Lee et al. 2003). It has been shown that the recruitment of HDAC occurs through general corepressor mSin3A, binding to α-helical mSin3- interacting domain [SID (371–387)] located within the central part of menin (Kim, Lee et al. 2003). Therefore this inhibitory mechanism is based on histone deacetylation and resulting silencing of promoters of JunD regulated genes. Confirming this hypothesis, increasing amount of p300 histone acetyltransferase restored menin- mediated repression of JunD activity in reporter gene assays (Kim, Lee et al. 2005).

The interaction with menin seems to be of a key importance in restraining the cancerogenic potential of JunD, and disruption of the JunD-FL–menin interaction is one of the mechanism leading to tumorigenesis for example in MEN 1 syndrome (Yazgan and Pfarr 2001). In normal cells JunD, in contrast to other AP-1 factors, has been shown to inhibit cell proliferation. It acts as a negative regulator of Ras-dependent cell growth and protects cells from p53- dependent senescence and apoptosis (Pfarr, Mechta et al. 1994; Weitzman, Fiette et al. 2000). It has been proposed that JunD might achieve its unique tumor suppressor properties through interaction with menin (Agarwal, Novotny et al.

2003). This is not the case for other members of Jun family (c-Jun, JunB), lacking the N-terminal menin binding domain (Agarwal, Guru et al. 1999; Yazgan and Pfarr 2001).
It is now commonly accepted that JunD in the context of normal cell exist always in the complex with menin, rather than in a 'free' form (Yazgan and Pfarr 2001), and this association appears to reverse the effect of JunD on cell growth. Binding to menin converts JunD to a growth suppressor, whereas it acts as a growth promoter when it is unable to bind menin (Agarwal, Novotny et al. 2003).

3.3.3. Inhibition of NF-κB pathway

The NF-κB transcription factors are major regulators of the cellular response to various types of stress. In unstimulated cells, NF-κB factors are present in the cytoplasm, bound in an inactive complex with the inhibitory protein IκB. Following stimulation, IκB is phosphorylated, and the NF-κB factors translocate to the nucleus (Siebenlist, Franzoso et al. 1994). Active NF-κB complexes are composed of homo- or heterodimers of the transcription factors p50, p52, p65 (RelA), c-Rel and RelB. The dimerization is mediated *via* N-terminal Rel- homolgy domain, which also mediates DNA-binding and is implicated in the nuclear targeting and binding to IκB (Baldwin 1996; Wulczyn, Krappmann et al. 1996).

Heppner *et al.* found that among NF-κB transcription factor family members, menin interacts with p50, p52 and p65 (RelA). The immunoprecipitation assays showed that amino acids 305 - 381 of menin are essential for this binding, and the interaction is mediated *via* N-terminal region of the Rel-homology domain of the NF-κB members (Heppner, Bilimoria et al. 2001). As a result of this interaction, an inhibition of p65-mediated transcriptional activation on NF-κB sites was observed, and this effect was specific to menin and occurred in a dose-dependent manner. As neither alterations in NF-κB protein expression levels nor protein compartmentalization was observed, it seems likely that menin acts directly on NF-κB in the nucleus (Heppner, Bilimoria et al. 2001). Since the Rel homology domain of NF-κB proteins is involved in DNA binding, it would be possible that menin interferes with the recruitment of the NF-κB complex to DNA (Malek, Huxford et al. 1998). Alternatively menin inhibitory role might be mediated by the histone

deacetylase complex recruited *via* mSin3A, however further studies are required to confirm this hypothesis (Kim, Lee et al. 2003).
NF-κB family members are involved in cellular functions such as cell proliferation, apoptosis, cell cycle regulation and are mediating positive signals on the cell growth. Therefore destabilization of those functions by menin may lead to cellular transformation and oncogenesis.

3.3.4. Maintenance of TGF-β signaling

Transforming growth factor β (TGF-β) signaling pathways cause growth inhibition in a variety of tissues, providing cytostatic signals that limit G1 progression and the cell proliferation. The critical components of the TGF-β pathway are the Smad transcription factors (Massague 1998). Activation of TGF-β stimulates Smad proteins, which transfer its effects to the nucleus. Receptor mediated phosphorylation of Smad2 or Smad3 induces their association with the common partner Smad4 followed by the translocation into the nucleus where these complexes activate transcription of specific genes (Massague 1998; ten Dijke, Miyazono et al. 2000). All Smad proteins share structural conservation of amino-terminal and carboxyl-terminal domains, designated MH1 and MH2, respectively. While the MH1 domain is important for DNA binding, the MH2 domain is involved in the interaction with other transcriptional regulators and therefore maintenance of the Smad transcriptional activity (Massague 1998).

Menin was shown to play an important role in supporting TGF-β signaling, and this effect occurs in Smad3 dependent manner. Menin interacts with Smad3 and promote Smad3-mediated transcription. The exact molecular mechanism has to be still confirmed, however it seems that menin does not affect the Smad3/Smad4 oligomerization, nor its nuclear translocation, but rather promotes the Smad3 binding to DNA at specific transcriptional regulatory sites (Kaji, Canaff et al. 2001). The exact domain in menin which is responsible for the interaction with Smad3, was not determined, however menin Δ(1–40) and menin Δ(278–477), which demonstrate nuclear localization but not JunD binding, retained the ability to bind Smad3. This suggests that the nuclear localization signals of menin are important for maintaining this interaction. At the same time a MH2 domain of

Smad3 was shown to be essential for this interaction (Kaji, Canaff et al. 2001). Additionally menin was also shown to bind Smad1 and Smad5 in the mouse bone marrow stromal and osteoblast cell lines (Sowa, Kaji et al. 2004). However the significance of this phenomenon is not yet well defined.

Consistent with the function of menin as a TGF-β activator, the expression arrays in menin-null MEFs revealed a decreased expression of several genes, including the one encoding fibulin 2 (Fbln2), periostin (Postn) and versican, known to be induced by TGF-β (Ji, Prasad et al. 2007). Moreover these cells showed poor response to TGF-β stimulation (Ji, Prasad et al. 2007). Similarly the loss of TGF-β responses was seen in parathyroid and anterior pituitary cells upon knock-down of menin (Kaji, Canaff et al. 2001; Sowa, Kaji et al. 2004). Therefore it indicates the critical role of menin, which seems to be required in TGF-β signaling. Thus disruption of menin function may result in blockage of TGF-β signaling disrupting the cellular steady state and pushing the cell toward inappropriate growth that ultimately may result in tumor formation (Sowa, Kaji et al. 2004).

Part 2

Results

Introduction

to the experimental section

The herein PhD work addresses the role of HBZ viral protein and menin tumor suppressor in the regulation of human telomerase reverse transcriptase (*hTERT*) gene expression in the context of the HTLV-1 infection and the adult T-cell leukemia development.

ATL cells are characterized by high *hTERT* expression and high telomerase activity (Uchida, Otsuka et al. 1999; Kubuki, Suzuki et al. 2005). The reactivation of telomerase observed in the late stages of ATL is in fact considered as one of the critical events during the HTLV-1-mediated leukemogenesis. Previous studies indicated that it occurs in an HBZ-dependent manner and involves JunD, a cellular AP-1 transcription factor, which allows for HBZ recruitment to the *hTERT* promoter (Kuhlmann, Villaudy et al. 2007).

In normal cells *hTERT* gene expression is inhibited by menin, and depleting menin in primary human fibroblasts resulted in the reactivation of telomerase and induction of cellular proliferation (Cong, Wright et al. 2002; Lin and Elledge 2003). Moreover in the cellular context menin occurs in the complex with JunD. This interaction leads to the repression of JunD-dependent transcription and converts JunD into a growth suppressor, whereas it acts as a growth promoter when menin is absent (Agarwal, Guru et al. 1999; Yazgan and Pfarr 2001; Agarwal, Novotny et al. 2003).

The objective of this PhD work was to determine the molecular mechanism of transcriptional regulation of the *hTERT* gene expression upon HTLV-1 infection and to address the possible interplay between menin and HBZ in this process.

The first chapter of the results section addresses the regulation of menin expression upon HTLV-1 infection. In particular I investigated the transcriptional regulation of *MEN-1* gene expression by Tax and HBZ viral proteins.

The second chapter presents the data concerning the HBZ-mediated deregulation of menin function as a transcriptional inhibitor of the *hTERT* gene. The results described in this section of experimental work, gave rise to the publication entitled "*HTLV-1 bZIP factor impedes the menin tumor suppressor and upregulates JunD-mediated transcription of the hTERT gene*" (Borowiak *et al.*, Carcinogenesis, 2013), which is attached in the appendix.

The third part of the experimental section is a comparative study concerning the *hTERT* promoter regulation by HBZ and the antisense proteins of HTLV-3 and HTLV-4. These results constitute a part of the research on the regulation of the transcriptional activity of Jun family members by antisense proteins encoded by viruses HTLV-1, HTLV-3 and HTLV-4, which is a subject of the publication entitled "*Human T-cell Leukemia virus type 3 (HTLV-3) and HTLV-4 antisense transcripts-encoded proteins interact and transactivate Jun family-dependent transcription via their atypical bZIP motif*" (Larocque *et al.*, currently under revision in the Journal of Virology).

I Regulation of *MEN-1* expression in adult T-cell leukemia

Among the HTLV-1 regulatory and accessory proteins, the Tax and HBZ are thought to play a central role in leukemogenesis. Both Tax and HBZ are known modulators of gene expression (Bex and Gaynor 1998). HBZ interacts *via* its C-terminal bZIP domain with host transcription factors from AP-1 and ATF/CREB families (Basbous, Arpin et al. 2003; Thebault, Basbous et al. 2004; Lemasson, Lewis et al. 2007), as well as with cellular coactivators p300/CBP *via* two N-terminal LXXLL-like motifs (Clerc, Polakowski et al. 2008). In addition, HBZ can bind to the p65 subunit of NF-κB and inhibit classical NF-κB pathway. (Zhao, Yasunaga et al. 2009). Similarly to HBZ, Tax also interacts with ATF/CREB family members and CBP/p300 coactivators, and interfere with NF-κB pathway, however opposite to HBZ, Tax activates this pathway, both *via* the classical and the alternative way (Suzuki, Hirai et al. 1995). Thus interfering with above-mentioned transcriptional pathways, both Tax and HBZ are potent transcriptional modulators, and are able to direct the expression of viral genes through viral LTRs, as well as to regulate the transcription of cellular genes *via* endogenous promoters.

As a first step to analyze the menin role in HTLV-1 infection and ATL development, I have checked its expression in the presence of Tax and HBZ.

1.1. Tax-mediated down-regulation of *MEN-1* transcription

In order to investigate the regulation of *MEN-1* gene expression in the presence of Tax, the menin protein and the *MEN-1* mRNA level were assessed in E12 and C50 Jurkat cells stably expressing Tax, described earlier by Escoffier *et al.* (Escoffier, Rezza et al. 2005). Both Jurkat E12 and C50 cells showed lower *MEN-1* mRNA level in comparison to Jurkat control cell line (respectively by 68% and 56%) (Figure 14A). A similar difference in menin expression was observed between Jurkat E12 and Jurkat control cell line also on the protein level, as shown by the Western blot (Figure 14B). Interestingly lower menin protein level in Jurkat E12 cells was accompanied by an increased JunD expression in this cell line.

Above-mentioned results are indicating that Tax could negatively regulate the *MEN-1* gene expression. To confirm this hypothesis Jurkat C50 cells were transfected with

siRNA directed against Tax (Figure 14C). Knock-down of Tax in those cells by 73%, resulted in an increase of *MEN-1* gene transcription by 34%.

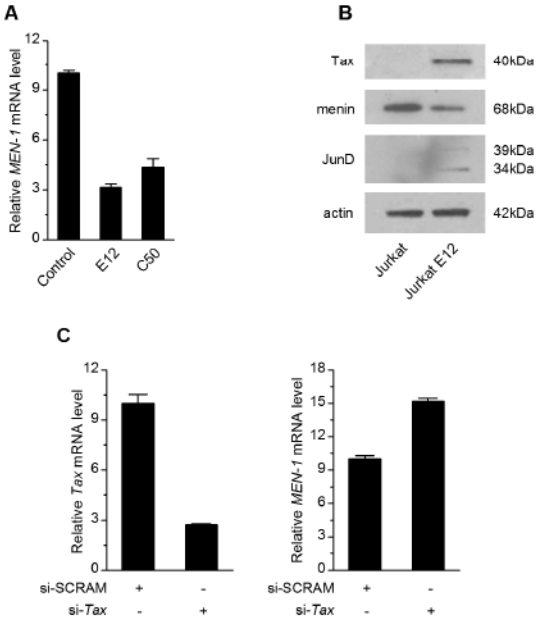

Figure 14. Tax down-regulated menin expression in T-lymphocytes. A) Comparison of *MEN-1* transcription level in control Jurkat cell line with Jurkat E12 and C50 Tax positive clones by real-time quantitative PCR. B) Western blot analysis of cell lysates from Jurkat and Jurkat E12 cells. C) *MEN-1* mRNA level in Jurkat C50 cells with a knock-down of Tax expression. Cells were transfected with siRNA directed against Tax or SCRAM siRNA as a negative control. Forty eight hours following the transfection, RNA was extracted, reverse transcribed and analyzed by real-time quantitative PCR. Results were normalized to actin expression levels. Graph represents the mean and standard deviation from one representative experiment performed in triplicate.

These results indicate that Tax is acting as an inhibitor of *MEN-1* gene expression. Considering the fact that Tax expression is frequently lost in leukemic cells from ATL patients (Tamiya, Matsuoka et al. 1996; Takeda, Maeda et al. 2004), this negative regulation of *MEN-1* gene will very likely refer only to the first stage of ATL development following the HTLV-1 infection. However, it has to be also considered that Tax may not retain its property in the context of viral infection, in the presence of other viral proteins, including HBZ.

1.2. HBZ-dependent up-regulation of *MEN-1* expression in ATL cells

The regulation of *MEN-1* gene expression in the presence of HBZ was first investigated in Jurkat cells transduced with HBZ. Stable expression of HBZ in those cells resulted in an increase of *MEN-1* mRNA level, indicating a possible role of this viral protein as a transcriptional activator of menin expression (Figure 15A).

To confirm this hypothesis in the context of the endogenous HBZ expression, the *MEN-1* transcription level was investigated in TL-Oml cell line. These cells were established from peripheral blood lymphocytes of an ATL patient (Sugamura, Fujii et al. 1984). Typically to the late stage leukemic ATL cells, they are characterized by the methylation of 5'LTR, which blocks the transcription from this viral promoter, including the transcription of Tax (Taniguchi, Nosaka et al. 2005). Thus in these cells the only viral protein expressed is HBZ transcribed from the 3'LTR. The HBZ knock-down in TL-Oml cells resulted in a decrease of *MEN-1* transcription level (Figure 15B). Namely the inhibition of HBZ expression by 45% induced by specific shRNA, resulted in a decrease in *MEN-1* transcription by 30% in those cells.

The lentiviral particles used for the infection of Jurkat cells, were bearing the coding sequence of HBZ-SP1, shown to be the most abundant HBZ spliced variant (Cavanagh, Landry et al. 2006; Murata, Hayashibara et al. 2006). Similarly the knock-down experiments performed in TL-Oml cells, and all following results presented in this manuscript also target the HBZ-SP1 splice variant, which will be referred in this work as HBZ.

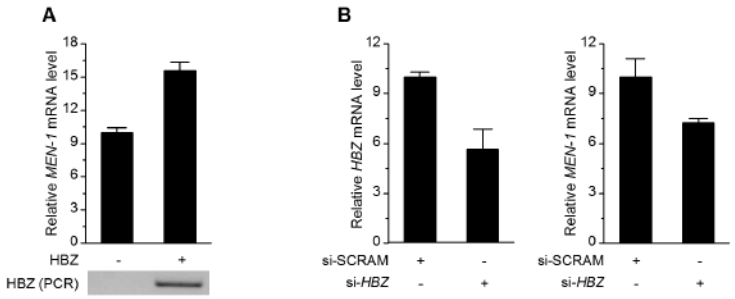

Figure 15. HBZ stimulates *MEN-1* expression in T lymphocytes. A) Ectopic expression of HBZ in Jurkat cells leads to an increase of endogenous *MEN-1* expression level. Jurkat cells were transduced using lentiviral particles bearing the coding sequence for HBZ or the control SCRAM one, containing scrambled nucleotide sequence designed to form a nonspecific shRNA. Following the selection with puromycin for 5 days, RNA was extracted, reverse transcribed and subjected to real-time quantitative PCR. Bottom panel indicates HBZ expression in transduced Jurkat cells (PCR). B) Silencing of *HBZ* expression results in a decrease of *MEN-1* expression level in TL-Oml ATL-derived cell line. TL-Oml cells were transduced with lentiviral particles bearing shRNA targeting *HBZ* spliced transcript or scrambled nonspecific sequence. Following the selection with puromycin, cells were harvested and subjected to RT-qPCR analysis.

In order to determine the HBZ-responsive sequence within the *MEN-1* promoter, luciferase reporter assay was performed in HeLa cells. The transcription from the previously described *MEN-1* truncated promoter constructs encompassing the residues -1416+49, -438+49 and -192+49, was assessed (Fromaget, Vercherat et al. 2003). Interestingly HBZ alone didn't have any stimulatory effect on *MEN-1* promoter (Figure 16). It can result from the fact that HBZ does not possess any known DNA-binding sequences, and it often relies on its partners for the recruitment to the DNA. One of such cellular partners allowing for HBZ targeting to the DNA is JunD transcription factor, already shown to be involved in HBZ-mediated up-regulation of *hTERT* gene expression (Kuhlmann, Villaudy et al. 2007), and stimulation of viral expression from the 3'LTR (Gazon, Lemasson et al. 2012). The luciferase reporter assay indicated that *MEN-1*

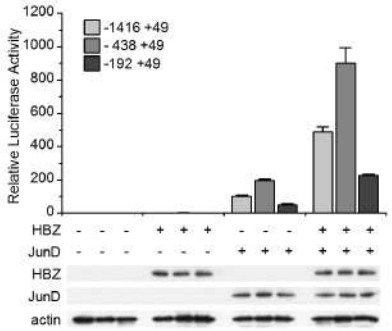

Figure 16. HBZ activates menin expression in a JunD-dependent manner *via* proximal promoter elements. HeLa cells were transiently transfected with reporter constructs bearing the luciferase gene under the control of *MEN-1* promoter regions (-1416+49; -438+49; -192+49, referring to the transcriptional start codon as +1) along with HBZ and JunD-encoding vectors. Luciferase activities were measured in cell lysates forty eight hours post-transfection, results represent the Firefly luciferase activity normalized with Renilla readout. Data (mean and standard deviation) are representative of three independent experiments. Lower panel: western blot analysis of the HBZ, JunD and actin protein levels in cell lysates.

induction mediated by HBZ is also JunD-dependent. Moreover among the constructs tested, the one encompassing the *MEN-1* promoter region between -438 and +49 showed the highest response rate.

Finally the analysis of peripheral blood mononuclear cells isolated from adult T-cell leukemia patients or healthy donors (Figure 17A) indicated a higher expression of *MEN-1* gene in leukemic cells. Importantly *MEN-1* mRNA level strongly correlated with the expression of *HBZ* (Figure 17B), with high statistical significance (linear regression and Spearman two-tailed test, P value < 0,0001). However there was no correlation between *MEN-1* and *JunD* mRNA levels (Figure 17C). It could suggest that the JunD endogenous expression is sufficient to maintain its helper function in HBZ-mediated up-regulation of the *MEN-1* gene. Effectively the ATL cells has been characterized by high expression and

elevated activity of the AP-1 family members, including JunD (Fujii, Iwai et al. 2000; Mori, Fujii et al. 2000). Therefore it seems that HBZ viral protein is the critical and limiting factor in *MEN-1* gene up-regulation in ATL cells.

Collectively, these results indicate that in the contrary to Tax, HBZ activates *MEN-1* gene expression in ATL cells. Moreover this effect seems to depend on JunD and relies on the sequence within the region spanning the residues between -438 and +49. However, further studies are required to determine the precise molecular mechanism implicated in this process, as well as its function and the physiological significance in the ATL cells.

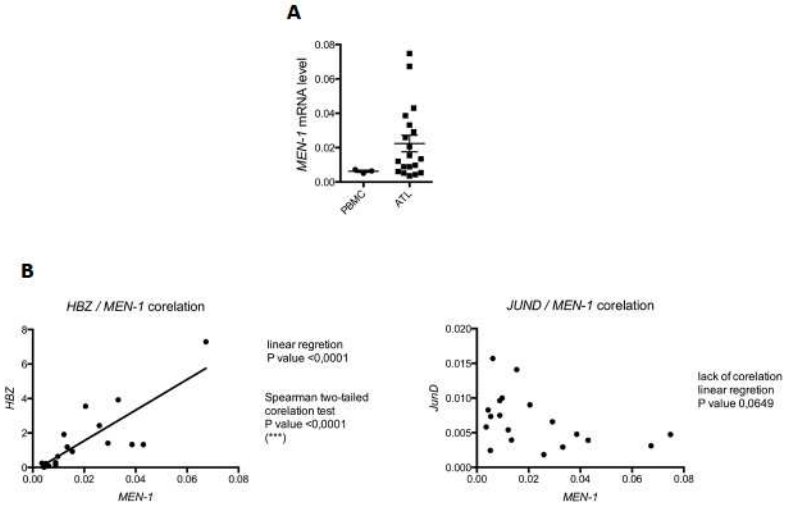

Figure 17. Leukemic cells from ATL patients express high levels of *MEN-1* mRNA which correlates with *HBZ* but not *JunD* transcription level. A) Analysis of *MEN-1* expression by the real-time quantitative PCR. B) Correlation analysis between the expression of *MEN-1* and *HBZ* or *JunD*. Peripheral blood mononuclear cells (PBMC) were purified from blood samples from ATL patients or healthy donors by Ficoll-Paque gradient centrifugation. RNA was extracted, reverse transcribed and subjected to the real-time quantitative PCR. Data represents mean value +/- SEM.

II HBZ-mediated menin loss-of-function as an inhibitor of *hTERT*

One of the key stages in HTLV-1 mediated leukemogenesis, which allows for bypassing the replicative senescence program and thus achieving long-term survival of leukemic cells, is the activation of telomerase. It has been previously shown in the laboratory that the HTLV-1 specific activation of the telomerase occurs *via* transcriptional activiation of its catalytic subunit hTERT, which is induced by HBZ protein (Kuhlmann, Villaudy et al. 2007).

The *hTERT* gene is tightly regulated in normal cells and menin tumor suppressor has been identified as one of the crucial factors inhibiting its expression (Lin and Elledge 2003). The results presented in the first part of the experimental section, indicate that menin expression is up-regulated in leukemic cells from ATL patients that are also characterized by high telomerase activity. In the second part of my work, I intended to determine the reasons of menin loss-of-function as an inhibitor of *hTERT* expression in those cells and the molecular mechanisms leading to the reactivation of *hTERT* in ATL cells.

2.1. Menin inhibitory role in the regulation of *hTERT* proximal promoter

Menin was reported as an inhibitor of *hTERT* gene for the first time ten years ago (Lin and Elledge 2003), however the exact mechanism of its action was not known so far. Importantly menin does not possess any DNA binding motifs, thus it will rely on other transcription factors for its recruitment to the promoters of target genes. In the cellular context menin often occurs in the complex with ubiquitously expressed member of AP-1 family of transcription factors, JunD (Agarwal, Guru et al. 1999; Gobl, Berg et al. 1999). Studies performed previously in the laboratory indicated JunD involvement in the *hTERT* gene regulation through the association with GC boxes located in the proximal part of the *hTERT* promoter (Kuhlmann, Villaudy et al. 2007). Thus it is possible that this factor could facilitate the recruitment of menin to the DNA and allow for exhibiting its inhibitory function.

In order to confirm the role of menin as an *hTERT* transcriptional inhibitor and challenge the hypothesis that its inhibitory role is exerted *via* JunD, I first aimed to

determine the binding of both JunD and menin to the endogenous *hTERT* promoter. Chromatin immunoprecipitation (ChIP) assay was performed in Jurkat cells using the control preimmune rabbit serum or the anti-menin or anti-JunD antibody and a pair of primers amplifying the proximal part of the *hTERT* promoter or the distal one as a specificity control, as indicated on the scheme (Figure 18A). Both JunD and menin were found to bind the proximal part of *hTERT* promoter but not the distal one (Figure 18B).

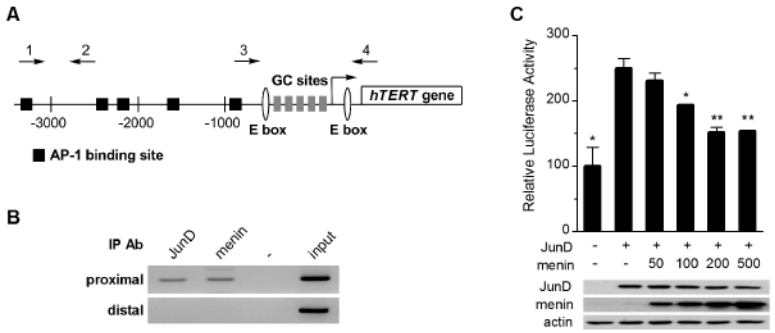

Figure 18. Menin inhibits JunD-mediated activation of *hTERT* promoter. A) Schematic representation of the *hTERT* promoter: binding sites for transcription factors are indicated; arrows labeled 1,2 and 3,4 indicate the locations of primers used to amplify sequences of the distal and proximal promoter, respectively. B) Menin is specifically recruited to proximal part of the *hTERT* promoter. Cell extracts from Jurkat cells were subjected to chromatin immunoprecipitation using control preimmune rabbit serum, anti-menin or anti-JunD antibody. Immunopreciptated DNA was amplified using primers spanning the *hTERT* proximal promoter (upper panel) or the distal one (lower panel). DNA corresponding to 1% of that used for immunoprecipitation, was amplified as input controls. Data presented are representative of three independent experiments. C) Menin inhibits JunD-mediated activation of *hTERT* proximal promoter. HeLa cells were transiently transfected with the p378 *hTERT*-Luc reporter plasmid along with JunD (50 ng) and indicated amounts of menin expression vector. Forty eight hours later, Firefly luciferase activity was analyzed and normalized to Renilla luciferase activity. Lower panel: Western blot analysis of JunD, menin and actin protein levels in cell lysates.

Thus indicating the possibility that menin effectively can be recruited to the proximal *hTERT* promoter *via* interaction with JunD. In order to confirm this hypothesis, a reporter assay using an expression vector encoding luciferase under the control of *hTERT* proximal promoter was performed. HeLa cells were transiently transfected with p378 *hTERT*-Luc construct along with JunD and menin expression vectors. As expected JunD stimulated the expression from *hTERT* proximal promoter and menin inhibited this effect in a dose-dependent manner (Figure 18C). This observation confirms that menin-mediated inhibition of *hTERT* promoter occurs in a JunD- dependent manner.

2.2. HBZ-mediated menin loss-of-function as an inhibitor of *hTERT*

As described in previous chapter, menin-mediated inhibition of *hTERT* expression occurs in a JunD-dependent manner. Importantly the same transcription factor cooperates with HBZ to activate the *hTERT* gene expression in ATL cells. Our results indicate that primary leukemic cells isolated from ATL patients express high level of both HBZ, JunD and menin (see Figure 28). Moreover previous reports indicate that menin inhibits JunD transcriptional activity (Agarwal 1999), while HBZ seems to exert opposite function (Thebault 2004). Thus I speculated that there could be a possible interference between menin and HBZ in the regulation of *hTERT* gene expression.

In order to assess JunD transcriptional activity in the presence of both menin and HBZ, transient transfection assays were performed using GAL4 transcription reporter system in which JunD is artificially tethered to DNA. I first evaluated the effect of menin on the transcriptional activity of JunD, in cells cotransfected with the reporter pG5-Luc vector (containing five GAL4-binding sites upstream of a minimal TATA box), along with a GAL4-JunD (JunD fused at its amino-termini to the yeast transcription factor GAL4 DNA-binding domain) expression vector and increasing amounts of a menin expression vector. Menin inhibited the transcriptional activity of JunD in a dose-dependent manner (Figure 19A), in line with previous reports (Agarwal, Guru et al. 1999). Subsequently, cells were cotransfected with the GAL4-JunD and the constant amount of menin expression vector, together with increasing amounts of the HBZ-encoding plasmid. As shown in Figure 19B,

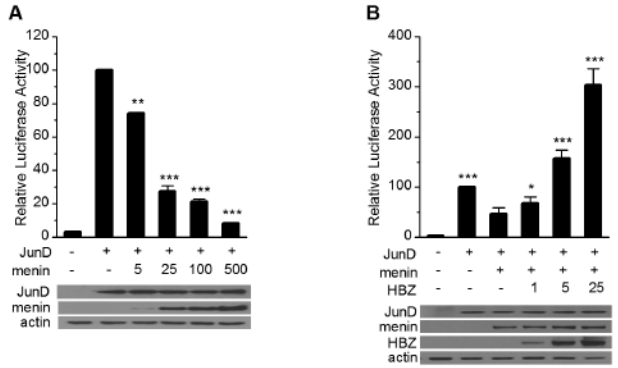

Figure 19. HBZ antagonizes the inhibitory effect of menin on the transcriptional activity of JunD. A) Menin represses JunD transcriptional activity. B) HBZ overcomes menin-mediated inhibition of JunD transcriptional activity. HeLa cells were cotransfected with the reporter construct pG5-Luc (100ng), and the expression vector for Gal4-JunD fusion protein (8ng) along with the indicated amounts of HBZ and menin (25ng in B) expression vectors. Firefly luciferase activity was measured forty eight hours post-transfection and normalized to Renilla luciferase activity. Data (mean and standard deviation) are representative of three independent experiments. Lower panel: western blot analysis of JunD, menin, HBZ and actin protein levels in cell lysates.

HBZ not only abolished the menin-mediated inhibition but also substantially increased JunD transcriptional activity despite the presence of its inhibitor.

Next this identified functional antagonism between HBZ and menin on JunD transcriptional activity was assessed in the context of the *hTERT* gene regulation. With this aim in view HeLa cells were transiently transfected with a reporter construct bearing a luciferase gene under the control of a proximal promoter of *hTERT*. Ectopic expression of menin significantly decreased the JunD-mediated activation of the *hTERT* proximal promoter, while overexpression of HBZ was able to overcome this inhibitory effect and resulted in the activation of *hTERT* promoter (Figure 20A). Moreover the HBZ stimulatory effect was even more pronounced in cells expressing JunD and HBZ only. The HBZ/JunD heterodimers activated the *hTERT* promoter approximately four times stronger than JunD

alone. Similar results were obtained upon transfection of Jurkat cells, confirming that the observed effect is not cell type-specific, and occurs also in T cells (Figure 20B). Moreover these results are coherent with the 3'LTR activation investigated by Gazon *et al*, who showed a three fold activation of viral expression by heterodimers HBZ/JunD (Gazon, Lemasson et al. 2012). It seems therefore that HBZ increase the JunD-transcriptional activity in the same manner, regardless of the cell type and target promoter.

Collectively, this data indicate that menin and HBZ exert opposite effects on the regulation of *hTERT* gene expression. While menin is a negative regulator of *hTERT* expression, HBZ acts as its transcriptional activator. Furthermore, those experiments provide evidence that HBZ acts as a functional antagonist of menin being able to overcome its repressive effect and activate *hTERT* transcription.

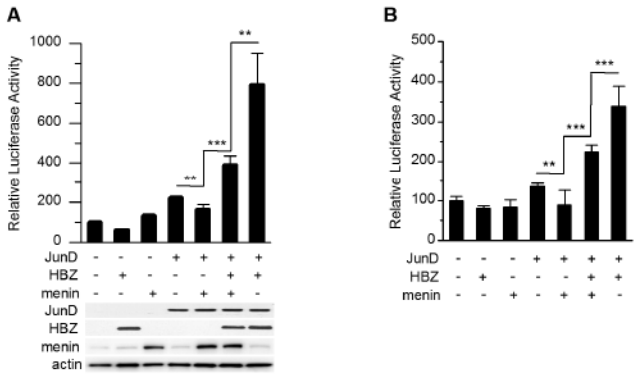

Figure 20. HBZ overcomes the menin-mediated inhibition of the *hTERT* promoter. HeLa (A) or Jurkat (B) cells were transiently transfected with the p378 *hTERT*-Luc reporter plasmid and the vector encoding JunD (25ng) and/or HBZ (200ng) and/or menin (200ng). Firefly luciferase activity was measured forty eight hours post-transfection and normalized to Renilla luciferase activity. Data (mean and standard deviation) are representative of three independent experiments. Lower panel: Western blot analysis of JunD, HBZ, menin and actin protein levels in cell lysates.

2.3. Molecular mechanism of HBZ-mediated menin-loss of function

2.3.1. Identification of menin/JunD/HBZ protein complex

In order to clarify the molecular mechanism by which HBZ is able to counteract the inhibitory effect of menin and thus enhances the activity of *hTERT* promoter, the subcellular localization of JunD, HBZ and menin was examined (Figure 21). HeLa cells were transfected with indicated expression vectors and following immunostaining the protein

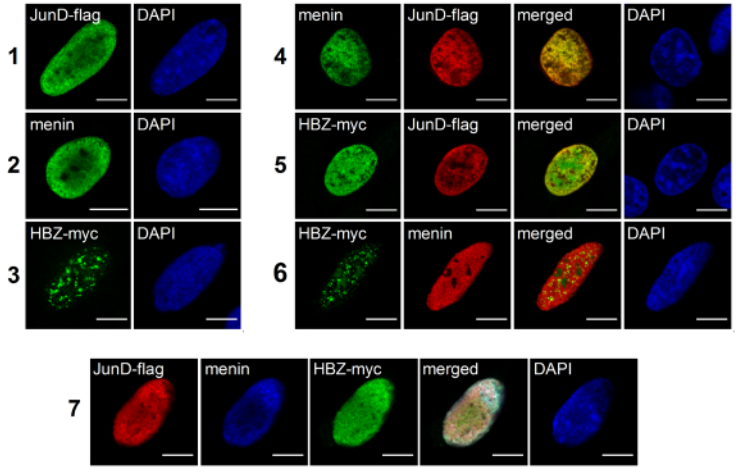

Figure 21. JunD, HBZ and menin colocalize in the nucleus when expressed together. HeLa cells were transiently transfected with JunD-flag (1), or menin (2) or HBZ-myc expression vector alone (3); or by two expression vectors (4-6) or all three plasmids together (7) as indicated. Following fixation with methanol, HBZ was stained with anti-myc antibody directly conjugated with FITC, JunD was visualized with anti-flag and anti-mouse Alexa 488-conjugated secondary antibody, and menin was detected using anti-menin and anti-rabbit Alexa 647-conjugated secondary antibody. DAPI was used for staining of the nucleus. The white bars correspond to a scale of 10 µm.

localization was investigated by confocal microscopy. As expected all of the proteins were localized to the nucleus (Guru, Goldsmith et al. 1998; Hipkaeo, Wakayama et al. 2004; Hivin, Frederic et al. 2005). Moreover when one expression vector was transfected at a time, as previously described (Gaudray, Gachon et al. 2002; Hivin, Frederic et al. 2005), HBZ exhibited nuclear localization with a speckled pattern (Figure 21, panel 3) whereas JunD and menin showed diffuse staining in the whole nucleus (Figure 21, panel 1 and 2, respectively). When expressed pairwise, menin and JunD strongly colocalized (Pearson's correlation coefficient PCC=0.965), while no co-localization was observed between HBZ and menin (PCC=0.571) (Figure 21, panel 4 and 6 respectively). The granular distribution of HBZ in nuclear specles was impaired upon cotransfection of JunD. HBZ relocalized to the whole nucleus (Figure 21, panel 5) and both proteins showed high degree of colocalization (PCC=0.938), confirming previous findings (Hivin, Basbous et al. 2007). This data suggest that the physical interaction with JunD alters the distribution of HBZ, as was similarly shown for c-Jun, another AP-1 transcription factor (Matsumoto et al., 2005). As a result, when all three proteins were coexpressed, all three colocalized with each other (PCC=0.938 for menin/JunD, PCC=0.964 for menin/HBZ and PCC=0.968 for JunD/HBZ) (Figure 21, panel 7).

Menin binding domain has been localized to the N-terminal part of JunD, while the leucine zipper, which allows for heterodimerization with HBZ is located on the C-terminus (Knapp, Heppner et al. 2000; Thebault, Basbous et al. 2004). In the light of the confocal microscopy studies I have next address a question whether HBZ and menin could interact simultaneously with JunD molecule. Although the binding sites for HBZ and menin do not overlap, it is possible that due to a steric hindrance in folded protein or a conformational change occurring upon the interaction of one partner with JunD, the binding of the other could be perturbed. As upon co-transfection of menin, HBZ did not reversed its localization to nuclear speckles, we could speculate that the interaction between HBZ and JunD is relatively stable and the above mentioned reasons could rather affect the recruitment of menin upon HBZ binding to JunD.

To address that possibility the co-immunoprecipitation studies were performed in HeLa cells cotransfected with a JunD-flag expression plasmid, together with either HBZ-myc or menin-encoding vector or with both. Equal amounts of cell lysates were immunoprecipitated with either an anti-flag or an anti-myc antibody (Figure 22). The anti-

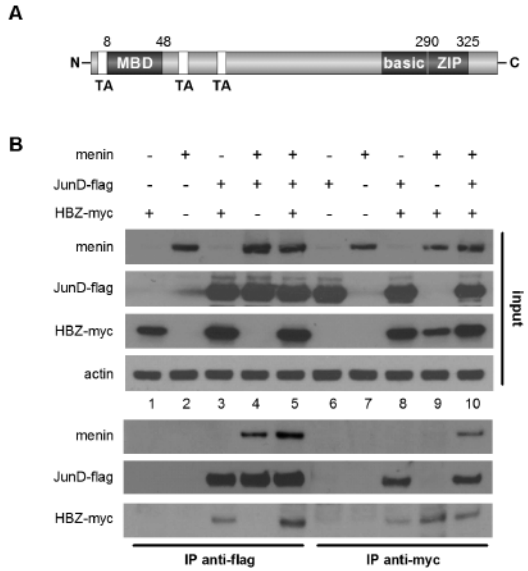

Figure 22. JunD, HBZ and menin form a ternary complex and JunD is a central scaffold protein. A) Schematic representation of identified functional domains of JunD (MBD, Menin Binding Domain; TA, Transcriptional Activity domain; ZIP, leucine zipper domain). B) HeLa cells were transfected with indicated expression vectors and equal amounts of cell lysates were immunoprecipitated with anti-flag or anti-myc antibody followed by immunoblotting with anti-myc, anti-flag or anti-menin antibodies. The results shown are representative of three independent experiments.

flag immunoprecipitation confirmed the JunD interaction with either HBZ or menin or both (lanes 3, 4 and 5). The anti-myc immunoprecipitation clearly indicated a lack of interaction between HBZ and menin in the absence of JunD (lane 9). However, when the three proteins were coexpressed, menin was also immunoprecipitated with anti-myc antibody (line 10). This proves a formation of JunD/HBZ/menin ternary complex where JunD plays a role of central scaffold protein concomitantly recruiting HBZ and menin.

Collectively, the results of co-immunoprecipitation and immunofluorescence studies indicate that the antagonism between menin and HBZ on the transcriptional activity of JunD does not depend on the competition for JunD binding, as all three proteins can co-exist in the same protein complex. Thus the HBZ-mediated impairment of menin function will depend on another mechanism than preventing menin binding to JunD, and possibly have a functional character.

2.3.2. Identification of p300 and HDACs as the effectors of menin- and HBZ-induced antagonism

Menin has been shown to control gene transcription indirectly, as a scaffold protein within chromatin remodeling complexes. The menin-mediated inhibition of JunD transcriptional activity has been linked to its association with the mSin3A-histone deacetylase (HDAC) complex and thus affecting the chromatin structure of the promoter regions of JunD-regulated genes (Kim, Lee et al. 2003). Interestingly HDAC have been also previously linked to the inhibition of *hTERT* expression in normal cells, as treatment with trichostatin A (TSA), the inhibitor of HDAC, induces the *hTERT* transcription (Takakura, Kyo et al. 2001).

Therefore in the next step the HDACs involvement in menin-mediated inhibition of *hTERT* expression was investigated. HeLa cells were transiently transfected with a reporter construct encoding luciferase under the control of the *hTERT* proximal promoter, along with JunD and menin expression vectors (Figure 23). To delineate the involvement of HDACs activity, I relied on trichostatin A (TSA), added to the culture medium. In TSA-untreated cells, menin decreased the JunD/HBZ-induced activation of the *hTERT* promoter by a factor of two. Conversely the addition of TSA relived the inhibitory effect of menin, underlying the involvement of HDACs in menin-mediated inhibition of the *hTERT* proximal promoter. However it is worth to keep in mind that the effect of TSA is unspecific, so it could also activate additional genes that directly or indirectly derepress the *hTERT* promoter, including for example c-Myc (Hou, Wang et al. 2002).

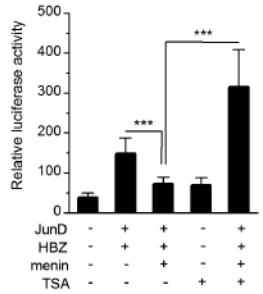

Figure 23. Trichostatin A (TSA) reverses the inhibitory effect of menin on the *hTERT* proximal promoter and reveals the stimulatory effect of HBZ. HeLa cells were transiently transfected with the p378 *hTERT*-Luc reporter plasmid and with the vector encoding JunD (25ng) and/or HBZ (200ng), together with 200ng of the expression vector for menin. Twelve hours after transfection 40 ng/mL of TSA was added to the culture medium. Twenty four hours later, Firefly luciferase activity was analyzed and normalized to Renilla luciferase activity. The values represent mean and standard deviation from triplicate out of one representative experiment of two independent assays.

At the same time I performed experiments aiming at elucidating the precise mechanism of HBZ-mediated activation of *hTERT* expression. HBZ is known to recruit the p300/CBP coactivator through two LXXLL-like motifs located on its N-terminal domain (Clerc, Polakowski et al. 2008). P300/CBP is a Histone Acetyl Transferase (HAT) implicated in transcriptional activation of gene expression, thanks to acetylation of histones H3 and H4, leading to an open chromatin structure thus facilitating the recruitment of transcription factors. In line with this p300/CBP has been linked with HBZ-mediated activation of eg. *DKK1* and *Foxp3* (Polakowski, Gregory et al. ; Zhao, Satou et al. 2011). Moreover its role was also recognized in the regulation of *hTERT* gene expression, (Kirch, Ruschen et al. 2002). And above all it was previously reported that the N-terminal domain of HBZ, containing LXXLL motifs interacting with KIX domain of p300/CBP (Cook, Polakowski et al. ; Clerc, Polakowski et al. 2008) is implicated in HBZ/JunD-mediated activation of *hTERT* promoter (Kuhlmann, Villaudy et al. 2007).

Therefore I sought to verify whether HBZ-mediated menin loss-of-function as an inhibitor

of *hTERT* promoter could depend on the recruitment of p300/CBP. With this aim in view I used previously described HBZ mutant, deficient in p300/CBP binding, in which LL in two LXXLL motifs ware changed for AA (Clerc, Polakowski et al. 2008). The luciferase reporter assay was performed in HeLa cells, using an *hTERT* proximal promoter-driven construct. As expected mutated HBZ showed a significantly reduced impact on the *hTERT* promoter in comparison to the wild type protein (Figure 24A).

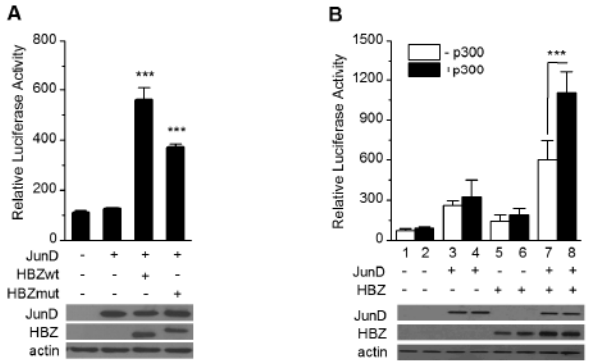

Figure 24. HBZ-mediated stimulation of the *hTERT* proximal promoter depends on the recruitment of p300 transcriptional coactivator. A) Mutated HBZ with perturbed ability to interact with p300, has a limited impact on the *hTERT* proximal promoter. HeLa cells were transiently cotransfected with the p378 *hTERT*-Luc reporter construct and with the plasmid encoding JunD (25ng) and the HBZ expression vector (200ng) encoding wild type or mutated protein (LXXLL motifs). Firefly luciferase activity was analyzed and normalized to Renilla luciferase activity forty eight hours post-transfection. Data (mean and standard deviation) from one representative experiment out of three independent trials are shown. B) Ectopic expression of p300 increases the JunD/HBZ-mediated activation of the *hTERT* proximal promoter. HeLa cells were transiently transfected with the p378 *hTERT*-Luc reporter plasmid and with the vector encoding JunD (25ng) and/or HBZ (200ng) along with p300 (400ng) expression vector (black bars) or without p300 (white bars). Firefly luciferase activity was analyzed and normalized to Renilla luciferase activity forty eight hours post-transfection. The mean and standard deviation are shown from one representative experiment out of three independent trials. Lower panels: western blot analysis of JunD, HBZ and actin protein levels in cell lysates.

In the same type of experiment in HeLa cells, the ectopic expression of p300 increased the stimulation of *hTERT* promoter by HBZ/JunD heterodimers by the factor of 2, as compared to condition without p300 (Figure 24B, compare lane 8 and 7). Due to high homology between p300 and CBP, only the impact of one of them was assessed in the overexpression experiment.

Collectively above-described luciferase reporter assays establish that the activation of *hTERT* proximal promoter by HBZ is dependent on the recruitment of p300/CBP transcriptional coactivator.

2.3.3. Opposite regulation of endogenous *hTERT* expression by menin and HBZ

Luciferase assays using the synthetic *hTERT* promoter, which I described above, are indicating that menin and HBZ exert opposite functions in the regulation of *hTERT* expression and that their effects depend on the respective recruitment of HDACs and p300/CBP. In order to assess the regulation of *hTERT* expression by menin and HBZ in the context of the endogenous promoter in adult T-cell leukemia cells, the *hTERT* mRNA level was quantified in TL-Oml cell line. These cells are characterized by high telomerase activity, and among viral proteins, they express only HBZ (Taniguchi, Nosaka et al. 2005). As Tax was shown to also interfere with *hTERT* gene expression, by the choice of TL-Oml cells lacking the expression of this viral protein we eliminate any possible non-HBZ related influence on the *hTERT* expression.

First a silencing of menin expression was performed in TL-Oml cells using lentiviral particles bearing specific shRNA. Following the transduction, a decrease of *MEN-1* mRNA level by 60% was observed in those cells. The efficiency of *MEN-1* knock-down observed on the protein level was similar, as shown by Western blot (Figure 25A). Menin depletion in TL-Oml cells, induced a 1.4-fold increase of *hTERT* transcription (Figure 25B). Moreover these qPCR results correlated well with telomerase activity measured by RQ-TRAP assay, indicating that the modulation of *hTERT* expression by menin has a direct functional impact on telomerase enzymatic activity (Figure 25C).

Subsequently the length of telomeric restriction fragment was assessed in those TL-Oml *MEN-1* knock-down cells (Figure 25D). Surprisingly the Southern blot using TTAGGG probe indicated no change in median length of telomeric restriction fragment between *MEN-1*

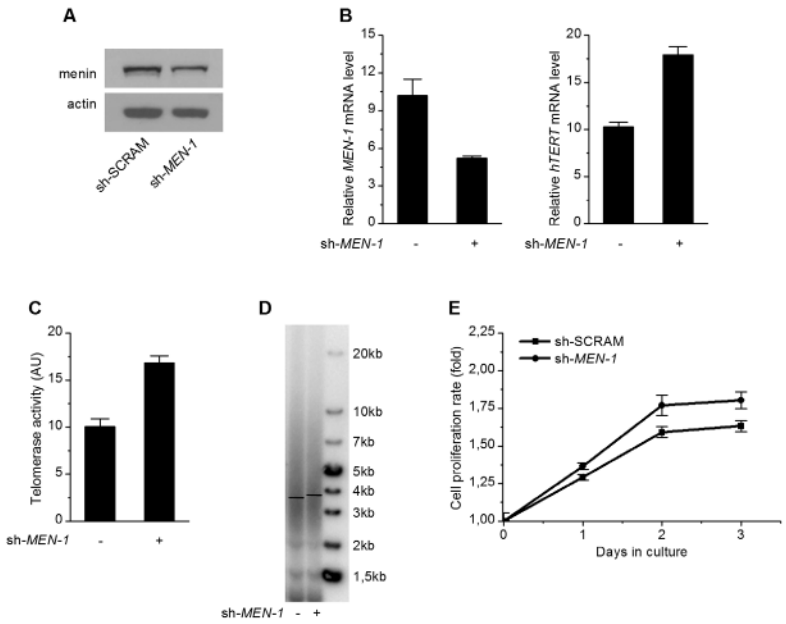

Figure 25. Menin inhibits endogenous *hTERT* expression and the telomerase activity in T cells. TL-Oml cells were infected with lentiviral particles bearing shRNA targeting the expression of *MEN-1* gene or the control SCRAM sequence. Cells were analyzed following the selection with puromycin. A) Analysis of menin protein expression level in shRNA *MEN-1* and shRNA SCRAM TL-Oml cells. Equal numbers of cells were lysed and cell lysates were subjected to Western blot analysis using anti-menin antibody and anti-actin one as a loading control. B) Analysis of *MEN-1* and *hTERT* mRNA level in shRNA *MEN-1* and shRNA SCRAM TL-Oml cells. RNA was extracted from equal number of cells, reverse transcribed and analyzed by real-time quantitative PCR. Results were normalized to the actin expression level. The mean value and standard deviation out of three independent experiments are shown. C) Analysis of telomerase activity in shRNA *MEN-1* and shRNA SCRAM TL-Oml cells. Equal amount of cell extracts were analyzed by RQ-TRAP assay as described in materials and methods. Telomerase activity is expressed in arbitrary unit (AU) per µg of cell extract relative to a standard calibration curve. D) Analysis of telomere length. Genomic DNA was isolated from shRNA *MEN-1* and shRNA SCRAM TL-Oml cells, digested with HinfI and RsaI and analyzed by Southern blotting using TTAGGG probe. The median length of telomeric restriction fragment and the molecular weight marker are indicated. E) Cell proliferation rate of shRNA *MEN-1* and shRNA SCRAM TL-Oml cells assessed by the MTT assay. Results are expressed as fold increase of optical density value relative to measurement at time point 0, and represent the mean values and standard deviations out of four independent experiments.

KD and control cells. On the other hand the *MEN-1* KD cells were also characterized by increased proliferation rate as shown by MTT proliferation assay (Figure 25E), which may explain no change in telomere length, despite clear decrease of *hTERT* expression and telomerase activity in those cells.

Summarizing, the experiments in TL-Oml cells confirmed the results obtained with luciferase reporter assays in HeLa cells, and proved the role of menin as an inhibitor of the endogenous *hTERT* expression.

In the next step in order to assess the role of HBZ in the regulation of endogenous *hTERT* expression in T cells, Jurkat cells were transduced with lentiviral particles bearing the coding sequence of HBZ. Stable expression of HBZ in those cells resulted in an increase of *hTERT* transcription as indicated by quantitative PCR (Figure 26A). Conversely when *HBZ* expression was knocked-down in TL-Oml ATL cell line, a strong reduction of *hTERT* endogenous transcription was observed (Figure 26B). More precisely the *HBZ* knock-down by 35%, resulted in a 2.5-fold decrease of *hTERT* mRNA level. That decrease correlated with an inhibition of cell growth (Figure 26C), confirming previous findings (Satou, 2006; Arnold, 2008).

Summarizing, the results obtained both in Jurkat and TL-Oml cells were comparable and confirmed the role of HBZ as an activator of *hTERT* expression in T cells. Interestingly *HBZ* silencing in TL-Oml cells seemed to have a greater effect on *hTERT* transcription than the silencing of *MEN-1*. As *HBZ* KD by only 35%, resulted in a 2.5-fold decrease of *hTERT* mRNA level, comparing to 60% inhibition of *MEN-1* mRNA level which induced a 1.4-fold increase of *hTERT* transcription. These may suggest that menin inhibitory function in HBZ-expressing cells is marginalized and underlines the critical role of HBZ in the regulation of *hTERT* expression in ATL cells.

In order to provide further information on the mechanism of HBZ-mediated *hTERT* up-regulation, the recruitment of transcription factors of interest to the *hTERT* promoter was assessed. The HBZ positive TL-Oml ATL cell line and Jurkat control leukemic T cells of non-ATL origin were used. The quantitative PCR indicates that the level of *hTERT* mRNA in TL-Oml cells is two times higher than in Jurkat cells (Figure 27B), despite comparable levels of Sp1, JunD and menin protein expression levels in both cell lines (Figure 27A), underlying once again the key function of HBZ in *hTERT* gene activation in ATL cells.

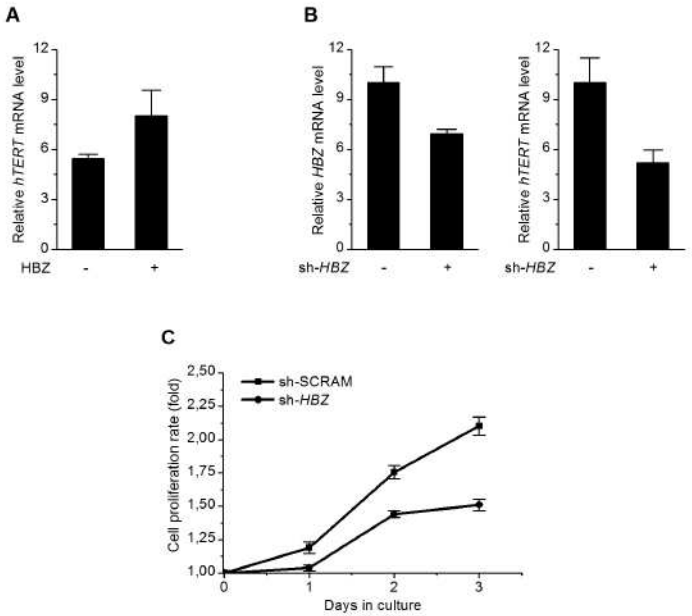

Figure 26. HBZ activates *hTERT* expression in T lymphocytes. A) Ectopic expression of HBZ in Jurkat cells leads to an increase of endogenous *hTERT* expression. Jurkat cells were transduced with lentiviral particles bearing the coding sequence of HBZ or the control empty one. After the selection with puromycin RNA was extracted, reverse transcribed and subjected to the real-time quantitative PCR. Data represents the mean values and standard deviations out of three independent experiments. B) *HBZ* knock-down leads to a decrease in endogenous *hTERT* expression in ATL cell line. TL-Oml cells were infected with lentiviral particles bearing shRNA targeting the expression of *HBZ* gene or the control SCRAM sequence. Following the selection with puromycin, RNA was extracted from equal number of cells, reverse transcribed and analyzed by real-time quantitative PCR to determine the *HBZ* and *hTERT* mRNA levels. Results were normalized to the actin expression level. The mean values and standard deviations out of three independent experiments are shown. C) TL-Oml cell proliferation rate is significantly reduced under *HBZ* knock-down condition. Cell proliferation rate was assessed in shRNA *HBZ* and shRNA SCRAM TL-Oml cells by the MTT assay. Results are expressed as fold increase of optical density value relative to the measurement at time point 0, and represent the mean values and standard deviations of four independent experiments.

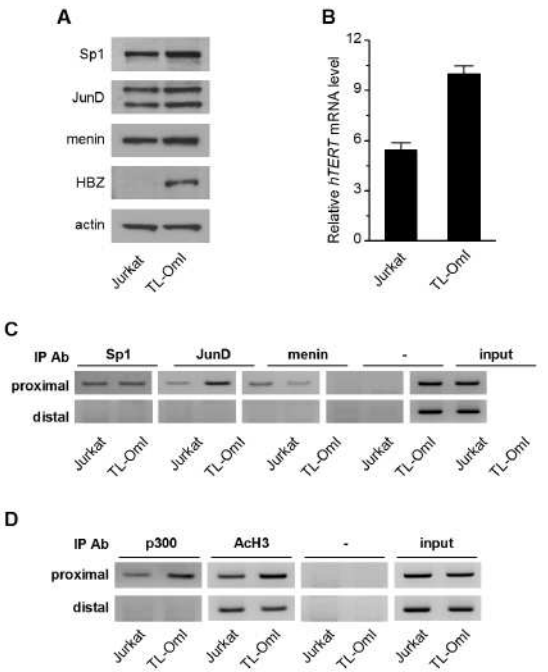

Figure 27. Characterization of the complex of transcription factors implicated in the *hTERT* promoter regulation in T lymphocytes. A) Comparison of the protein levels and B) *hTERT* mRNA expression in control Jurkat T-cells and in TL-Oml ATL cell line. C) Recruitment of the transcription factors implicated in the regulation of *hTERT* proximal promoter in HBZ-positive and HBZ negative cells. Cell extracts from Jurkat and TL-Oml cells were subjected to chromatin immunoprecipitation using control preimmune rabbit serum or anti-Sp1, anti-JunD or anti-menin antibody. Immunoprecipitated DNA was amplified using primers spanning the *hTERT* proximal promoter (upper panel) or the distal one (lower panel). DNA recovered from samples before IP, which corresponds to 1% of that used for immunoprecipitation, was also PCR amplified as input controls. Data shown are representative of three independent experiments. D) Analysis of the p300 recruitment and the resulting histone acetylation within the *hTERT* proximal promoter. Chromatin immunoprecipitation was performed on Jurkat and TL-Oml cell extracts using anti-p300 and anti-AcH3 antibodies or control preimmune rabbit serum, and analyzed as described in C.

Chromatin immunoprecipitation (ChIP) assay was performed with a pair of primers amplifying the proximal part of *hTERT* promoter or the distal one as a specificity control. Whereas there was no difference in Sp1 recruitment to the *hTERT* proximal promoter between both cell lines, a substantial increase of JunD binding and reduced recruitment of menin in TL-Oml cells was obserwed (Figure 27C). This could suggest that in the presence of HBZ, the JunD binding to *hTERT* promoter is stabilized, while the menin access is partially restrained.

Taking into account that menin and HBZ-mediated opposite functions depend on their respective recruitment of HDACs and p300, known histone acetylation modifiers, I have subsequently evaluated the p300 recruitment and the histone acetylation status of the endogenous *hTERT* promoter in HBZ-positive and negative cells. ChIP assay was performed, using an anti-p300 or anti-acetylated histone H3 (AcH3) antibody or the control preimmune rabbit serum (Figure 27D). An increase of p300 recruitment to the proximal *hTERT* promoter was observed in TL-Oml ATL cells in comparison to the control Jurkat cell line, and this effect was not observed for the distal promoter, indicating the specificity of the experiment. Quantification of the bands intensities using ImageJ software following their normalization to the input, indicated that p300 is recruited more preferentially to the *hTERT* promoter in TL-Oml than in Jurkat cells by the factor of 1.96. Consistent with this data an increase by the factor of 1.5 in the histone acetylation within the proximal *hTERT* promoter in TL-Oml cells was observed. While the amounts of AcH3 at the distal region of the *hTERT* promoter were found to be constant.

These results confirmed the previously obtained data from the luciferase reporter assays, and agree with the hypothesis that menin- and HBZ-mediated opposite regulation of *hTERT* gene expression depend on their contrary effect on histone acetylation of *hTERT* proximal promoter, *via* the respective recruitment of HDACs and p300. Additionally they suggest that menin loss of function mediated by HBZ results from reversing its effect on histone deacetylation within the *hTERT* proximal promoter, thanks to p300 causing histone acetylation of this region, as a result allowing for increased *hTERT* expression.

2.4. Menin loss-of-function as an *hTERT* inhibitor in ATL cells

The transcription profiles of circulating leukemic cells from 19 patients, clinically displaying an acute form, were analyzed. The quantitative RT-PCR showed a high *hTERT* and *JunD* mRNA levels in those cells in comparison to primary T lymphocytes isolated from healthy individuals (Figure 28A), confirming previous findings (Uchida, Otsuka et al. 1999; Fujii, Iwai et al. 2000; Mori, Fujii et al. 2000). Moreover also *MEN-1* expression level

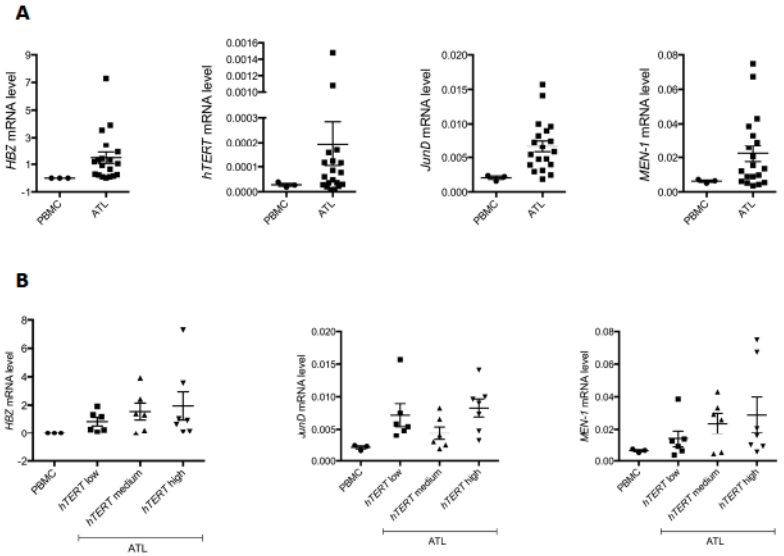

Figure 28. Endogenous *hTERT*, *HBZ*, *JunD* and *MEN-1* mRNA levels in leukemic cells from ATL patients. Primary peripheral blood mononuclear cells (PBMC) were purified from blood samples from 19 ATL patients and 3 healthy individuals by Ficoll-Paque gradient centrifugation. RNA was extracted, reverse transcribed and subjected to the real-time quantitative PCR. Data were normalized to actin expression level and represent mean values +/- SEM. A) Mean *hTERT*, *HBZ*, *JunD* and *MEN-1* expression levels in samples from ATL patients versus healthy donors. B) Mean *HBZ*, *JunD* and *MEN-1* mRNA levels in primary PBMCs from healthy individuals (n=3) and ATL patients divided for groups of low (n=6), medium (n=6) and high (n=7) *hTERT* expression level.

was found to be elevated in those ATL cells. This directly indicates menin loss-of-function in leukemic cells, as despite its high expression level it didn't inhibit the *hTERT* transcription.

Moreover analysis of the transcription profiles in groups of patients divided arbitrary depending on *hTERT* expression level for low, medium and high, showed no correlation of *hTERT* expression level with *JunD* transcription (Figure 28B). This is possibly due to the fact that JunD is expressed endogenously on the saturating level, sufficient to maintain its role as a scaffold protein on the *hTERT* promoter. In the contrary a positive correlation was observed between the *hTERT* and *HBZ* expression levels, indicating therefore that the HBZ is a critical and limiting factor responsible for *hTERT* gene activation in ATL cells.

Interestingly enough qRT-PCR analysis of above-mentioned leukemic samples, revealed as well a positive correlation between *hTERT* and *MEN-1* expression levels. However this result may come from the fact that *MEN-1* expression is strongly regulated by HBZ, as shown in the first part of the experimental section. Thus high *MEN-1* transcription level in the group of high *hTERT* expression could come from the fact that this group is also characterized by high HBZ abundance.

Summarizing, it seems therefore that HBZ being a potent transcription factor, activates unspecifically the expression of *MEN-1* gene, but at the same time inhibits its tumor suppressor function, rendering it inactive. Thus despite its abundance menin does not exert its function as an inhibitor of *hTERT*.

III *hTERT* promoter regulation by HBZ, APH3 and APH4 – comparative studies

The HTLV retrovirus family is composed of the well-known HTLV-1 and HTLV-2 viruses and the most recently discovered HTLV-3 and HTLV-4 (Calattini, Chevalier et al. 2005; Wolfe, Heneine et al. 2005; Calattini, Chevalier et al. 2006). All the members of the HTLV strain produce the antisense proteins, sharing certain molecular characteristics. APH-3 and APH-4 (antisense proteins of HTLV-3 and HTLV-4 respectively), similarly to HBZ interact with AP-1 factors, despite the fact that they are devoid of a typical bZIP domain. The interaction occurs *via* a basic amino acid-rich region showing irregularly spaced leucines or isoleucines, predicted to form a leucine zipper-like coiled-coil domain (Larocque *et al*, in preparation).

Taking into account that APH-3 and APH-4 are able to interact with AP-1 family members, I intended to check their impact on the *hTERT* gene expression. The comparative study was performed between APH-3/APH-4 and HBZ, which is known to activate *hTERT* proximal promoter in cooperation with JunD (Kuhlmann, Villaudy et al. 2007).

APH-3 and APH-4, similarly to HBZ, were able to increase the JunD-mediated activation of *hTERT* proximal promoter as shown by the luciferase assay (Figure 29A). Moreover the effect of APH-3 and APH-4 was stronger than the one exerted by HBZ, and the APH-4 showed the highest degree of transactivation. Additionally as shown in Figure 29B and 29C, opposite to HBZ, APH-3 and APH-4 stimulated the *hTERT* proximal promoter also in the presence of JunB and c-Jun. However to obtain the activation of *hTERT* promoter comparable with the one observed with JunD, higher expression levels of JunB and c-Jun were necessary. Thus 8 times more of the expression plasmids encoding JunB and c-Jun were used (200ng of JunB and c-Jun expression plasmids in comparison to 25ng of JunD vector used for the transfection of HeLa cells grown in 1 well of 24-well plate).

Summarizing, in line with previous findings HBZ increased the transcriptional activity of JunD and inhibited the one of c-Jun and JunB (Basbous, Arpin et al. 2003; Thebault, Basbous et al. 2004; Kuhlmann, Villaudy et al. 2007). Conversely both APH-3 and APH-4 activated the transcription from the proximal *hTERT* promoter in the presence of all Jun family members tested. Moreover a moderate activation of the *hTERT* promoter was also observed when APH-3 and APH-4 were expressed alone, and presumably this

effect depended on the transcriptional potential of the endogenous Jun factors.

Similarly to the effect of APH-3 and APH-4 on the *hTERT* proximal promoter observed here, those factors activated also Jun-dependent transcription through the AP-1 binding sites present in the collagenase promoter (Larocque *et al*, in preparation). Therefore it seems that APH-3 and APH-4 are increasing the transcriptional activity of Jun family factors independently from the mode of the recruitment to the promoter, so both if the binding occurs directly to the DNA (*via* AP-1 binding sites) or indirectly *via* Sp1 factors recruited to the GC boxes. Moreover as APH3 and APH4 contain LXXLL and LXXLL-like motifs, we may speculate that the stimulatory effect may be dependent on the p300/CBP recruitment (Larocque, Halin et al. 2011).

Further studies are required to prove whether identified increased activity of *hTERT* proximal promoter mediated by APH-3 and APH-4, indeed result in an increase of endogenous hTERT protein levels and lead finally to higher telomerase activity in HTLV-3 and HTLV-4 infected individuals. However presented study raised a concern that APH-3 and APH-4 has a potential to activate the *hTERT* expression even stronger than HBZ.

For the moment an infection with HTLV-3 or HTLV-4 has not been yet associated with any disease, however a relatively low number of infected individuals have been reported so far (Thomas, Perzova et al. ; Duong, Jia et al. 2008). Therefore the infection cases with HTLV-3 and HTLV-4 should not be neglected and the pathogenicity of those viruses should be considered.

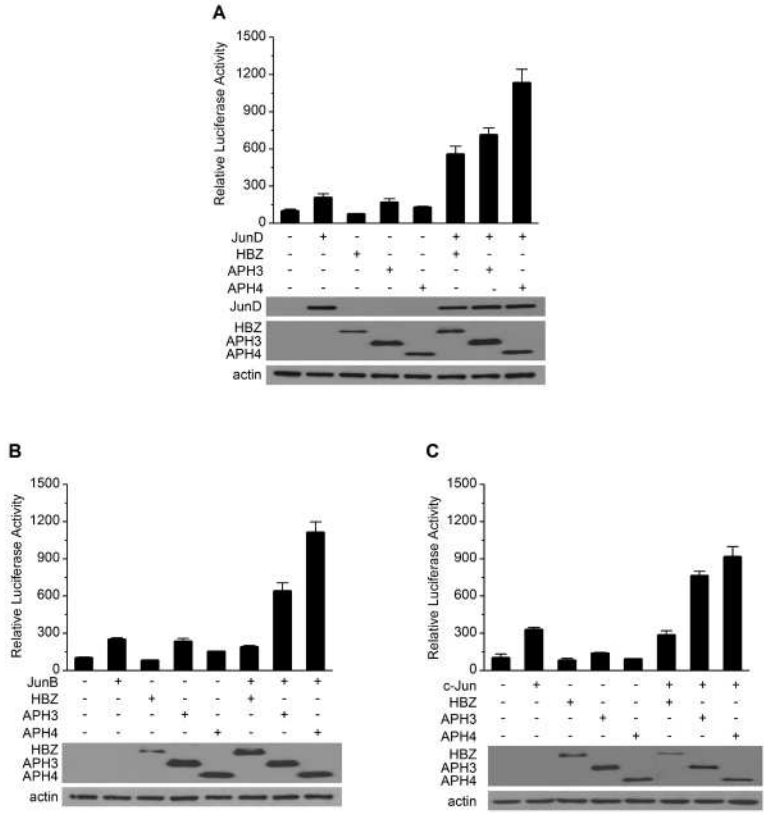

Figure 29. Transcriptional activation of the *hTERT* promoter by heterodimers of Jun factors with antisense proteins of HTLV family. HeLa cells were transiently transfected with the p378 *hTERT*-Luc reporter plasmid together with HBZ or APH3 or APH4 expression vector (200ng) along with the plasmid encoding JunD – 25ng (A) or JunB – 200ng (B) or c-Jun – 200ng (C). Firefly luciferase activity was analyzed and normalized to Renilla luciferase activity forty eight hours post-transfection. The mean values and standard deviations from one representative experiment out of three independent trials are shown. Lower panels: western blot analysis of JunD, HBZ, APH3, APH4 and actin protein levels in transfected cell lysates.

Part 3

Discussion and perspectives

Discussion

I Differential regulation of menin expression by Tax and HBZ

Results presented in the first part of the experimental section in herein manuscript indicate that *MEN-1* expression is differentially regulated by Tax and HBZ. While Tax inhibits *MEN-1* transcription in T-cells (Figure 14), HBZ exerts opposite effect (Figure 15).

Importantly Tax and HBZ exhibit different expression kinetics throughout the leukemogenic process induced by HTLV-1. While HBZ is constantly expressed, the Tax expression is frequently lost in ATL cells, due to genetic and epigenetic modifications within the 5'LTR or the Tax coding sequence (Tamiya, Matsuoka et al. 1996; Takeda, Maeda et al. 2004). Consistent with this, the analysis of the leukemic cells from ATL patients, indicated high *MEN-1* transcription level in those cells, which correlated well with the transcription of the *HBZ* gene (Figure 17). My experiments therefore clearly indicate that in the late stages of ATL development, when HBZ is the only expressed viral protein, the *MEN-1* transcription is kept on the high level. However it is still open how the transcription of *MEN-1* gene will be regulated in the initial stages following the HTLV-1 infection, when both Tax and HBZ are present. Further studies are also required to determine the molecular mechanism standing behind the differential regulation of *MEN-1* promoter by Tax and HBZ.

Preliminary results involving luciferase reporter assays on 293 cells, obtained previously in our laboratory, indicate that the Tax responsive sequence is located within the region of *MEN-1* promoter encompassing residues between -438 and +49. The same

sequence of the proximal promoter was also responsible for HBZ-mediated activation of *MEN-1* promoter, as shown by the luciferase assay (Figure 16). This part of *MEN-1* promoter contains few putative transcription factor binding sites including: three GC boxes, an E box and a NF-κB site, as well as a large CpG island (described in details in the 1.1. section of the bibliographical introduction).

Importantly the HBZ stimulatory effect is dependent on JunD (Figure 16). As HBZ cannot efficiently bind DNA, it is possible that JunD, heterodimerizing with HBZ *via* its leucine zipper domain, may facilitate HBZ recruitment to the *MEN-1* promoter. The responsive region is devoid from canonical AP-1 sites, however, JunD may be also recruited to the promoter of target genes indirectly, binding to NF-κB or Sp1 transcription factors (Mensah-Osman, Veniaminova et al. ; Stein, Baldwin et al. 1993; Toualbi-Abed, Daniel et al. 2008). Further analysis of the promoter activity, involving mutagenesis of the transcription binding sites, is necessary co confirm the proposed regulatory mechanism.

Previous studies linked the HBZ role in the transcriptional activation, with its N-terminal activatory domain recruiting the p300 transcriptional co-activator. It would be interesting therefore to verify whether *MEN-1* gene regulation also involves the same mechanism.

Interestingly Tax was also shown to interact with p300, and through its sequestration Tax can participate in transcriptional silencing of certain cellular genes involving for example the ones regulated by Myo-D (Riou, Bex et al. 2000). It would be therefore possible that the differential role of Tax and HBZ in the *MEN-1* gene regulation, depends on their opposite function in the p300 shuttling from/to the *MEN-1* promoter.

Tax was also reported to play a role in the epigenetic regulation of expression, silencing for example the *SHP-1* tumor suppressor gene. In the presence of Tax, dissociation of transcription factors from core promoter of *SHP-1* gene was observed, followed by HDAC1 binding and final DNA methylation (Nakase, Cheng et al. 2009).

Taking into account that the Tax responsive sequence within the proximal *MEN-1* promoter contains a CpG island (residues -194/+73), a possible involvement of Tax in the DNA methylation within this region should be considered. Especially that this region seems to be susceptible for that kind of regulation and its methylation was previously

shown to be responsible for *MEN-1* gene silencing in the context of Pancreatic Ductal Adenocarcinoma (Cavallari, Silic-Benussi et al. 2009).

Taking together my results presented in the first chapter, show that *MEN-1* gene expression is inhibited by Tax, while HBZ acts as a strong activator of its expression. The responsive sequence has been mapped to the proximal part of the promoter, however further studies are necessary to determine the precise molecular mechanism of the *MEN-1* gene regulation by both Tax and HBZ.

II HBZ-mediated inhibition of menin function and the activation of telomerase

A) Menin inhibits *hTERT* expression in normal cells

Our studies on menin function focused on its role in the inhibition of the human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase. For the first time menin was identified as a repressor of *hTERT* expression by Lin *et al*, who performed a genetic screening employing the enhanced retroviral mutagenesis (ERM). Menin role has been then confirmed in primary human fibroblasts, where knock-down of menin resulted in the stimulation of *hTERT* expression, reactivation of telomerase and an induction of cellular proliferation (Lin and Elledge 2003). Until now however the exact molecular mechanism of *hTERT* gene regulation by menin, was not known.

Menin does not contain any DNA-binding motifs, so it requires another transcription factor for its recruitment to the promoters of target genes. In the cellular context it occurs in the complex with JunD, and represses JunD-regulated genes thanks to the recruitment of mSin3A-HDAC histone deacetylase complex (Agarwal, Guru et al. 1999; Gobl, Berg et al. 1999; Kim, Lee et al. 2003). Previous work performed in our laboratory indicated JunD involvement in the regulation of *hTERT* expression *via* GC boxes located in the proximal part of the promoter (Kuhlmann, Villaudy et al. 2007).

On the basis of my experiments, I propose that in normal cells menin inhibits *hTERT* expression *via* interaction with JunD binding to Sp1 sites, and that its mode of action is dependent on the histone deacetylation mediated by HDACs. Interestingly and analogical mechanism of transcriptional regulation has been also proposed in the context

of gastrin gene, where JunD is recruited to the gastrin promoter also through Sp1, and the menin-mediated inhibition can be restored upon treatment with trichostatin A (TSA), the inhibitor of HDACs (Mensah-Osman, Veniaminova et al.). Many previous studies shown that Jun family members can be actively recruited not only to the canonical AP-1 sites, but also to GC boxes (Kardassis, Papakosta et al. 1999; Chen and Chang 2000; Wang, Tsao et al. 2000; Wu, Zhang et al. 2003). However studies on menin-mediated inhibition of JunD transcriptional activity reported this effect so far only in the context of direct DNA binding through the AP-1 sites (Agarwal, Guru et al. 1999; Gobl, Berg et al. 1999). My results together with the one of Mensah-Osman *et al*, show therefore for the first time that menin inhibits JunD target genes regardless of the mode of its recruitment to the promoter, and can exerts its inhibitory role also within JunD/Sp1 complexes recruited to the GC boxes.

Data presented in this manuscript, showed that menin inhibits JunD-mediated activation of the *hTERT* promoter (Figure 18). In agreement with my results, Takakura *et al*, showed previously that overexpression of JunD together with c-Jun or c-Fos leads to the inhibition of the *hTERT* promoter activity (Takakura, Kyo et al. 2005). This stay in line with the hypothesis raised in this manuscript that in normal cells JunD recruits menin to the *hTERT* promoter, and in this protein complex acts as transcriptional inhibitor of *hTERT*.

Moreover, my experiments indicate that menin-mediated repression of *hTERT* promoter depends on HDACs, as its effect was rescued by TSA, the specific histone deacetylase inhibitor (Figure 23). These results are consistent with previous reports, which showed the activation of *hTERT* gene transcription upon treatment with trichostatin A (Takakura, Kyo et al. 2001). In this report the proximal 181bp promoter, which contains two E boxes and five GC boxes, was determined to be the responsive TSA sequence. Additionally the overexpression of Sp1 enhanced responsiveness to TSA and mutation of Sp1, but not c-Myc sites, of the core *hTERT* promoter abrogated this activation (Takakura, Kyo et al. 2001). These results indicate thus that TSA HDACs inhibitor activates the *hTERT* promoter in normal cells in an Sp1-dependent manner, standing therefore in line with the hypothesis presented in herein manuscript.

One of the previous studies suggested that menin can bind *hTERT* promoter in a

sequence independent manner (La, Silva et al. 2004; Hashimoto, Kyo et al. 2008). Potentially this direct dsDNA binding could occur *via* nuclear localization signals (NLSs) containing multiple positively charged lysine and arginine residues, therefore potentially susceptible to establish the interaction with negatively charged DNA (La, Desmond et al. 2006). In the contrary to this report, my chromatin immunoprecipitation results indicate that menin is recruited to the *hTERT* promoter in a sequence specific manner. As menin was only found to bind to the proximal promoter, containing the GC boxes, and not to the distal part of the promoter deprived from AP-1 and Sp-1 binding sites (Figure 18B and 27C).

In conclusion, this part of my work brings new insights into the understanding of the regulation of *hTERT* gene expression in non-cancerous cells, revealing for the first time the mechanism of menin-mediated inhibition. My results indicate that menin inhibits *hTERT* thanks to the histone deacetylase activity of HDACs, and its effect is dependent on JunD, allowing for the recruitment of menin/HDACs inhibitory complex to the GC boxes within the proximal *hTERT* promoter.

B) HBZ induces menin loss-of-function and activates *hTERT* in ATL cells

In contrast to normal cells, the leukemic T-lymphocytes isolated from ATL patients exhibit high *hTERT* expression levels and consequent high telomerase activity. It has been previously demonstrated in our laboratory that HTLV-1-induced activation of the *hTERT* expression depends on HBZ. This process is JunD-dependent, as JunD interacting with Sp1 factors bound to GC boxes within the proximal promoter of *hTERT*, allows for HBZ recruitment to the target sequence (Kuhlmann, Villaudy et al. 2007).

My experiments indicate that the ATL cells, which are characterized by high *hTERT* expression, exhibit also high levels of *MEN-1* transcription (Figure 28). Taking into account the menin role as an inhibitor of *hTERT*, it is surprising that in this case, despite its abundance, it does not down-regulate *hTERT* transcription. It might therefore suggest the functional impairment of menin in ATL cells. Such an effect could result from preventing menin recruitment to the promoter or blocking/reversing its activity in HTLV-1 infected cells.

The experiments presented in herein manuscript show that menin-mediated inhibition of *hTERT* promoter is counteracted by HBZ. Moreover this effect of HBZ seems not to depend on the possible uncoupling menin from the binding to the common partner JunD. As the results of co-immunoprecipitation supported by the immunofluorescence colocalization studies, indicated the possibility of simultaneous interaction of JunD with both HBZ and menin (Figure 21 and 22). Nevertheless it is worth to point out, that in the context of their recruitment to the chromatin, a small reduction was observed in the menin binding to the endogenous *hTERT* promoter in the TL-Oml cells, as shown by ChIP assay (Figure 27C). This might suggest that upon formation of the ternary protein complex on the chromatin, which also involves Sp1 factors, HBZ could partially affect the binding of menin to JunD and thus menin recruitment to the promoter. However this change seems not to be significant enough to account for complete menin loss-of-function leading to the re-expression of *hTERT* gene in ATL cells.

The main mechanism responsible for HBZ-mediated menin loss-of-function concerns its opposite role on the status of histone acetylation within the *hTERT* proximal promoter. The luciferase reporter assays indicated that while menin inhibitory effect was dependent on the HDACs activity relieved by TSA treatment (Figure 23), the HBZ-mediated activation of the *hTERT* promoter was dependent on the recruitment of p300 histone acetyltransferase (Figure 24). The HBZ mutant exhibiting lack of the ability to bind p300, showed significantly lower activity on the *hTERT* promoter in comparison to the wild type protein. At the same time the overexpression of p300 increased markedly the HBZ-induced stimulation of the promoter. In agreement with these luciferase reporter assays, the chromatin immunoprecipitation confirmed the increased recruitment of p300 and resulting increase of histone acetylation status of the *hTERT* endogenous promoter in HBZ expressing TL-Oml ATL cells (Figure 27D).

Therefore it can be concluded that the HBZ-mediated functional impairment of the menin inhibitory role on *hTERT* expression results from reversing the menin-induced post-translational modification of histones. Similarly it was previously shown in the context of Nur77 promoter, that p300 overexpression relieves the menin-mediated repression of JunD activity, presumably by reversing HDACs-mediated histone deacetylation (Kim, Lee et al. 2005).

Interestingly Jun family members are able to interact directly with CBP/p300 histone acetyltransferase, what results in an increase of the Jun transcriptional activity (Bannister and Kouzarides 1995; Bannister, Oehler et al. 1995; Kim, Lee et al. 2005). Among this family of transcription factors, also JunD can interact directly with p300 *via* its N-terminal domain, as shown by Kim *et al.* (Kim, Lee et al. 2005). We can thus hypothesize that HBZ binding p300 with high affinity by its LXXLL domains, and at the same time having a possibility to interact with JunD by its leucine zipper, could facilitate the recruitment of p300 to JunD. Therefore potentially two p300 molecules could be recruited to the *hTERT* promoter, one bound to JunD and the other to HBZ. However considering the size of p300, an effect of steric hindrance might exclude that possibility.

Of note, HBZ was shown in another study to inhibit histone acetyltransferase activity of CBP and p300 coactivators (Wurm, Wright et al.). *In vitro* HAT assays indicated that the inhibitory effect of HBZ depend on its basic-leucine zipper (bZIP) region, interacting with HAT domain of CBP/p300 and perturbing its function. Importantly this inhibitory function of HBZ on CBP/p300 HAT activity was blocked by the bZIP domain of c-Jun. Thus it is likely that repression of CBP/p300 HAT activity and interaction with bZIP factors are mutually exclusive functions of HBZ. Therefore upon interaction with JunD, we may expect that p300 will be fully functional and therefore participate in the up-regulation of JunD-mediated transcription.

It is also worth to point out that HBZ-mediated histone acetylation within the proximal *hTERT* promoter, may not only reverse the function of menin, but also counteract the effect of other repressors recruiting HDACs to this part of the promoter. One of such candidates identified so far, is the TAL1 (T-cell acute lymphoblastic leukemia 1), which recruits HDAC1 histone deacetylase complex to the *hTERT* promoter presumably also *via* Sp1 binding sites (Terme, Mocquet et al. 2009). In this study JunD/HBZ heterodimers were shown to block the repressive effect of TAL1 (Terme, Mocquet et al. 2009). It is therefore possible that this effect occurs thanks to the active recruitment of p300 to this region and reversing TAL1 effect on the histone deacetylation. However in this case it is not the only mechanism involved, as HBZ was also shown to decrease the intracellular levels of TAL1 inducing its proteasomal degradation.

In conclusion, I explain here the molecular mechanism standing behind the HBZ-mediated activation of *hTERT* gene expression. My results indicate that HBZ recruiting

p300 histone acetyltransferase reverses the effect of menin-recruited HDACs (Figure 30). Therefore despite high menin expression level in ATL cells, menin does not exhibit its function as an *hTERT* inhibitor. In agreement with the results presented in herein manuscript, all HTLV-1 transformed cell lines and the leukemic cells from ATL patients were shown to exhibit increased *hTERT* expression level (Uchida, Otsuka et al. 1999; Kubuki, Suzuki et al. 2005). Consistent with the fact that the hTERT subunit is the limiting factor in the assembly of the active telomerase holoenzyme, the HBZ-induced re-expression of *hTERT*, results in the activation of telomerase activity. This event is a key step in the establishment of the cancerous phenotype of HTLV-1 infected cells and a necessary element in the progression of Adult T-cell leukemia (Uchida, Otsuka et al. 1999; Kubuki, Suzuki et al. 2005).

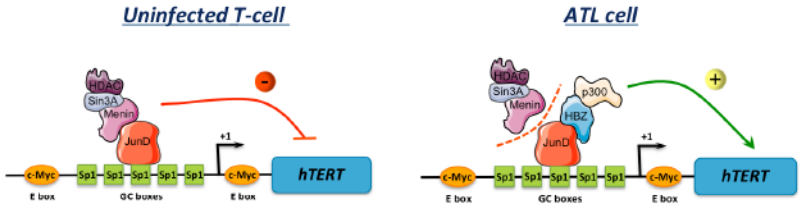

Figure 30. Schematic representation of deciphered molecular mechanism of *hTERT* gene regulation. In uninfected cells the *hTERT* expression is inhibited by menin recruiting histone deacetylase complex (HDACs). In ATL cells, HBZ is able to overcome this effect by recruiting p300 histone acetyltransferase.

C) JunD- both repressor and activator of *hTERT* depending on the recruited partner

The results presented in this manuscript indicate that menin inhibits *hTERT* expression in HTLV-1 negative cells, while HBZ activates this gene, employing a mechanism reversing the action of menin. However neither menin, nor HBZ can bind directly the *hTERT* promoter and their function is dependent on JunD, which allows for their recruitment and mediate their action. On the basis of data presented herein we can

hypothesize that in normal (i.e. HTLV-1-uninfected) cells, the transcriptional activity of JunD is restrained due to its interaction with menin, resulting in transcriptional inhibition of *hTERT* expression. Conversely in HTLV-1 transformed cells, HBZ being able to abrogate the inhibitory function of menin would change the character of this AP-1 factor and shift it towards transcriptional activator.

Indeed JunD can mediate both positive and negative effects on signaling events, which is a unique property among AP-1 transcription factors. JunD was shown to act both as an activator and as a repressor of gene expression depending on its cellular context, protein partner and gene target (Mensah-Osman, Veniaminova et al. ; Weitzman, Fiette et al. 2000).

Therefore it seems that the primordial event during transcriptional regulation of *hTERT* gene is the assembly of the JunD/Sp1 scaffold on the *hTERT* promoter, which is followed by the recruitment of menin or HBZ, and the formation of HDACs- or HAT-containing complex respectively, determining the character of JunD-dependent regulation of the *hTERT* expression.

D) Histone acetylation – general mechanism of oncogenic virus-specific activation of telomerase?

The transcriptional activation of the *hTERT* gene is considered as a key step in HTLV-1 induced leukemogenesis, endowing HTLV-I-infected cells with extended life span and allowing for clonal expansion of infected cells, finally leading to their immortalization and the development of the Adult T-cell leukemia. In fact the activation of telomerase is a common mechanism during cellular transformation and carcinogenesis provoked by multiple human DNA and RNA tumor viruses, including Epstein- Barr virus, Kaposi sarcoma–associated herpesvirus, human papillomavirus, hepatitis B and hepatitis C viruses (Bellon and Nicot 2008). However the exact mechanism and the factors implicated in the activation of the *hTERT* gene expression in the context of viral infection remains to be elucidated.

Interestingly the studies on *hTERT* activation by human Adenovirus, identified a similar mechanism to the one described in this manuscript. It has been shown that viral

E1A protein is recruiting CBP/p300 coactivator to the *hTERT* promoter and the responsive sequence has been mapped to the region located downstream from the position -149, so the region that contains the Sp1 binding sites (Kirch, Ruschen et al. 2002). The activation of *hTERT* promoter by E1A protein was clearly dependent on the histone acetyltransferase activity of the recruited CBP/p300, as its effect was blocked by roscovitine - an inhibitor of CBP/p300 phosphorylation (thus HAT activity) and reversed by HDAC-1 overexpression. This studies stay in accordance with the report of Takakura *et al,* which indicates the acetylation of the histones within the proximal promoter of *hTERT*, to play an important role in the regulation of the expression of this gene. The TSA treatment resulted in the de-repression of *hTERT* promoter, and this effect was similarly dependent on the GC boxes (Takakura, Kyo et al. 2001).

It is therefore possible that the histone acetylation occurring within the proximal *hTERT* promoter, may be a powerful mechanism leading to the transcriptional activation of this gene. The molecular mechanism of *hTERT* gene activation identified by our group for HBZ, and the one identified by Kirch *et al.* for human Adenovirus E1A protein, potentially may also refer to other oncogenes. We could speculate that the recruitment of p300 and resulting increase in histone acetylation of the proximal promoter of *hTERT*, presumably reversing menin inhibitory function, may be a general mechanism for oncogenic virus-specific activation of telomerase.

III Fine regulation of menin expression and function during the HTLV-1-induced leukemogenesis

The results presented in herein manuscript indicate that HBZ exerts a bimodal function in the regulation of menin. On one hand HBZ leads to the up-regulation of *MEN-1* gene expression and on the other hand functionally impairs its protein product, menin.

Importantly my co-immunoprecipitation results (Figure 22) show that HBZ does not interact with menin directly. In order to antagonize the function of menin, HBZ requires heterodimerization with an intermediate partner – the AP-1 factor JunD, which acts as a scaffold and links both factors together. Therefore HBZ seems to affect menin

function only in the context of AP-1 signaling, through reversing its action on JunD-transcriptional activity.

It is worth to consider that potentially another intermediate partner linking HBZ and menin together could play a role in affecting menin function on another cellular pathways. However the knowledge acquired until now, seems to exclude that kind of possibility. HBZ is known to interact with Smad3 through its MH2 domain (Zhao, Satou et al. 2011) and with the p65 NF-κB factor *via* its Rel homology domain (Zhao, Yasunaga et al. 2009). Menin is also able to bind p65 and Smad3, however exactly the same regions are implicated in the establishment of the interaction (Heppner, Bilimoria et al. 2001; Kaji, Canaff et al. 2001). Nevertheless this hypothesis has to be still experimentally confirmed, it is very likely that binding of those factors to menin and HBZ will be therefore mutually exclusive. Thus JunD, being able to interact at the same time with HBZ *via* its C-terminal leucine zipper, and menin through its N-terminal domain, seems to be the only factor being able to link both proteins together.

Taking into account, that JunD seems to be the only effector of HBZ-mediated impairment of menin function, the HBZ effect on menin would be very selective and concern only the AP-1 signaling. Implying therefore that remaining functions of menin, including the inhibition of classical NF-κB pathway (Heppner, Bilimoria et al. 2001) and the enhancement of TGF-β signaling (Kaji, Canaff et al. 2001), will stay intact. Moreover HBZ-induced up-regulation of menin expression, could even lead to the enhancement of the menin effect on those cellular pathways. Interestingly HBZ has been also shown to inhibit the classical NF-κB pathway *via* p65, and to selectively stimulate the TGF-β signaling through Smad2/3 (Zhao, Yasunaga et al. 2009; Zhao, Satou et al. 2011). It is therefore tempting to assume that menin might be one of the effectors of HBZ action on those pathways, or at least contribute to the enhancement of the HBZ effect. However this hypothesis still requires a support of the experimental data.

Taking into account the role of TGF-β and NF-κB in innate immunity and inflammatory response, it is believed that modulation of their action mediated by HBZ, contributes to the suppression of immune response and therefore enhance the virus persistence by enabling the escape from viral clearance (Zhao and Matsuoka 2012). If, as assumed above, menin effect on TGF-β and NF-κB is retained in the late stages of ATL

development, it will similarly to HBZ, contribute to the development of the adult- T-cell leukemia, ensuring the virus survival and successful spread of infection.

In addition to above described effect of HBZ, also Tax seems to play an important role in the regulation of *MEN-1*. The results presented in herein manuscript indicate that in the contrary to HBZ being an activator of *MEN-1* expression, Tax acts as its inhibitor (Figure 14).

In fact Tax and HBZ are known to exert opposite functions on many processes involved in HTLV-1 induced leukemogenesis. Beginning with differential regulation of viral expression from viral LTRs and finishing with the opposite regulation of many cellular signaling pathways (Currer, Van Duyne et al. 2012; Zhao and Matsuoka 2012). Concerning the fact that Tax and HBZ exhibit distinct expression kinetics, their differential effects endow the virus with a tool for fine regulation of different cellular processes.

Menin, being a subject of a complex regulation by Tax and HBZ, both on the level of the gene expression and the regulation of the protein function, is likely to contribute to the HTLV-1-induced leukemogenesis. On the basis of the initial experiments presented in herein manuscript, we may attempt to build a model of the leukemogenic process taking into account the possible involvement of menin.

- Tax, which is expressed mostly in the initial stages following the viral infection, inhibits *MEN-1* expression. Therefore it deprives cells from the menin-mediated cell cycle control and induces genome instability presumably contributing to the establishment of the cancerous phenotype of the HTLV-1 positive cells. Following the initiation of the leukemogenic process, when Tax expression is lost, and HBZ is the only viral protein expressed, the *MEN-1* transcription can be reactivated. At the same time HBZ inhibits selectively menin function on the AP-1 signaling, leading to the activation of telomerase. Additionally the remaining functions of menin, not affected by HBZ, will be very likely profitable in this late stage of ATL development contributing to immunosuppression and viral persistence.

General conclusions

The work presented in this manuscript concerns the regulation of menin expression and function in the context of the HTLV-1 infection. I showed that *MEN-1* transcription is differentially modulated by the HTLV-1 regulatory proteins: Tax and HBZ. While Tax inhibits *MEN-1* expression, the HBZ protein serves as its activator.

Additionally I showed that HBZ is able to functionally impair menin and cancel its effect as the JunD transcriptional inhibitor. This is achieved by reversing the menin-mediated histone deacetylation, thanks to p300 histone acetyltransferase recruited by LXXLL motifs located at the N-terminus of HBZ.

The acetylation of histone amino termini by histone acetyltransferases, is a well known mechanism implicated in the activation of gene expression (Davie and Spencer 1999). It leads to the chromatin remodeling into more loose structure with increased accessibility for the transcription factors. This posttranslational modification of nucleosomal histones dictates the dynamic transition between transcriptionally inactive chromatin states into the active ones. As shown in herein manuscript, HBZ employing this mechanism is able to activate the *hTERT* gene expression, which is repressed by menin in the absence of HBZ. Consequently HBZ allows for telomerase reactivation, which is an important step in the development and progression of adult T-cell leukemia.

Summarizing, my work brings new insights into the understanding of the transcriptional regulation of the *hTERT* gene expression in normal cells and upon HTLV-1 infection. They also open new perspectives for further research on the consequences of the functional inhibition of menin tumor suppressor and perturbation of AP-1 signaling in order to further understand the deregulation of endogenous transcription mediated by HTLV-1 and finally complete the knowledge on the molecular basis of adult T-cell leukemia development.

Perspectives

I Analysis of JunD phosphorylation upon heterodimerization with HBZ

Work presented herein indicates that HBZ impedes menin function as an inhibitor of JunD transcriptional activity thanks to the recruitment of p300 histone acetyltransferase and resulting modulation of the histone acetylation status within the promoter of target gene. However menin in addition to the recruitment of HDACs is known to repress JunD transcriptional activity by blocking its phosphorylation at the N-terminus by JNK and ERK MAPK kinases (Gallo, Cuozzo et al. 2002; Kim, Lee et al. 2003) (for more details look in the chapter 1.3.2.). It would be possible therefore that HBZ heterodimerizing with JunD could interfere also with this function of menin.

This hypothesis is supported by the luciferase reporter assay using HBZ mutant, which is not able to recruit p300 (Figure 24A). In comparison with wild type HBZ, this mutant, as expected, exerts an impairment in the activation of the *hTERT* promoter. However it still retained the ability to stimulate the luciferase expression on the higher level than JunD alone. Therefore indicating that HBZ-mediated activation of JunD transcriptional activity may not only depend on the recruitment of the p300, but also on another complementary mechanism. It is therefore possible that observed stimulatory effect of HBZmut may stem from blocking menin repression of JunD phosphorylation and as a result activation of JunD-mediated transcription.

Confirmation of this hypothesis would complete the molecular mechanism of HBZ-mediated menin loss-of-function and enlarge our understanding of the perturbation of menin tumor suppressor function, presumably contributing to the HTLV-1 pathogenicity.

II Identification of other target genes up-regulated by HBZ

This PhD work showed that HBZ by impeding the function of menin up-regulates the transcriptional activity of JunD, what results in an increase of the expression of JunD-regulated gene. Our studies focused on the human telomerase reverse transcriptase (*hTERT*), which is the first identified target for the heterodimers JunD/HBZ. However, JunD is an ubiquitous transcription factor participating in the regulation of many cellular genes implicated in a variety of cellular processes including proliferation, survival, differentiation, growth, apoptosis, cell migration, and transformation. In particular, JunD in cooperation with menin has been shown to regulate, among others, the expression of osteoblast differentiation markers (Naito, Kaji et al. 2005), the gastrin endocrine factor (Mensah-Osman, Veniaminova et al.), and the Nur77 nerve growth factor IB (Kim, Lee et al. 2005). Potentially those genes may also undergo the HBZ-mediated transcriptional activation, resulting from menin loss-of-function, which molecular mechanism has been described in this manuscript.

Initial studies performed in the laboratory, already identified the *CCND1* gene encoding the cyclin D1, as another target gene for heterodimers JunD/HBZ. Interestingly, in this case the recruitment of HBZ occurs *via* the NF-κB site located in the proximal part of the promoter, as the IκBα inhibitor restrains the JunD/HBZ-mediated activation of the *CCND1* transcription (unpublished data).

Importantly, JunD forms homo- and heterodimers with b-ZIP factors from Jun and Fos families, which can be recruited to TRE (5′-TGA(C/G) TCA-3′) and CRE motifs (5′-TGACGTCA-3′), or their variants of a related sequence (Curran and Franza 1988). Additionally the heterodimerization with Nrf factor allows for the JunD recruitment to ARE elements (a/gTGACnnnGC) within the promoters of target genes (Venugopal and Jaiswal 1998). Moreover, in addition to direct DNA binding, JunD can be also recruited to the GC boxes and NF-κB sites, indirectly, thanks to the respective interaction with Sp1 and p65 (Mensah-Osman, Veniaminova et al. ; Stein, Baldwin et al. 1993; Toualbi-Abed, Daniel et al. 2008). Furthermore an interaction with other structurally unrelated DNA binding proteins has been reported within the composite regulatory elements, where the AP-1 sites are immediately adjacent to regulatory elements, which binds factors like NFAT, Ets and Smad family members. This cooperative interaction enhances the DNA

binding affinity and the transcriptional activity of the ternary complex compared to each of the factors alone (Chinenov and Kerppola 2001).

The above mentioned interactions with accessory proteins as well as the composition of AP-1 dimer itself, determines the affinity of DNA binding and the sequence specificity of JunD and therefore a set of target genes (Chinenov and Kerppola 2001).

HBZ heterodimerizing with JunD may therefore influence the JunD recruitment, or by modulation of its affinity of direct DNA binding, or by changing its dynamics of interaction with other transcription factors responsible for the recruitment to the non AP-1 sites. As HBZ have low DNA-affinity, it is likely to negatively affect the direct DNA binding and thus increase the sequence specificity, limiting the number of recognized alternative sites. It is therefore worth to consider that the spectrum of target genes for the couple JunD/HBZ may differ from the set of genes regulated by JunD heterodimers with other JunD and Fos family members.

The genome wide analysis of the regions occupied by heterodimers HBZ/JunD by the ChIP-seq technology, revealing the set of genes potentially affected by this dimer of transcription factors, would broaden the research initiated in presented study. Such an approach could greatly contribute to the better understanding of the deregulation of transcription by HBZ, finally leading to better understanding of the mechanisms of HTLV-1 mediated leukemogenesis.

Part 4

Materials and methods

Constructs

The pcDNA-HBZ-Myc encoding the SI spliced isoform of HBZ and the mutated version HBZmut-LL27/28AA-LL47/48AA, lacking the possibility to interact with p300 (Clerc, Polakowski et al. 2008), as well as the hTERT promoter-Firefly luciferase reporter construct (p378 hTERT-Luc) (Takakura, Kyo et al. 2005) and the JunD expression vector (pCMV-JunD-Flag) used in this study were described previously (Kuhlmann, Villaudy et al. 2007). The expression vector of menin (pCI-M1-Menin-S) was a generous gift from Dr. C.X. Zhang (Lyon, France). The pG5-Luc reporter construct containing five tandem binding sites for the yeast protein Gal4 upstream of a minimal TATA box, the pBind vector containing the yeast Gal4 DNA-binding domain upstream of a multiple cloning region and the tk-Renilla plasmids were purchased from Promega, France. To generate the pBind-JunD plasmid that encodes the mouse JunD fused to the Gal4-binding domain, a JunD coding sequence was PCR amplified from pGAD10-JunD mouse vector (a generous gift from Dr JM Mesnard, Montpellier France) using forward (5'-CGGGATCCAGGAAACGCCCTTC-3') and reverse primer (5'-GGATCCTCAGTACGCCGGGACCTGG-3), creating BamHI sites at both ends of the JunD PCR product, and inserted into linearized pBind plasmid (Promega, Charbonnières, France).

Cell culture, transient transfection, luciferase assays

HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Life Technologies, Frederick, MD, USA) supplemented with 10% heat-inactivated fetal calf serum and 100 IU/ml penicillin, 50 µg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were transfected with p378 hTERT-driven Firefly luciferase and tk-driven Renilla luciferase reporter constructs by using PolyFect Transfection Reagent (Qiagen). At 48h post-transfection, reporter gene analysis was performed using the Dual Luciferase Reporter Assay System (Promega) on a Turner Biosystem Luminometer. Transfection efficiencies were normalized to the activity of Renilla luciferase.

Jurkat cells were maintained in RPMI 1640 (Gibco) medium supplemented with 10% heat-inactivated fetal calf serum and 100 IU/ml penicillin, 50 µg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Transfection of Jurkat cells was carried out using the Nucleofector

kit, according to the manufacturer's conditions (Amaxa, Köln, Germany).

Peripheral blood mononuclear cells were isolated from healthy volunteers using Ficoll-Paque density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden), and activated with Dynabeads CD3/CD28 (Invitrogen). Jurkat and TL-Oml (Sugamura, Fujii et al. 1984) cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal calf serum and 100 IU/ml penicillin, 50 µg/ml streptomycin.

Viral particle production and cell infection

Lentiviral vector encoding HBZ spliced transcript pBabe-HBZ (SI), was prepared using pBabe-puro backbone (Morgenstern and Land 1990). The lentiviral vectors bearing the short hairpin (sh) RNA targeting HBZ (SI) - V4, or the one containing scrambled nucleotide sequence designed to form a nonspecific hairpin shRNA (SCRAM) - V5, were described previously (Arnold, Zimmerman et al. 2008). The lentiviral particles bearing shRNA directed against *MEN-1* mRNA were purchased from Santa Cruz Biotechnology (sc-35922-V). Remaining helper-free recombinant lentiviruses were produced upon transient cotransfection of lentiviral plasmid vectors and packaging plasmids: pCMVgag-polΔR8.2 (Gag-Pol plus accessory proteins of HIV-1) and pCMV-ENV_{VSVG} (the vesicular stomatitis virus G protein) at a ratio of 8:4:8. 293T cells were transfected using the calcium phosphate method (Chen and Okayama 1987). Forty eight hours post-transfection, the supernatant was harvested, filtered through a 0.45-µm membrane and purified by ultracentrifugation through a 25% [wt/vol] sucrose cushion. Titres were determined by infection of HeLa cells with serially diluted stock of viral particles. Transductions were carried out for 2 h on 10^5 cells. The percentage of transduced GFP-positive cells was assessed 3 days post-transduction by flow cytometry (FACScalibur, Becton Dickinson). Jurkat or TL-Oml cells were infected with lentiviral particles at multiplicity of infection MOI=5, following the incubation for 1 hour in the presence of polybrene (8µg/ml). Jurkat cells were subjected to RT-qPCR analysis, following the selection in growth medium containing puromycin for 5 days. TL-Oml cells were analyzed two to four weeks following the transduction. Selection of TL-Oml cells was also carried out in the growth medium containing puromycin.

Proliferation assay

Proliferation rate was examined in control and (shRNA)-transduced TL-OmI cells by measuring the mitochondrial activity of the cells determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Sigma, St Louis, MO, USA). Measurement of mitochondrial dehydrogenase cleavage of MTT to formazan dye provides an indication of the level of cell proliferation. Briefly, cells were plated on day 0 and monitored over a 3-day period. MTT was added to each well for the last three hours of incubation and absorbance at 570 nm was measured. Results are expressed as fold increase of optical density value relative to measurement in time point 0 and represent means for n=4.

Western blot and Immunoprecipitation assay

Prior Western blot and immunoprecipitation assays the protein concentration in the cell lysates was determined by Lowry assay using RC DC Protein Kit (BioRad) according to the manufacturer's instructions. Total protein concentration was determined using BSA standard calibration curve.

For Western blot analysis, protein extracts (3 µg) were diluted in Laemmli sample buffer, resolved by an SDS-PAGE in a 10 or 12% polyacrylamide gel and transferred onto a 0.22µm nitrocellulose membrane (Schleicher & Schuell). The primary antibodies used for Western blot included anti-myc TAG (clone 4A6, 05-724, Millipore), anti-flag M2 (F3165, Sigma, L'Isle d'Abeau, France), anti-actin (A2066, Sigma), anti-JunD (sc-74, Santa Cruz Biotechnology), anti-Sp1 (sc-14027, Santa Cruz Biotechnology) and anti-menin (A300-105A, Bethyl Labolatories) or polyclonal anti-HBZ antibody (Gaudray, Gachon et al. 2002). The horseradish peroxidase-conjugated secondary antibodies were purchased from GE Healthcare (anti-mouse, NA9310V and anti-rabbit, NA9340V). Proteins were visualized using an enhanced chemiluminescence detection system (Western Lightning Chemiluminescence Reagent Plus, Perkin Elmer, Courtaboeuf, France).

To examine protein-protein interaction in HeLa cells, subconfluent cells were transfected with the indicated combinations of expression vectors. Cells were lysed in 50 mM Tris pH 8.0, 150 mM NaCl, 0.5% Nonidet-P40 buffer supplemented with complete

protease inhibitors (Roche Diagnostics) and equal amounts of cell extracts (900 µg) were immuno-precipitated with anti-flag M2 (F3165, Sigma, L'Isle d'Abeau, France) or anti-myc TAG (clone 4A6, 05-724, Millipore) antibodies using Protein G Plus Protein A Agarose Suspension (IP05, Calbiochem). Bound proteins were eluted with flag peptide (F3290, Sigma) or c-myc peptide (M2435, Sigma) respectively, resolved on 10% SDS-PAGE and analyzed by Western blot using anti-myc, anti-flag and anti-menin antibodies.

Confocal microscopy

HeLa cells were directly grown on coverslips and transiently transfected with the indicated expression vectors using Effectene transfection reagent (Qiagen). Cells were fixed with methanol 24h post-transfection and permeabilized with 0.5% Triton X-100 in PBS. Immunostaining was performed using anti-myc antibody directly conjugated with FITC (F2047, Sigma), or anti-flag M2 (F1804, Sigma) and anti-menin (A300-105A, Bethyl Labolatories) primary antibodies followed by incubation with secondary antibodies: anti-mouse Alexa 488 and anti-rabbit Alexa 647 (4408, Cell Signalling and A-31573, Invitrogen, respectively). Cells were mounted in Vectashield 4', 6-diamidino-2-phenylindole (DAPI) (1.5 mg/ml) (Vector Laboratories). Following immunostaining, images were acquired using spectral Leica sp5 microscope and analyzed using the ImageJ program (National Institutes of Health).

Quantitative real-time Polymerase Chain Reaction (qPCR)

Total RNAs were isolated from cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Contaminating DNA was removed using RNase-free DNase (Qiagen). RNA (1 µg) was reverse transcribed for 50 min at 42°C using Superscript II (Invitrogen) and oligo(dT)12-18 as primers. The qPCRs were performed using the FastStart Universal SYBR Green Master Mix (Roche) according to the manufacturer's instructions. The nucleotide sequence of the primers used are as follows: MEN-1 sense, 5'-AGGATCATGCCTGGGTAGTG-3' and antisense, 5'-GTCAATGGAAGGGTTGATGG-3', hTERT sense, 5'-TGTTTCTGGATTTGCAGGTG-3' and antisense, 5'-GTTCTTGGCTTTCAGGATGG-3',

actin sense, 5'-TGAGCTGCGTGTGGCTCC-3' and antisense, 5'-GGCATGGGGGAGGGCATACC-3', JunD sense, 5'-CGCCTGGAAGAGAAAGTGAA-3' and antisense, 5'-GTTGACGTGGCTGAGGACTT-3', hbz sense 5'-GCAGAACGCGACTCAACCG-3' and antisense, 5'-GCCGATCACGATGCGTTTCCC-3'. Polymerase chain reaction was performed using StepOnePlus Real-Time PCR System as follows: denaturation for 10 min at 95°C, followed by 45 cycles of 95°C for 10 s, 60°C for 60 s and 72°C for 10 s. The levels of actin transcripts were used to normalize the amount of cDNA in each sample.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed according to the manufacturer's instructions (Upstate Biotechnology Inc) with minor modifications (Terme, Mocquet et al. 2009). Briefly, formaldehyde cross-linked and sonicated chromatin was incubated with antibody recognizing Sp1 (sc-14027X, Santa Cruz Biotechnology), JunD (sc-74X, Santa Cruz Biotechnology), menin (A300-105A Bethyl Labolatories), p300 (sc-585X, Santa Cruz Biotechnology) or acetylated histone H3 (sc-34262, Santa Cruz Biotechnology) followed by protein A-agarose IP. Eluted protein-DNA cross-links were reversed by heating at 65°C overnight, and 25% of the recovered DNA was used in PCR reaction to amplify the 278-bp region of the hTERT proximal promoter with the Phusion high-fidelity DNA polymerase (Ozyme) using the forward n°3 (-190/-171) 5'-CACAGACGCCCAGGACCGCG-3' and the reverse n°4 primer (+69/+88) 5'-GCGCGCGGCATCGCGGGGGT-3'. A negative control PCR, leading to a 150-bp fragment, was performed using forward n°1 (-2916/-2897) 5'-GGCAGGCACGAGTGATTTTA-3' and reverse n° 2 primer (-2782/-2763) 5'-CTGAGGCACGAGAATTGCTT-3' flanking a region deprived from Sp1 or AP1 sites within the distal region of hTERT. DNA recovered from chromatin samples before IP, which corresponds to 1% of that used for immunoprecipitation, was also PCR amplified as loading control. The PCR reactions for hTERT promoter were performed as follows: 32 cycles of 98°C for 10 sec and 71°C for 30 sec, followed by 7 min at 72°C in GC buffer for proximal promoter and 30 cycles of 98°C for 30 sec, 60°C for 30 sec and 72°C for 15 sec, followed by 7 min at 72°C in HF buffer for distal promoter. PCR products were separated on 2% agarose gel and visualized with ethidium bromide staining.

Real-time quantitative telomeric repeat amplification protocol (RQ-TRAP)

Telomerase activity was assessed in Tl-OmI cells by RQ-TRAP, a modified version of the TRAP assay (Wege, Chui et al. 2003). Cells transduced with MEN-1 shRNA or the control SCRAM one were lysed in CHAPS buffer containing 10mM Tris-HCl pH7.5, 1mM $MgCl_2$, 1mM EGTA, 0.5% CHAPS 3-((3-cholamidopropyl)-dimethylammonio)-1-propane-sulfonate, 10% glycerol, 0.1mM PMSF, 5mM β-mercaptoethanol. Following lysis of cells for 30min at 4°C, cell lysates were centrifuged at 12,000g for 30 min at 4°C, and the supernatant collected. The protein concentration in the cell lysate was determined by Lowry assay using RC DC Protein Kit (BioRad) according to the manufacturer's instructions. Telomerase activity was determined in cell extracts (0.1µg) using LC-FastStart DNA Master SYBR Green I mix (Roche Diagnostics) and the following primers: TS (5'-AATCCGTCGAGCAGAGTT-3'), ACX (5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3'). Following telomere elongation for 30min at 30°C, a polymerase chain reaction was performed as follows: 10mn at 95°C and 40 cycles of denaturation at 95°C for 30s, annealing at 60°C for 90s and extension at 72°C for 8s, using the LightCycler (Roche Diagnostics). Telomerase activity was expressed in arbitrary unit (AU) per µg of cell extract relative to a standard calibration curve performed using protein lysate from 293T cells. Samples were serially diluted to verify the linearity of the RQ-TRAP reaction. RNAse-treated and no-template-reactions were also assayed to assure the specificity of the assay.

Telomere length analysis – Southern blot

The methods for measurement of mean terminal restriction fragment (TRF) length have been described elsewhere (Allsopp, Vaziri et al. 1992; Vaziri, Schachter et al. 1993). In brief, telomere length was determined by standard TRF analysis, which is based on Southern blotting. Following the transduction of TL-OmI cells with vectors bearing shRNA targeting the expression of *MEN-1* gene or the control SCRAM sequence and subsequent selection process with puromycin, genomic DNA was isolated using DNeasy Blood and tissue kit (Qiagen). DNA (10µg) was digested overnight with HinfI and RsaI restriction endonucleases at 37°C. Following the digestion DNA (4µg) was resolved on 1% agarose

gel maintained at 13°C, using a CHEF DR-II pulsed-field apparatus (Bio-Rad, Hercules, CA). Separation was obtained within 17h at 160V (forward pulse) and 100V (reverse pulse) with a switch time of 0,1 – 0,8. Following electrophoresis, DNA was subjected to in situ denaturation and transferred overnight by capillary transfer to Hybond N+ nylon membrane (GE Healthcare Life Sciences). Following the DNA crosslinking (120J), hybridization was carried out using dCTPα P32-labeled T2AG3 oligo. Subsequently membrane was washed as follows: 10min in 2xSSC for 10min, 30min in 2xSSC/1% SDS and 2 x 30min in 0.2xSSC/1%SDS. Membrane were exposed to PhosphorImager screens for 3h. The signal intensity was determined over the lane as a function of the distance from the slot and the median telomere length was calculated referring to the molecular weight marker assuming the Gaussian distribution. Data were analyzed by ImageQuant and Microsoft Excel.

Statistical Analysis

Data were reported as mean ± standard deviation (SD). The Student t test (two tailed) was used to assess significance of differences between two groups, and P values were considered as significant when $*P \leq 0.05$, and highly significant when $**P \leq 0.01$, $***P \leq 0.001$. Graphical representations and statistical analysis of the data were performed using OriginPro Data Analysis and Graphing Software. Pearson's correlation coefficient (PCC) and Mander's overlap coefficient (MOC) for confocal microscopy images were calculated using JACoP application in ImageJ program (National Institutes of Health).

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Appendix

Publications and conference abstract that originated from this PhD work:

- **HTLV-1 bZIP factor impedes the menin tumor suppressor and upregulates JunD-mediated transcription of the *hTERT* gene**

Borowiak M, Kuhlmann AS, Girard S, Gazzolo L, Mesnard JM, Jalinot P, Duc Dodon M.

2013 – Carcinogenesis (Epub ahead of print on the 19th of Jun 2013)

- **HBZ impedes the Menin function and up-regulates the transcription of the *hTERT* gene in leukemic cells**

Borowiak M, Kuhlmann AS, Girard S, Gazzolo L, Duc Dodon M.

2011 - Meeting abstract, *Retrovirology*, 8 (Suppl 1): A182

- **Human T-cell Leukemia virus type 3 (HTLV-3) and HTLV-4 antisense transcripts-encoded proteins interact and transactivate Jun family-dependent transcription via their atypical bZIP motif**

Larocque E, André-Arpin C, Borowiak M, Lemay G, Switzer WM, Duc Dodon M, Mesnard JM, Barbeau B.

2013 - Journal of Virology (under revision)

OXFORD JOURNALS
Carcinogenesis

HTLV-1 bZIP factor impedes the menin tumor suppressor and upregulates JunD-mediated transcription of the hTERT gene.

Journal:	*Carcinogenesis*
Manuscript ID:	CARCIN-2012-01348.R2
Manuscript Type:	Review
Date Submitted by the Author:	04-Jun-2013
Complete List of Authors:	Borowiak, Malgorzata; CNRS-UMR5239-ENS, Laboratoire de Biologie Moléculaire de la Cellule Kuhlmann, Anne-Sophie; CNRS-UMR5239-ENS, Laboratoire de Biologie Moléculaire de la Cellule Girard, Sophie; CNRS-UMR5239-ENS, Laboratoire de Biologie Moléculaire de la Cellule Gazzolo, Louis; CNRS-UMR5239-ENS, Laboratoire de Biologie Moléculaire de la Cellule Mesnard, Jean-Michel; CNRS, UM5236, Université de Montpellier Jalinot, Pierre; CNRS-UMR5239-ENS, Laboratoire de Biologie Moléculaire de la Cellule DUC DODON, Madeleine; CNRS-UMR5239-ENS, Laboratoire de Biologie Moléculaire de la Cellule
Keywords:	Telomerase, Cancer, HTLV-1, Menin, JunD transcription factors

HTLV-1 bZIP factor impedes the menin tumor suppressor and upregulates JunD-mediated transcription of the *hTERT* gene.

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Running head: Menin and HBZ in JunD-mediated *hTERT* transcription
Key words: Telomerase, cancer, HTLV-1, menin, HBZ, regulation, transcription factors

Text: 4781 words
6 figures
Abstract: 208 words
49 references

Abstract

Telomerase activity in cancer cells is dependent on the transcriptional regulation of the human telomerase reverse transcriptase (*hTERT*) gene, encoding the catalytic subunit of human telomerase. We have previously shown that HBZ (HTLV-1 bZIP), a viral regulatory protein encoded by the human retrovirus, HTLV-1 (Human T cell Leukemia virus, type 1) cooperates with JunD to enhance *hTERT* transcription in ATL (Adult T-cell Leukemia) cells. Menin, the product of the tumor suppressor *MEN-1* gene, also interacts with JunD, represses its transcriptional activity and down-regulates telomerase expression. The main objective of the present study was to examine how menin and HBZ get involved in the regulation of *hTERT* transcription. Here, we report that JunD and menin form a repressor complex of *hTERT* transcription in HBZ-negative cells. Conversely, in HBZ-positive cells, the formation of a JunD/HBZ/menin ternary complex and the recruitment of p300 histone acetyl transferase activity by HBZ lead to a decreased activity of the JunD-menin suppressor unit, that correlates with the activation of *hTERT* transcription. Silencing HBZ or menin expression in ATL cells confirms that these proteins are differentially involved in telomerase regulation. These results propose that HBZ, by impeding the tumor suppressor activity of menin, functions as a leukemogenic cofactor to up-regulate gene transcription and promote JunD-mediated leukemogenesis.

Introduction

The human telomerase is a ribonucleoprotein complex that consists of an RNA subunit, a catalytic subunit, hTERT (human telomerase reverse transcriptase) and telomerase-associated proteins. This complex elongates telomeres by adding multiple TTAGGG repeats to the chromosomal ends [1,2]. While telomerase is repressed in most normal somatic cells with a finite proliferative capacity, telomerase activity is observed in germ and stem cells [3,4]. Likewise, in tumor cells, permanent activation of telomerase allows stabilization of their telomere length, leading ultimately to their immortalization. As transcriptional regulation of the *hTERT* gene is the major mechanism for cancer-specific activation of telomerase, high *hTERT* expression is related to clinical aggressiveness and poor prognosis in a variety of malignancies, including lymphomas and leukemia. Thus, in leukemic cells from patients with adult T-cell leukemia (ATL), a highly aggressive malignancy associated with HTLV-1 (Human T-cell Leukaemia Virus, type 1) infection, the level of hTERT transcripts increased with the advancing ATL stage, underlining that reactivation of telomerase activity may serve as a key molecular prognostic marker of the development and progression of this malignant lymphoproliferative disease [5-9].

The cloning of the *hTERT* promoter has revealed that a region encompassing the 283-bp upstream of the transcription site, designated as the proximal core promoter, is essential for *hTERT* transcriptional activation [10,11]. Binding specific sites of the activating transcription factors c-Myc and Sp1 (two E-boxes and five GC-boxes respectively) have been clearly identified as activators of transcription in these regulatory sequences [12]. In particular, Sp1 is an essential actor in the up-regulation of telomerase expression, mainly through its interaction with transcriptions factors such as NFAT, c-Myc and the activator protein-1 (AP-1) JunD [12-14]. We have previously reported an increase of *hTERT* transcription when JunD associates with both Sp1 tethered on GC boxes and the HTLV-1 basic leucine Zipper (HBZ) factor, encoded by the antisense strand of the HTLV-1 proviral genome [15]. These observations indicated that the limited effect of JunD on telomerase activation increased through the intervention of JunD/HBZ dimers, in line with the known ability of the viral protein to increase the transcriptional activity of JunD. Interestingly, JunD is the only AP-1 protein to interact with menin, an ubiquitously expressed cellular protein, encoded by the multiple endocrine neoplasia type 1 (*MEN-1*) tumor suppressor gene. Menin has been shown to repress the transcriptional activity of JunD as inactivation of menin switched JunD from a downstream action of growth suppression to growth promotion [16,17]. Thus, in normal cells, JunD and menin together might form a complex restraining *hTERT* transcription. Therefore, increased levels of telomerase activity observed in ATL cells might be either related to a down-regulation of menin expression or to a functional impairment.

In this study, we examined the molecular mechanisms by which menin and HBZ are implicated in the JunD-mediated *hTERT* transcriptional regulation. We report that in HBZ-negative cells, the activation of the *hTERT* proximal promoter by JunD is repressed by menin through the recruitment of histone deacetylases activity. Conversely, in HBZ expressing cells, we observe the formation of a JunD/HBZ/menin complex in which the repressive effect exerted by menin is restrained by HBZ in a

p300 dependent manner. We then identified HBZ as a key viral protein in the reactivation of telomerase. Indeed, results from *HBZ*- or *MEN-1*-silenced TL-Oml ATL cells expressing elevated *MEN-1* expression levels provide evidence that HBZ participates to the up-regulation of *hTERT* transcription. These observations underline that HBZ, by its ability to switch the suppressor JunD/menin unit into an activator JunD/HBZ/menin unit, is playing a pivotal role in the up-regulation of *hTERT* transcription and support a unique and novel role of HBZ as a functional competitor of menin.

Materials and methods

Constructs

The pcDNA-HBZ-myc encoding the HBZ-SI spliced isoform [18], the mutated version HBZmut $[LXXLL]_2$ [19], the hTERT promoter-*Firefly* luciferase reporter construct (p378 hTERT-Luc) [20] and the JunD expression vector (pCMV-JunD-flag) used in this study were described previously [15]. The expression vector of menin (pCI-M1-Menin-S) was a generous gift from Dr. CX Zhang (Lyon, France). The pG5-Luc reporter construct containing five tandem binding sites for the yeast protein Gal4 upstream of a minimal TATA box, the pBind vector containing the yeast Gal4 DNA-binding domain upstream of a multiple cloning region and the tk-Luc plasmid encoding *Renilla* luciferase were purchased from Promega, France. To generate the GAL4-JunD construct, JunD cDNA was ligated into the BamHI-EcoRV sites of pBind vector.

Cell culture, transfection, luciferase assays and RNA interference

HeLa cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Life Technologies, Frederick, MD, USA) supplemented with 10% heat-inactivated fetal calf serum and 100 IU/ml penicillin, 50 µg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Cells were transfected with pG5- or p378 *hTERT*-driven *firefly* luciferase and tk-driven *renilla* luciferase reporter constructs by using calcium phosphate precipitation method [21]. At 48h post-transfection, reporter gene analysis was performed using the Dual Luciferase Reporter Assay System (Promega) on a Turner Biosystem Luminometer. Transfection efficiencies were normalized to the activity of *Renilla* luciferase. Peripheral blood mononuclear cells were isolated from healthy volunteers using Ficoll-Paque density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden), and activated with Dynabeads CD3/CD28 (Invitrogen). Jurkat (HTLV-1 uninfected T-cell line) and TL-Oml (ATL derived T-cell line) [22] cells were maintained in RPMI medium supplemented with 10% heat-inactivated foetal calf serum and 100 IU/ml penicillin, 50 µg/ml streptomycin. Jurkat cells were transfected using the Nucleofector kit, according to the manufacturer's conditions (Amaxa, Köln, Germany). To silence menin or HBZ, TL-Oml cells were infected with lentiviral particles bearing short hairpin (sh) RNA directed against *MEN-1* mRNA (Santa Cruz Biotechnology, sc-35922-V) or targeting HBZ spliced transcript (V4) or scrambled nonspecific sequence (V5) produced as described [23]. Cells were selected in growth medium containing puromycin for 5 days. Two to four weeks following the transduction, cells were harvested and subjected to RT-qPCR analysis.

Lentiviral vector construction and transduction of recombinant lentivirus

The pWPIR-GFP HIV derived vector, obtained from D. Trono, contained the IRES-GFP under the control of the EF1 (human elongation factor 1 alpha) promoter [24]. To generate the HBZ encoding lentiviral construct, sHBZ cDNA was cloned into the BamH1-Xho1 sites located upstream of the IRES in order to allow individual translation of the bicistronic mRNA containing both HBZ and GFP. Recombinant lentiviruses were produced and titrated as described [25]. Jurkat cells cultured for 1 hour in presence of polybrene (8µg/ml) were then incubated overnight with virus stocks at a multiplicity of infection of 2. Seven days later, cells were sorted on the basis of GFP expression by fluorescence-activated cell sorting (FACS) on a FACSVantage (Becton Dickinson).

Proliferation assay

Proliferation rate was examined in control and (shRNA)-transduced TL-Oml cells by measuring the mitochondrial activity of the cells determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Sigma, St Louis, MO, USA). Measurement of mitochondrial dehydrogenase cleavage of MTT to formazan dye provides an indication of the level of cell proliferation. Briefly, cells were monitored over a 3-day period after plating. MTT was added to each well for the last three hours of incubation and absorbance at 570 nm was measured. Results are expressed as fold increase of optical density value relative to measurement in time point 0 and represent means for n=4.

Immunoblot and Immunoprecipitation assays

For immunoblot analysis, protein extracts (3 µg) were diluted in Laemmli sample buffer, resolved by an SDS-PAGE in a 10 or 12% polyacrylamide gel and transferred onto a 0.22µm nitrocellulose membrane (Schleicher & Schuell). The primary antibodies used for western blot included anti-myc (clone 9E10, Roche Diagnostics, Germany), anti-flag (F3165, Sigma, L'Isle d'Abeau, France), anti-βactin (A2066, Sigma), anti-JunD (sc-74, Santa Cruz Biotechnology) anti-Sp1 (sc-14027, Santa Cruz Biotechnology) and anti-menin antibodies (A300-105A Bethyl Labolatories) or polyclonal HBZ antibodies [18]. The horseradish peroxidase-conjugated secondary antibodies were purchased from GE Healthcare (anti-mouse, NA9310V and anti-rabbit, NA9340V). Proteins were visualized using an enhanced chemiluminescence detection system (Western Lightning Chemiluminescence Reagent Plus, Perkin Elmer, Courtaboeuf, France). For immunoprecipitation assays, HeLa cells were transfected with the indicated expression vectors. Cells were lysed in 50 mM Tris pH 8.0, 150 mM NaCl, 0.5% Nonidet-P40 buffer supplemented with complete protease inhibitors (Roche Diagnostics) and equal amounts of cell extracts (900 µg) were immuno-precipitated with anti-FLAG.M2 (F3165, Sigma, L'Isle d'Abeau, France) or anti-myc.TAG (clone 4A6, 05-724, Millipore) antibodies using Protein G Plus/Protein A Agarose Suspension (IP05, Calbiochem). Bound proteins were eluted with FLAG Peptide (F3290, Sigma) or c-Myc Peptide (M 2435, Sigma) respectively and resolved on 10% SDS-PAGE and analyzed by Western blot assay using anti-myc, anti-flag and anti-menin antibodies.

Confocal microscopy

HeLa cells were directly grown on coverslips and transiently transfected with the indicated expression vectors using Effectene transfection reagent (Qiagen). Cells were fixed with methanol 24h post-transfection and permeabilized with 0.5% Triton X-100 in PBS. Immunostaining was performed using anti-myc antibody directly conjugated with FITC (F2047, Sigma, L'Isle d'Abeau, France), or anti-FLAG.M2 (F1804, Sigma, L'Isle d'Abeau, France) and anti-menin (A300-105A, Bethyl Labolatories) primary antibodies followed by incubation with secondary antibodies: anti-mouse Alexa 488 and anti-rabbit Alexa 647 (4408, Cell Signalling and A-31573, Invitrogen, respectively). Cells were mounted in Vectashield 4', 6-diamidino-2-phenylindole (DAPI) (1.5 mg/ml) (Vector Laboratories). After immunostaining, images were acquired using a spectral Leica sp5 microscope and analyzed using the ImageJ program (National Institutes of Health).

Quantitative real-time Polymerase Chain Reaction (qPCR) analysis of gene expression

Total RNAs were isolated from cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Contaminating DNA was removed using RNase-free DNase (Qiagen). RNA (1 µg) was reverse transcribed for 50 min at 42°C by using Superscript II (Invitrogen) and oligo(dT)12-18 as primers. The qPCRs were performed using the FastStart Universal SYBR Green Master Mix (Roche) according to the manufacturer's instructions. The nucleotide sequence of the primers used are the following: *MEN-1* sense, 5'-AGGATCATGCCTGGGTAGTG-3' and antisense, 5'-GTCAATGGAAGGGTTGATGG-3', *hTERT* sense, 5'-TGTTTCTGGATTTGCAGGTG-3' and antisense, 5'-GTTCTTGGCTTTCAGGATGG-3', *actin* sense, 5'-TGAGCTGCGTGTGGCTCC-3' and antisense, 5'-GGCATGGGGGAGGGCATACC-3', *JunD* sense, 5'-CGCCTGGAAGAGAAAGTGAA-3' and antisense, 5'-GTTGACGTGGCTGAGGACTT-3', *hbz* sense 5'-GCAGAACGCGACTCAACCG-3' and antisense, 5'-GCCGATCACGATGCGTTTCCC-3'. Polymerase chain reaction was performed using StepOnePlus Real-Time PCR System as follows: denaturation for 10 min at 95°C, followed by 45 cycles of 95°C for 10 s, 60°C for 60 s and 72°C for 10 s. The levels of *actin* transcripts were used to normalize the amount of cDNA in each sample. To check that the primers used for amplification are giving a specific PCR product, melting curve analysis has always been performed and confirmed on agarose gel.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed according to the manufacturer's instructions (Upstate Biotechnology Inc) with minor modifications [8]. Briefly, formaldehyde cross-linked and sonicated chromatin was incubated with antibody recognizing Sp1 (sc-14027X, Santa Cruz Biotechnology), JunD (sc-74X, Santa Cruz Biotechnology), menin (A300-105A Bethyl Laboratories), p300 (sc-585X, Santa Cruz Biotechnology) or acetylated histone H3 (sc-34262, Santa Cruz Biotechnology) followed by protein A-agarose IP. Eluted protein-DNA cross-links were reversed by heating at 65°C overnight, and 25% of the recovered DNA was used in PCR reaction to amplify the 278-bp region of the hTERT proximal

promoter with the Phusion high-fidelity DNA polymerase (Ozyme) using the forward n°3 (-190/-171) 5'-CACAGACGCCCAGGACCGCG-3' and the reverse n°4 primer (+69/+88) 5'-GCGCGCGGCATCGCGGGGGT-3'. A negative control PCR, leading to a 150-bp fragment, was performed using forward n°1 (-2916/-2897) 5'-GGCAGGCACGAGTGATTTTA-3' and reverse n° 2 primers (-2782/-2763) 5'-CTGAGGCACGAGAATTGCTT-3' flanking a region deprived from Sp1 or AP1 sites within the distal region of *hTERT*. DNA recovered from chromatin samples before IP, which corresponds to 1% of that used for immunoprecipitation, was also PCR amplified as loading control. The PCR reactions for *hTERT* promoter were processed using Phusion high-fidelity DNA polymerase (Ozyme) through 32 cycles of 98°C for 10 sec and 71°C for 30 sec, followed by one cycle for 7 min at 72°C in GC buffer for proximal promoter and through 30 cycles of 98°C for 30 sec, 60°C for 30 sec and 72°C for 15 sec, followed by one cycle for 7 min at 72°C in HF buffer for distal promoter. PCR products were separated on 2% agarose gel and visualized with ethidium bromide staining.

Statistical Analysis

Data were reported as mean ± standard deviation (SD). The Student *t* test (two tailed) was used to assess significance of differences between two groups, and P values were considered as significant when $*P \leq 0.05$, and highly significant when $**P \leq 0.01$, $***P \leq 0.001$. Graphical representations and statistical analysis of the data were performed using OriginPro Data Analysis and Graphing Software. Pearson's correlation coefficient (PCC) for confocal microscopy images were calculated using JACoP application in ImageJ program (National Institutes of Health).

Results

HBZ impedes the inhibitory effect of menin on the transcriptional activity of JunD

The effect of menin on the transcriptional activity of JunD was first evaluated in HeLa cells co-transfected with the reporter pG5-Luc vector (containing five GAL4-binding sites upstream of a minimal TATA box), with a GAL4-JunD vector along with increasing amounts of a menin expression vector. As shown in Figure 1A, menin inhibited the transcription of luciferase, in a dose-dependent manner, in line with previous data [16]. Thus, a menin level of 25 ng correlated with a 70% inhibition. To investigate how HBZ protein influences the inhibitory effect of menin, cells were co-transfected with the reporter pG5-Luc and the GAL4-JunD vectors together with a constant amount (25ng) of the menin expression vector and with increasing amounts of an HBZ expression vector. As shown in Figure 1B, the transcription of luciferase was increasingly activated in a dose-dependent manner. An amount between 1 to 5 ng of the HBZ plasmid was sufficient to overcome the inhibitory effect of menin, whereas at the highest one (25 ng) a 10-fold increase was observed.

Both HBZ and menin interact with JunD to form a JunD/HBZ/menin ternary complex in vivo

HBZ interacts with the leucine zipper domain in the C-terminal part of JunD, whereas menin binds to the N-ter domain (Figure 2A) [16,26,27]. To clarify the molecular mechanism by which HBZ is

restraining the inhibitory effect of menin and thus enhances the JunD-mediated transcriptional activity, the subcellular localization of JunD, HBZ and menin proteins was first examined by confocal microscopy (Figure 2B). When coexpressed, menin and JunD strongly colocalized (Pearson's correlation coefficient PCC=0.965)(panel 4). As expected, upon cotransfection with JunD, HBZ relocalized to the whole nucleus and both proteins showed high degree of co-localization (PCC=0.938) (panel 5)[28]. Interestingly, upon the coexpression of HBZ and menin, these two proteins did not colocalize (PCC=0.571) (panel 6). Finally, when JunD, HBZ and menin were cotransfected, the three proteins colocalized within the nucleus (PCC=0.938 for menin/JunD, PCC=0.964 for menin/HBZ and PCC=0.968 for JunD/HBZ) (panel 7).

We next explore whether JunD, HBZ and menin could form a ternary complex. Cells were cotransfected with a JunD-flag expression plasmid, either with an HBZ-myc or a menin-encoding vector or with both. Equal amounts of cell lysates were immunoprecipitated with either an anti-flag or an anti-myc antibody (Figure 2C). These immunoprecipitates were then subjected to western blot analysis. The anti-flag immunoprecipitate confirmed the JunD interaction with either HBZ or menin or both (lanes 3, 4 and 5). The anti-myc immunoprecipitate clearly indicated a lack of interaction between HBZ and menin in absence of JunD (lane 9). However, when the three components were coexpressed, menin was detected, underlining the formation of a specific ternary complex (lane 10). Thus, JunD behaves as a scaffold protein recruiting simultaneously both HBZ and menin. Collectively, these results propose that in HBZ-expressing cells, the increase of the transcriptional activity of JunD is dependent on the formation of JunD/HBZ/menin complexes.

Opposite effects of HBZ and menin on the JunD-mediated transcriptional activation of the hTERT proximal promoter depend on p300 and on HDAC recruitment, respectively

We next investigated how HBZ and menin are operating at the level of the *hTERT* proximal promoter. The above observations imply that in absence of HBZ, the JunD-mediated transcriptional activation of the *hTERT* promoter is restrained because of its interaction with menin. Indeed, expression of menin significantly decreased the activation of the *hTERT* proximal promoter, in a dose-dependent manner (Figure 3A). Conversely, the activation of the *hTERT* proximal promoter in HeLa cells and in Jurkat T-cells co-transfected with HBZ and JunD was significantly enhanced (Figures 3B, 3C). Collectively, these data indicate that the cooperation of JunD and HBZ restrained the repressing effect of menin on *hTERT* activation. They further suggest that menin and HBZ are exerting opposing functional effects in the regulation of telomerase activity.

The menin-mediated inhibition of JunD transcriptional activity has been linked to its association with a mSin3A-histone deacetylase (HDAC) complex [29]. Indeed, trichostatin A (TSA), an HDAC inhibitor induces hyperacetylation of histones at the *hTERT* proximal promoter, and upregulates the *hTERT* gene in telomerase-positive tumor cells [29-31]. Consistent with these observations, we observed that in TSA-untreated cells, menin decreased by two-fold the JunD/HBZ-induced activation of the *hTERT* promoter and that this effect was relieved by TSA (Figure 3D).

HBZ has been shown to activate gene expression though its interaction with the Histone Acetyl Transferase (HAT) coactivator p300 [32,33]. We have previously reported that the N-terminal activation domain of HBZ is responsible for the stimulation of the JunD-mediated *hTERT* transcription [15]. This domain binds with high affinity to the KIX domain of p300, inferring that p300 is functionally required for the enhancement of JunD-mediated transcription by HBZ [19,34]. Indeed, luciferase assays performed with HBZ mutated in both LXXLL motifs showed a decrease of the luciferase activity, compared to that with the wild type HBZ (Fig 3E). In addition, in presence of p300, a two-fold activation of the *hTERT* proximal promoter is observed in cells expressing JunD and HBZ, when compared to cells without p300 (Figure 3F, compare lane 8 to lane 7).

We then tested the histone acetylation status of the endogenous *hTERT* promoter sequences in cells stably expressing HBZ and JunD. Chromatin immunoprecipitation (ChIP) assay was performed using an anti-acetylated H3 (AcH3) antibody and a pair of primers amplifying the proximal part of the *hTERT* promoter or the distal one as control (Figure 3G). Whereas the amounts of AcH3 at the distal region of the *hTERT* promoter were found to be quite similar, a marked increase of AcH3 was observed at the proximal promoter of cells co-expressing HBZ and JunD (Figure 3H, upper panel, compare lane 4 to lane 5). That increase was less prominent, when menin was overexpressed (compare lane 6 to lane 5). Collectively, these data underline that HBZ recruits p300 and abrogates the inhibitory effect of menin by increasing histone acetylation at the *hTERT* proximal promoter.

HBZ increases JunD-mediated hTERT transcription in leukemic T cells

As underlined above, JunD-mediated activation of *hTERT* transcription in HBZ-negative cells is repressed by menin. Consequently, it may be expected that in those cells an increase of *hTERT* correlated with a decrease of menin expression. To verify that assumption, we first evaluated the levels of *hTERT*, *JunD* and *MEN-1* transcripts in human peripheral blood T lymphocytes isolated from healthy individuals, either resting or activated after a two-days incubation with anti-CD3 plus anti-CD28-mAbs conjugated magnetic beads. As illustrated in Figure 4A, a 10-fold increase in the amount of *hTERT* mRNAs was observed in activated T lymphocytes, when compared to that in resting cells, in agreement with previous results [13,35]. No modification in the level of *JunD* transcripts was observed in both cell types. However, the amount of *MEN-1* transcripts significantly decreased in activated cells, revealing an inverse correlation between transcription of *hTERT* and that of *MEN-1*, in resting as well as in activated lymphocytes. The WB analysis of the corresponding cell lysates confirms these results (Figure 4B).

In contrast, preliminary observations indicated that in leukemic cells obtained from ATL patients, increased amounts of *hTERT* transcripts were detected together with those of *MEN-1* and *HBZ* mRNAs (data not shown). Such a profile was observed in TL-Oml cells established from peripheral blood lymphocytes of an ATL patient. These cells do not express Tax but retain the HBZ transcription [36]. Furthermore, in leukemic T cells of non-ATL origin, such as Jurkat cells that do not express HBZ, both *hTERT* and *MEN-1* transcripts were detected (Figure 5B and data not shown). Despite similar amounts of menin and JunD in both cell lines (Figure 5A), the amount of hTERT transcripts in TL-Oml

cells is higher than that in Jurkat cells (Figure 5B). Interestingly, a level of *hTERT* transcription similar to that observed in TL-Oml cells is observed in Jurkat cells transduced with HBZ (Figure 5C). Accordingly, the expression of HBZ is enhancing *hTERT* transcription underlining that this viral protein is participating to the maintenance of a high level of *hTERT* transcription in ATL cells.

To investigate how HBZ is affecting the binding of JunD/Sp1/menin to DNA, we performed ChIP assays in TL-Oml and in Jurkat cells (Figure 5D). Each of these 3 proteins was found to be exclusively associated to the proximal sequences of the *hTERT* promoter. Whereas there was no difference in Sp1 recruitment to the *hTERT* proximal promoter between both cell lines, we observed a substantial increase of JunD binding and reduced recruitment of menin in TL-Oml cells, suggesting that in presence of HBZ, the JunD binding to *hTERT* promoter was stabilized, while the access of menin was restrained. ChIP assays were then performed using antibodies directed to p300 and AcH3. The band intensities were quantified using ImageJ software (National Institutes of Health) and normalized to those of the input. Thus, the band corresponding to p300 recruitment to *hTERT* proximal promoter in TL-Oml cells is more intense than the one in Jurkat cells by a factor of 1.96. Likewise, the band corresponding to AcH3 is more intense by a factor of 1.50 in TL-Oml than in Jurkat cells, indicating that p300 recruited on the proximal promoter leads to an increase of histone acetylation in TL-Oml cells (Figure 5E).

To further assess the role of HBZ in the regulation of endogenous *hTERT* transcription in ATL cells, knockdown of menin and HBZ expression by specific shRNA was performed in TL-Oml cells. As shown in Figures 6A and 6C, a 60% inhibition of MEN-1 mRNA level induced a 1.4-fold increase of *hTERT* transcripts, which was proportional to telomerase activity (Figure S1). As expected, proliferation of these cells increased, confirming that menin is indeed involved in the down-regulation of cell proliferation (Figure 6D). Conversely, when HBZ was knocked-down by 35%, a 2.5-fold decrease of the level of *hTERT* mRNAs was observed (Figures 6B and 6C). That decrease correlated with an inhibition of cell growth, indicating that the induction of telomerase expression by HBZ promotes cell proliferation (Figure 6E). Altogether, these results indicate that HBZ switches the JunD/menin inhibitory complex into a JunD/HBZ activator complex of *hTERT* transcription.

Discussion

Telomerase activation, a critical step in cellular immortalization and carcinogenesis, depends on the transcriptional activity of the *hTERT* gene. As reported in other cancers, a high amount of *hTERT* transcripts is observed in ATL cells, in which HBZ transcripts are always detected [37,38]. The proximal core promoter was identified as responsible for the transcriptional activity of the *hTERT* gene [39]. Furthermore, various transcription factors have been shown to play both direct and indirect roles in the activation of *hTERT* transcription, suggesting that the level of telomerase expression is determined by the sum of the effects of these factors [4].

We have previously reported that HBZ through its interaction with JunD critically participates to the transcriptional activation of the *hTERT* proximal promoter. This synergistic activity of the JunD/HBZ dimers is dependent on the tethering of JunD to the *hTERT* promoter via the interactions of

JunD with Sp1 bound to the GC boxes present in the proximal sequences. [2,4,15]. Likewise, the upregulation of HTLV-1 antisense transcription has been found to depend on the cooperation of JunD and HBZ [40]. In the present study, we have shown that HBZ contributes to hinder the inhibitory effects exerted by menin on the JunD-mediated activation of the *hTERT* proximal promoter.

The divergent effects of these cellular and viral proteins are dependent on their recruitment by JunD and on the subsequent formation of a ternary complex in HTLV-1-infected cells. The opposite effects of menin and HBZ on the transcriptional activity of JunD are further dependent on the respective recruitment of HDAC and p300. Both TSA an HDAC inhibitor, and the ectopic expression of p300 are enhancing the JunD/HBZ activity on the *hTERT* proximal promoter. Conversely, a mutated HBZ unable to recruit p300 does not stimulate JunD transcriptional activity, confirming the implication of p300 on *hTERT* transcription (Fig 3E). In addition, the higher JunD-mediated transcriptional activity in cells expressing HBZ correlates with an increase of *hTERT* transcription. This observation provides another example of the ability of HBZ to upregulate the expression of genes through its interaction with p300/CBP, such as those coding for Dkk1 and TGFβ [32-34]. Furthermore when HBZ is over-expressed, an increase of histone acetylation is observed at the *hTERT* proximal promoter as the consequence of the increase in the amount of p300 recruited by HBZ. Consequently, the interaction of JunD with Sp1 may represents a primordial event allowing the assembly of a transcription-regulating complex on the *hTERT* core promoter upon recruitment of menin/HDAC and HBZ/p300.

A mechanism of menin/JunD/Sp1-mediated inhibition of gastrin gene has been previously reported [41]. New insights into the role of menin on the inhibition of *hTERT* transcription are provided by the present data. Indeed, menin has been identified as a potent repressor of *hTERT* expression in a variety of cell lines, implying that loss of its repressive effect on *hTERT* transcription is contributing to the tumor development observed in *MEN-1*-deficient patients [42]. As menin does not contain known DNA-binding motifs, its repressing effect appears to be linked to its interaction with JunD and to the recruitment of repressive chromatin-modifying protein complexes involved in reducing histone acetylation. Likewise, it has previously been shown that *MEN-1* mediated-repression of JunD activity in T cells reduces transcription of the Nur77 locus by recruitment of mSin3A-HDAC [29,43]. These results contrast with observations indicating that menin represses *hTERT* transcription via direct binding to the *hTERT* promoter that may disturb the interactions of transcription factors with the promoter [44,45]. Nevertheless, association with the promoter may be stabilized by the low-affinity sequence-independent interaction of menin with DNA [45].

As underlined in Introduction, it has been proposed that the loss of function of the growth suppressor unit (formed by the growth promoter, JunD and the growth suppressor, menin) results from either menin loss or disabling of menin's ability to bind to JunD [17]. Indeed, in normal (HTLV-1 uninfected) resting T lymphocytes, *hTERT* transcripts are scarce in resting T cells that express both JunD and menin mRNAs. After activation, a significant increase of *hTERT* transcripts is detected, that is accompanied by a decrease of *MEN-1* transcription. These observations indicate that *hTERT* transcription is fully operational when menin levels are decreasing. However, in contrast to normal activated T cells, ATL cells exhibit high levels of *hTERT* transcripts, despite expressing high levels of

menin. The respective role of menin and HBZ in the *hTERT* transcriptional regulation in ATL cells has therefore been analysed in shRNA-based inhibition experiments using the TL-Oml ATL cell line. *MEN-1* knockdown results in an increase of *hTERT* transcription that correlates with an increased proliferation. In contrast, the decrease of HBZ expression is followed by a decrease of *hTERT* transcripts and a decreased proliferation. Likewise, the ectopic expression of HBZ in HTLV-1-uninfected T cells induces an increase of *hTERT* transcription. Thus in ATL cells, an original molecular mechanism is operating, as HBZ through its interaction with JunD and p300 induces the loss of function of the growth suppressor JunD/menin unit. Likewise, HBZ may also intervene in upgrading the functional cooperation of JunD and NF-κB in the transcriptional regulation of cyclin D1 [46]; Levy & Duc Dodon, unpublished data). Consequently, in ATL cells, JunD may recover its functional property as a growth promoter and be able to fully contribute to the development of the HTLV-1-induced leukemogenic process. We propose that HBZ, by impeding the tumor suppressor activity of menin, functions as a leukemogenic cofactor to up-regulate gene transcription and promote JunD-mediated leukemogenesis.

As both JunD and Sp1 are similarly expressed in uninfected Jurkat T cells and in TL-Oml cells, it is tempting to speculate that these two proteins are readily available for regulation of *hTERT* transcription by menin or HBZ, and possibly others transcription factors with no DNA binding ability. Indeed, HBZ, similarly to menin, does not contain known DNA-binding sequences, therefore relies on JunD for access to promoter DNA. HBZ also serves as a link between JunD and stimulating chromatin-modifying proteins, thereby potentiating the transcriptional activity of this AP-1 factor. Thus, the stimulation of JunD transcriptional activity by HBZ may favour the growth-promoting property of JunD on one hand, and increase the telomerase activity on the other hand, which is in agreement with the involvement of HBZ in T cell proliferation [23,47,48]. Overall, these observations stress the essential implication of HBZ in the establishment and maintenance of the leukemic process through *hTERT* and telomerase activation, making this viral protein a pertinent therapeutic target. Finally it is worth pointing out that the transcriptional activation of *hTERT* is a common mechanism during cellular transformation and carcinogenesis provoked by multiple human DNA and RNA oncogenic viruses [49]. It is therefore tempting to speculate that the inhibition of menin function in the up-regulation of *hTERT* transcription may be considered as a general mechanism for the activation of telomerase during the tumor development.

Funding

This work was supported in part by INSERM (Institut National de la Santé et de la Recherche Médicale) and a grant from Association pour la Recherche sur le Cancer (n° SFI20101201784 to MDD). ASK was supported by a fellowship from the Association pour la Recherche sur le Cancer. MB was supported by a fellowship "Mobilité Européenne" from the Ministère de l'Enseignement Supérieur et de la Recherche, and from Association pour la Recherche sur le Cancer.

Conflict of interest statement: None declared

Acknowledgements

We thank Fatima Mechta-Grigoriou (Institut Curie, Paris, France), Eric Gilson and Adelaïde Saint-Leger (LBMC, Lyon, France) and Antoine Gross (CPBS, Montpellier, France) for helpful discussions; M. Matsuoka (Kyoto university, Japan) for providing TL-Oml cells; D. Rigal (Etablissement Français du Sang, Lyon, France) for providing human peripheral blood. We thank M. Takakura for providing p378 *hTERT*-Luc, C.X. Zhang for that of pCI-M1-Menin-S plasmid.

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FIGURES LEGENDS

Fig. 1. Menin and HBZ exert opposite effects on the transcriptional activity of JunD. (**A**) Menin represses JunD-mediated transcriptional activity. HeLa cells were cotransfected with the reporter construct pG5-Luc (100 ng), and the expression vector Gal4-JunD fusion proteins (8 ng) together with the indicated amounts of an expression vector for menin. Luciferase expression in the absence of menin is arbitrarily set at 100 on the y-axis. Lower panel: western blot analysis of the levels of JunD, menin and actin in cell lysates. (**B**) HBZ overcomes menin-mediated inhibition of JunD transcriptional activity. HeLa cells were cotransfected with the reporter construct pG5-Luc (100ng) together with the expression vector for Gal4-JunD fusion proteins (8ng), along with menin (25 ng) and indicated amounts of the HBZ expression vector. Luciferase activity in the absence of HBZ and menin is arbitrarily set at 100 on the y-axis. Luciferase activity was normalized to tk-luc activity. Data (mean and standard deviation) are representative of three independent experiments. Results of Student's t-test are indicated on the graph, Lower panel: western blot analysis of the levels of JunD (arrow), menin, HBZ and actin in cell lysates.

Fig. 2. *In vivo* interaction between JunD, menin and HBZ proteins. (**A**) Schematic representation of the identified functional domains of JunD (MBD, Menin Binding Domain; TA, Transcriptional Activity domain; ZIP, Leucin zipper domain. (**B**) JunD, menin and HBZ colocalize in the nucleus when expressed together. HeLa cells were transfected with JunD-flag (1), or menin (2) or HBZ-myc expression vector alone (3) or JunD-flag together with menin (4), or JunD-flag together with HBZ-myc (5), or menin and HBZ-myc (6), or JunD-flag, menin, and HBZ-myc expresion vectors (7). Following fixation with methanol, HBZ was stained with anti-myc antibody directly conjugated with FITC, JunD was visualized by anti-flag and anti-mouse Alexa 488 conjugated secondary antibody, and menin was detected using anti-menin and anti-rabbit Alexa 647-conjugated secondary antibody. DAPI was used for staining of the nucleus. The white bars correspond to a scale of 10 µm. (**C**) HeLa cells were transfected with the indicated expression vectors and equal amounts of lysates were immunoprecipitated with anti-flag or anti-myc antibody followed by immunoblotting with anti-myc, anti-flag or anti-menin antibodies. The results shown are from one representative assay out of three independent experiments.

Fig. 3. Menin and HBZ exert opposite effects on the transcriptional activation of the *hTERT* proximal promoter. (**A**) Menin inhibits JunD-mediated activation of *hTERT* proximal promoter. HeLa cells were transiently transfected with the p378 *hTERT*-Luc reporter plasmid along with JunD (25 ng) and indicated amounts of menin expression vectors. Forty-eight hours later, luciferase activity was analyzed and normalized to tk-Luc activity. Lower panel: western blot analysis of the levels of JunD, menin and actin in cell lysates. (**B**) HBZ overcomes the repressive effect of menin on the *hTERT* proximal promoter. HeLa cells were transiently transfected with the p378 *hTERT*-Luc reporter plasmid and with the vector encoding JunD (25 ng) and/or HBZ (200ng) and/or menin (200 ng). Forty-eight hours later, luciferase activity was analyzed and normalized to tk-Luc activity. Data (mean and

standard deviation) are representative of three independent experiments. Lower panel: western blot analysis of the levels of JunD, HBZ, menin and actin in cell lysates. (**C**) HBZ overcomes the repressive effect of menin in T cells. Jurkat cells were transiently transfected with the p378 *hTERT*-Luc reporter plasmid and with the vector encoding JunD (25 ng) and/or HBZ (200ng) and/or menin (200 ng). Twenty-four hours later, luciferase activity was analyzed and normalized to tk-Luc activity. (**D**) Trichostatin A (TSA) reversed the inhibitory effect of menin and revealed the stimulatory effect of HBZ on the *hTERT* promoter. HeLa cells were transiently transfected with the p378 *hTERT*-Luc reporter plasmid and with the vector encoding JunD (25 ng) and/or HBZ (200 ng), together with 200 ng of the expression vector for menin. Twelve hours after transfection, the medium was changed by the addition of 40 ng/mL of TSA. Twenty-four hours later, luciferase activity was analyzed and normalized to tk-Luc activity. The values are those obtained in triplicate, from one experiment representative of two independent experiments. (**E**) Effect of HBZ mutated in LXXLL motifs on the *hTERT* promoter. HeLa cells were transiently transfected with the p378 hTERT-Luc reporter plasmid and with the vector encoding JunD (25ng) and/or HBZ (200ng) either wild type (wt) or mutated (mut) in LXXLL-motifs. Luciferase activity was analyzed and was normalized to tk-Luc activity. The values are representative of three independent experiments. Lower panel: western blot analysis of JunD, HBZ and actin protein levels in cell lysates. (**F**) Involvement of p300 in the activation of the *hTERT* proximal promoter. HeLa cells were transiently transfected with the p378 hTERT-Luc reporter plasmid and with the vector encoding JunD (25 ng) and/or HBZ (200 ng) with p300 (400 ng) expression vector (black bars) or without (open bars). Luciferase activity was analyzed and normalized to tk-Luc activity. The values are representative of three independent experiments. Lower panel: western blot analysis of the levels of JunD, HBZ and actin in cell lysates. (**G**) Schematic representation of the *hTERT* promoter sequences: binding sites for transcription factors are indicated; the arrows 1,2 and 3,4 indicate the locations of primers used to amplify sequences of the distal and proximal promoter, respectively. (**H**) HBZ and menin affect the histone acetylation status of the endogenous *hTERT* promoter. HeLa cells stably overexpressing HBZ and JunD and transfected with a menin expression vector were subjected to chromatin immunoprecipitation using anti-AcH3 antibody or control preimmune rabbit serum. Purified DNA was analyzed by PCR using primers spanning the *hTERT* proximal promoter (primers 3 and 4, upper panel) or the *hTERT* distal promoter (primers 1 and 2, lower panel). DNA recovered from chromatin samples before immuno-precipitation, which corresponds to 1% of that used for immuno-precipitation, was also PCR amplified as input.

Fig. 4. Endogenous *hTERT*, *MEN-1* and J*unD* mRNA levels in T lymphocytes. **(A)** mRNA levels were assessed by real-time quantitative PCR in peripheral blood T lymphocytes before (resting) and after activation with anti-CD3, -CD28 monoclonal antibodies. Histograms represent the results from two independent experiments that are expressed as the level of related mRNA expression normalized to actin expression. **(B)** Western blot analysis of the levels of menin, JunD, sp1 and actin in cell lysates.

Fig. 5. Expression of HBZ stimulates *hTERT* expression in T lymphocytes. (**A**) Comparison of protein levels and (**B**) *hTERT* mRNA expression in the non-infected Jurkat T-cell line and in the TL-Oml ATL cell line. (**C**) Ectopic expression of HBZ in Jurkat cells leads to an increase of endogenous *hTERT* expression. Jurkat cells were transduced with lentiviral particles bearing the coding sequence of HBZ or the control empty one. After cell sorting, RNAs were extracted, reverse transcribed and subjected to real-time quantitative PCR. Data represent the mean out of three independent experiments. (**D**) Analysis of the cellular proteins implicated in the regulation of the *hTERT* proximal promoter in HBZ-positive and HBZ-negative cells. Cell extracts from Jurkat and TL-Oml cells were subjected to chromatin immunoprecipitation using control pre-immune rabbit serum or anti-Sp1, anti-JunD or anti-menin antibody. Immunoprecipitated DNA was amplified using primers spanning the *hTERT* proximal promoter (upper panel) or the *hTERT* distal promoter (lower panel). DNA corresponding to 1% of that used for immunoprecipitation, was amplified as inputs. Data presented are representative of three independent experiments. (**E**) Analysis of the p300 recruitment and the resulting histone acetylation within the *hTERT* proximal promoter. Chromatin immunoprecipitation was performed on Jurkat and TL-Oml cell extracts using anti-p300 and anti-AcH3 antibodies or control preimmune rabbit serum, and processed as described in **D**.

Fig. 6. Effect of *MEN-1* or *HBZ* knock-down in ATL cells. TL-Oml cells were infected with lentiviral particles bearing shRNA targeting the expression of *HBZ* or *MEN-1* gene or shRNA control (SCRAM) as indicated. Following selection with puromycin, RNA was extracted from equal number of cells, reverse transcribed and analysed by real-time quantitative PCR to determine the *MEN-1* (**A**), *HBZ* (**B**) and *hTERT* (**C**) mRNA levels. Results are expressed as the indicated mRNA level relative to that of actin; the mean and standard deviations out of three independent experiments are shown. (**D**) Proliferation of TL-Oml cells is significantly increased under *MEN-1* knockdown condition, as assessed by the MTT assay. (**E**) TL-Oml cell proliferation is significantly reduced under *HBZ* knockdown condition. In **D** and **E**, results are expressed as fold increase of optical density value relative to measurement at time point 0, and represent the means and standard deviations of four independent experiments.

Abbreviations: AP-1, activator protein-1; AcH3, acetylated histone H3; ATL, Adult T-cell leukemia; ChIP, chromatin immunoprecipitation; HAT, histone acetyl transferase; HBZ, HTLV-1 basic leucine zipper; HDAC, histone deacetylase complex; hTERT, human telomerase reverse transcriptase; HTLV-1, human T-cell leukemia virus type 1; MEN-1, multiple endocrine neoplasia type 1; MBD, menin binding domain; PCC, Pearson's correlation coefficient; PCR, polymerase chain reaction; RT, reverse transcription; shRNA, small hairpin RNA; TSA, trichostatin A; ZIP, leucine zipper domain.

Additional files

Additional file 1. Figure S1. Effect of MEN-1 knock-down on endogenous telomerase activity in TL-Om1 cells. Cells were transfected with MEN-1 shRNA as described in material and methods. (A) western blot analysis of cell lysates; (B) Telomerase activity was assessed by RQ-TRAP.

Additional file 2. Methods

Real-time quantitative telomeric repeat amplification protocol (RQ-TRAP)

Telomerase activity was assessed in TL-Om1 cells by RQ-TRAP, a modified version of the TRAP assay *. Cells transduced with *MEN-1* shRNA or the control one were lysed in 10mM Tris-HCl pH7.5 buffer containing 1mM $MgCl_2$, 1mM EGTA, 0.5% 3-((3-cholamidopropyl)-dimethylammonio)-1-propane-sulfonate, 10% glycerol, 0.1mM PMSF, 5mM β-mercaptoethanol. Cell extracts (0.1µg) were analyzed using LC-FastStart DNA Master SYBR Green I mix (Roche Diagnostics) and the following primers: TS (5'-AATCCGTCGAGCAGAGTT-3'), ACX (5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3'). After a 30min incubation at 30°C, a polymerase chain reaction was performed as follows: 10mn at 95°C and 40 cycles of 95°C for 30s, 60°C for 90s and 72°C for 8s, using the LightCycler (Roche Diagnostics). Telomerase activity was expressed in arbitrary unit (AU) per µg of cell extract relative to a standard calibration curve performed using protein lysate from 293T cells. Samples were serially diluted to verify the linearity of the RQ-TRAP reaction and RNAse-treated to check specificity of this assay.

*Wege H, Chui MS, Le HT, Tran JM, Zern MA. SYBR Green real-time telomeric repeat amplification protocol for the rapid quantification of telomerase activity. *Nucleic Acids Res*. 2003;31:E3-3.

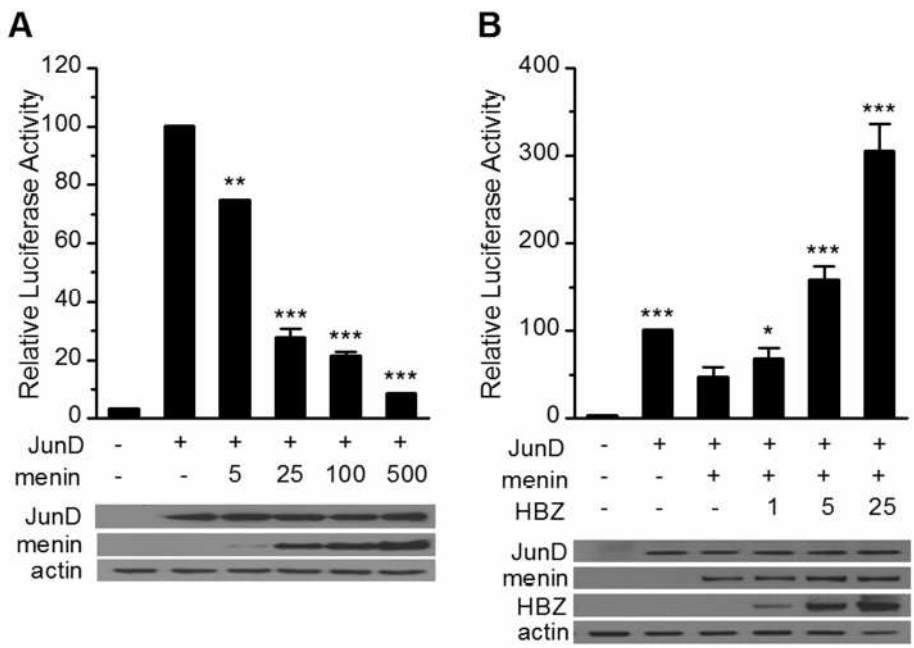

Figure 1.
Menin and HBZ exert opposit effects on the transcriptional activity of JunD
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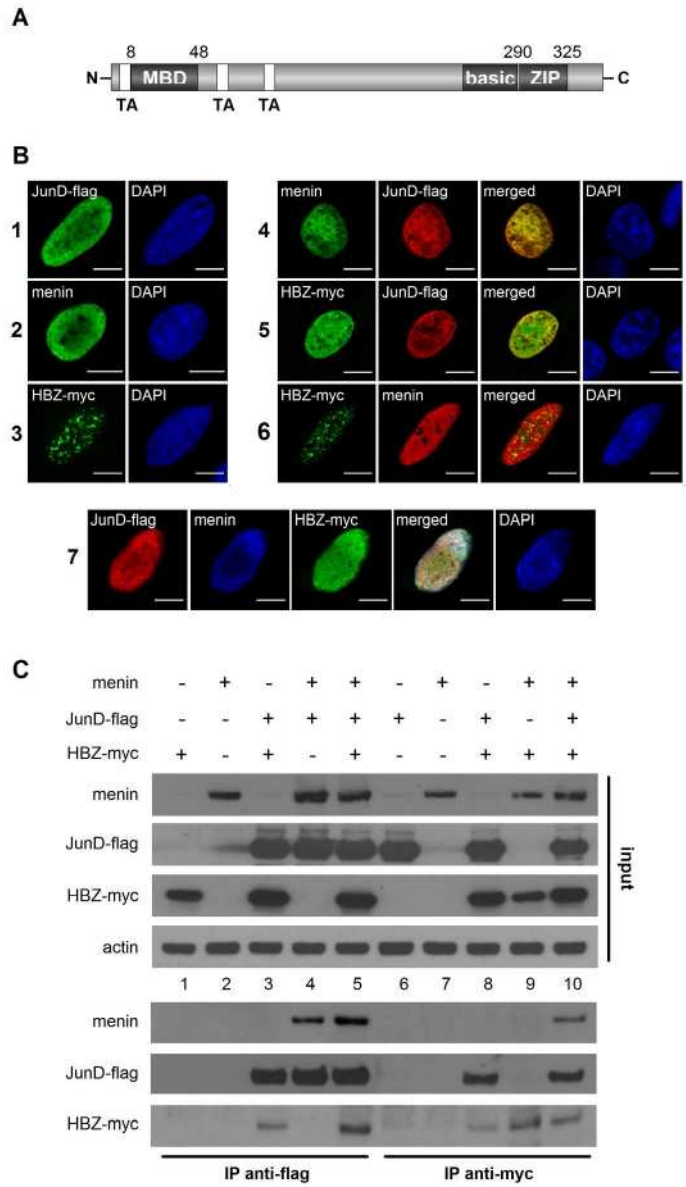

Figure 2
In vivo interaction between JunD, menin and HBZ proteins
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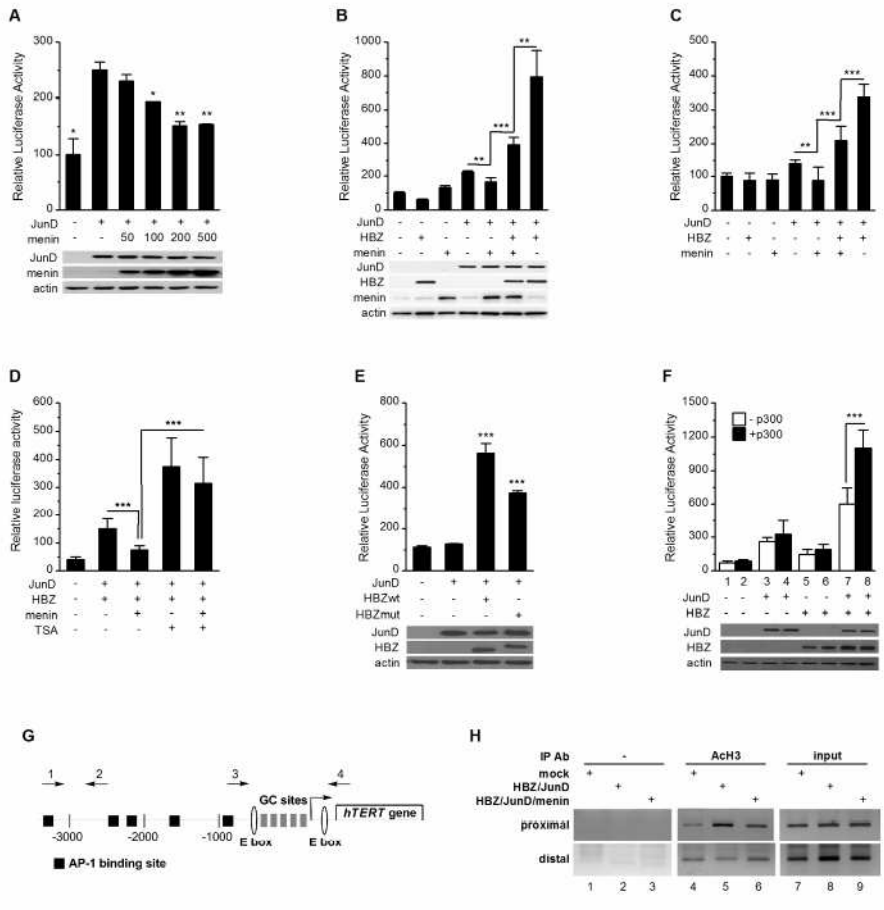

Figure 3.
Menin and HBZ exert opposite effects on the transcriptional activation of the hTERT proximal promoter
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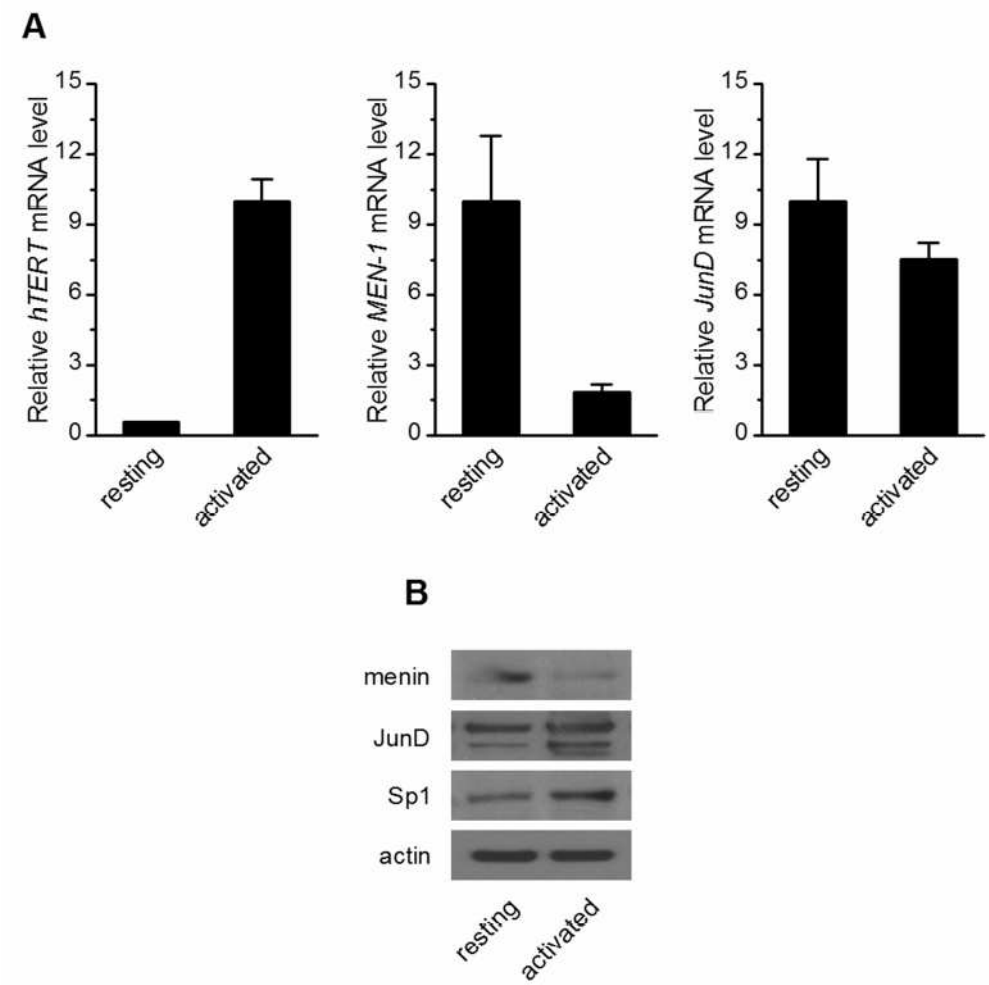

Figure 4.
Endogenous hTERT, Men-1 and JunD levels in T lymphocytes
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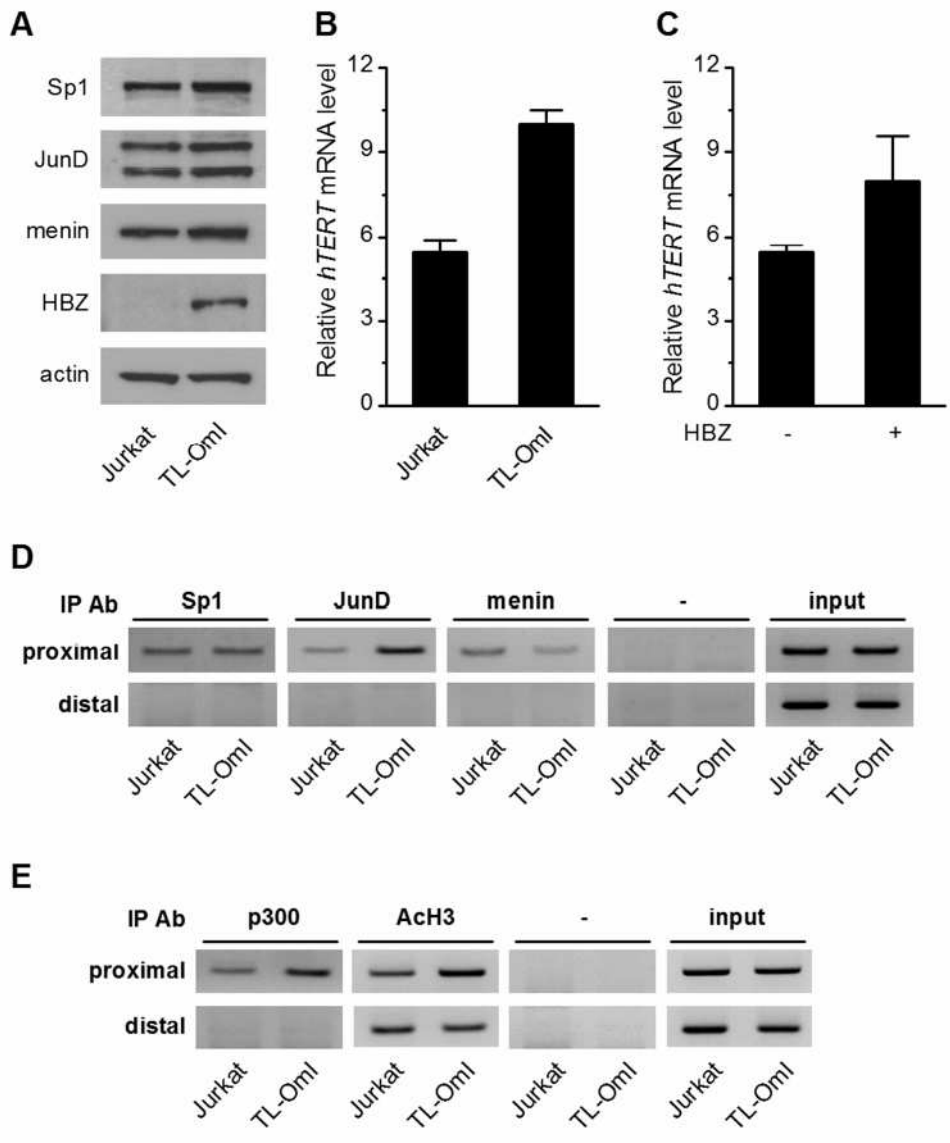

Figure 5.
Expression of HBZ stimulates hTERT expression in T lymphocytes
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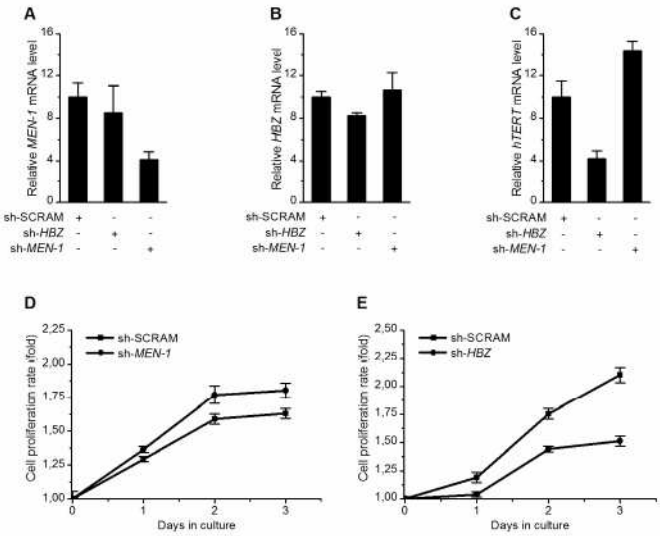

Figure 6.
Effect of Men-1 or HBZ knock-down in ATL cells
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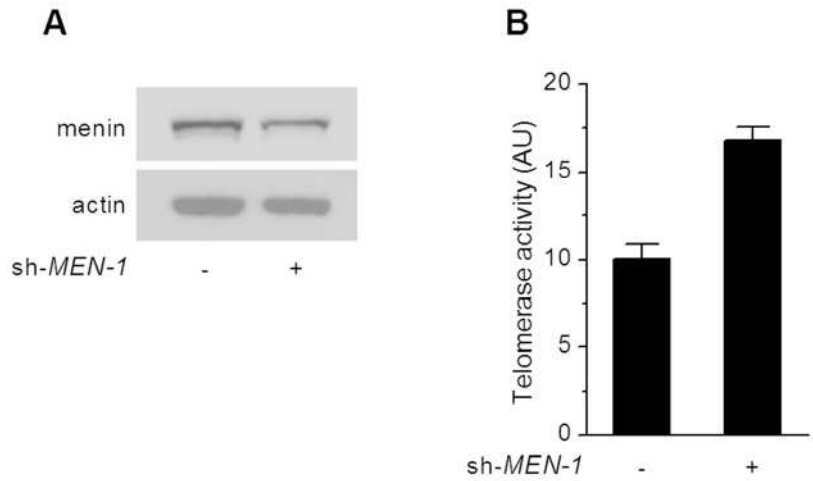

Figure S1.
Effect of Men-1 knock-down on endogenous telomerase activity in TL-OmI cells
87x46mm (300 x 300 DPI)

Additional file 2. Methods

Real-time quantitative telomeric repeat amplification protocol (RQ-TRAP)

Telomerase activity was assessed in TL-Om1 cells by RQ-TRAP, a modified version of the TRAP assay *. Cells transduced with *MEN-1* shRNA or the control one were lysed in 10mM Tris-HCl pH7.5 buffer containing 1mM $MgCl_2$, 1mM EGTA, 0.5% 3-((3-cholamidopropyl)-dimethylammonio)-1-propane-sulfonate, 10% glycerol, 0.1mM PMSF, 5mM β-mercaptoethanol. Cell extracts (0.1µg) were analyzed using LC-FastStart DNA Master SYBR Green I mix (Roche Diagnostics) and the following primers: TS (5'-AATCCGTCGAGCAGAGTT-3'), ACX (5'-GCGCGGCTTACCCTTACCCTTACCCTAACC-3'). After a 30min incubation at 30°C, a polymerase chain reaction was performed as follows: 10mn at 95°C and 40 cycles of 95°C for 30s, 60°C for 90s and 72°C for 8s, using the LightCycler (Roche Diagnostics). Telomerase activity was expressed in arbitrary unit (AU) per µg of cell extract relative to a standard calibration curve performed using protein lysate from 293T cells. Samples were serially diluted to verify the linearity of the RQ-TRAP reaction and RNAse-treated to check specificity of this assay.

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RETROVIROLOGY

MEETING ABSTRACT Open Access

HBZ impedes the Menin function and up-regulates the transcription of the hTERT gene in leukemic cells

Malgorzata Borowiak, Anne-Sophie Kuhlmann, Sophie Girard, Louis Gazzolo, Madeleine Duc Dodon*

From 15th International Conference on Human Retroviruses: HTLV and Related Viruses
Leuven and Gembloux, Belgium. 5-8 June 2011

Leukemic cells from Adult T-cell leukemia (ATL) patients display elevated telomerase activity, resulting mainly from transcriptional up-regulation of the human telomerase catalytic subunit (hTERT). We have previously shown that HBZ (HTLV-1 bZip) protein cooperates with JunD transcription factor to enhance hTERT expression after JunD anchoring to Sp1 bound to Sp1 sites within the hTERT proximal promoter. In normal somatic cells, telomerase expression is negatively regulated by tumor suppressor gene products, such as Menin, encoded by the multiple endocrine neoplasia type 1 (MEN-1) gene. Interestingly, the interaction of Menin with JunD has been shown to repress its transcriptional activity.

We report here that, in HBZ-expressing cells, Menin and HBZ exert opposite effects on JunD-mediated regulation of hTERT transcription. Chromatin immuno-precipitation as well as functional assays demonstrate that this antagonism is linked to the recruitment of p300 by HBZ and HDACs by Menin. Furthermore, knock-down of Menin in the ATL Tl-Om1 (only expressing HBZ) cells results in an increase of hTERT expression and of telomerase activity, whereas a knock-down of HBZ exerts opposite effects. Interestingly, primary leukemic cells isolated from ATL patients that express high amounts of hTERT transcripts, are also characterized by an elevated expression of HBZ and MEN-1 genes. Thus, in leukemic cells, HBZ behaves as a key factor impeding the Menin function and sustaining hTERT transcription. These findings underline the critical role of HBZ as a tumor-promoting protein during the development of the HTLV-1-induced leukemogenic process.

Published: 6 June 2011

doi:10.1186/1742-4690-8-S1-A182
Cite this article as: Borowiak *et al.*: HBZ impedes the Menin function and up-regulates the transcription of the hTERT gene in leukemic cells. *Retrovirology* 2011 **8**(Suppl 1):A182.

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