

**Analysis of enhanced retinoblastoma protein (pRb)  
degradation in HPV-positive cells after histone  
deacetylase inhibition**

Dissertation  
submitted to the  
Combined Faculties for the Natural Sciences and for Mathematics  
of the Ruperto-Carola University of Heidelberg, Germany  
for degree of  
Doctor of Natural Sciences

**presented by**  
**Handan Karaduman**  
**from Trabzon, Turkey**

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Diplom-Biologin      Handan Karaduman

born in:      Karlsruhe, Germany

Oral examination: .....

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inhibition**

Referees: PD. Dr. Dieter Kübler

Prof. Dr. Frank Rösl

## THE MILL, THE STONE, AND THE WATER

All our desire is a grain of wheat.  
Our whole personality is the milling-building.  
But this mill grinds without knowing about it.

The millstone is your heavy body.  
What makes the stone turn is your thought-river.  
The stone says: I don't know why we do all this,  
but the river has knowledge!

If you ask the river, it says,  
I don't know why I flow,  
All I know is that a human opened the gate!

And if you ask the person, he says:  
All I know, oh gobble of bread, is that if this  
stone stops going around, there'll be no bread for your bread-soup!  
All this grinding goes on, and no one has any  
knowledge!  
So just be quiet, and one day turn to God, and say:  
"What is this about bread-making?"

**Hz. Mevlana Celaleddin Rumi**

## INDEX

<b>Index</b> .....	<b>1</b>
<b>Abbreviations</b> .....	<b>5</b>
<b>Abstract</b> .....	<b>7</b>
<b>Zusammenfassung</b> .....	<b>9</b>
 <b>I. INTRODUCTION</b> .....	 <b>11</b>
1.1 Human papillomavirus (HPV) onfection can cause benign and malignant tumors on squamous epithelial cells .....	11
1.2 Cervical cancer is causally linked with HPV infection .....	11
1.3 Functional organization of the HPV Genome .....	12
1.4 HPV oncoprotein and their transformation capacity .....	13
1.4.1 The high-risk E6 oncoprotein and its biological activities .....	14
1.4.2 The high-risk HPV E7 oncoprotein and its biological activities .....	15
1.4.2.1 Control of the cell cycle and its disruption by high-risk HPV E7 .....	16
1.4.2.2 Binding of High-risk HPV E7 to tumor suppressor protein pRb abrogates the control for transition from G1 to S Phase during the cell cycle ...	18
1.5 The tumorsuppressor protein “Retinoblastoma Protein” .....	20
1.5.1 The tumor suppressor protein pRb and Cancer .....	20
1.5.2 pRb belongs to the pocket protein family and exerts different function .....	20
1.5.3 Regulation of the E2F transcription factors by pRb .....	22
1.5.4 Transcriptional repression by the pRb/HDAC/E2F complex .....	23
1.6 Chromatin regulation and the role of HDAC inhibitors .....	25
1.6.1 Chromatin regulation by HATs and HDACs .....	25
1.6.1 Inhibitors of HDAC molecules .....	26
1.6.3 HDAC inhibitors induced cellular effects .....	26
1.6.3.1 Influence of HDAC inhibition on gene transcription .....	27
1.6.3.2 HDAC and cell cycle .....	28
1.6.3.3 HDAC inhibitor induced apoptosis .....	28
1.6.4 HDAC inhibitors and treatment of cancer .....	29
 <b>Aims of this study</b> .....	 <b>32</b>

<b>II. MATERIALS AND METHODS</b>	<b>33</b>
<b>2.1 Materials</b>	<b>33</b>
2.1.1 Chemicals and Reagents	33
2.1.2 Laboratory equipment	35
2.1.3 Solutions and Buffers	36
2.1.4 Cell Lines	40
2.1.5 Antibodies	42
2.1.6 PCR primers	43
2.1.7 Plasmids	43
2.1.8 Oligonucleotids for Northern Blot analyses	44
2.1.9 Size markers for DNA/RNA electrophoresis	44
2.1.10 Kits	44
2.1.11 Others	45
<b>2.2 Methods</b>	<b>46</b>
2.2.1 Cell culture techniques	46
2.2.1.1 Cell culture	46
2.2.1.2 Thawing and freezing of eukaryotic cells	46
2.2.1.3 Cell Counting	46
2.2.1.4 Cell treatment with sodium butyrate, MG-132, TLCK	47
2.2.2 Preparation of DNA probes	47
2.2.2.1 Transformation of Component bacteria	47
2.2.2.2 Plasmid –DNA restriction analyses	47
2.2.2.3 DNA extraction from agarose gel (QIAquick gel extraction kit)	47
2.2.3 Protein analysis	48
2.2.3.1 Preparation of nuclear and cytoplasmic proteins	48
2.2.3.2 SDS-total protein extracts	49
2.2.3.3 SDS-Polyacrylamide gel electrophoresis	49
2.2.3.4 Western Blot “Semidry”	49
2.2.3 Nucleic acid analysis	50
2.2.4.1 RNA extraction (Absolutely RNA <sup>TM</sup> RT-PCR Miniprep Kit)	50
2.2.4.2 DNA extraction from eukaryotic cells	50
2.2.4.3 DNA restriction analysis	51
2.2.4.4 DNA electrophoresis and Southern Blot	51
2.2.4.4 RNA electrophoresis and Northern Blot	52

2.2.4.6 Probe labelling, “Random Priming” and hybridization .....	52
2.2.4.7 Reverse transcription and <i>Polymerase Chain Reaction</i> (PCR):	
Semi-quantitative RT-PCR .....	52
2.2.5 Transfection analysis .....	53
2.2.5.1 Stable transfection protocol “Effectene” .....	53
2.2.5.2 Stable transfection by Calcium-Phosphate co-precipitation .....	54
2.2.6 DNA staining with DAPI and SR 101 for flow cytometry and cell cycle	
analysis .....	55
<b>III. RESULTS .....</b>	<b>56</b>
3.1 Analysis of HPV-positive cell lines after HDAC inhibition .....	56
3.1.1 HDAC inhibitors induce pRb degradation only in the presence of HPV	
oncogenes .....	56
3.1.2 HPV E7 is selectively responsible for the degradation of pRb in the presence	
of HDAC inhibitors .....	58
3.1.3 Degradation releases negatively regulated genes from pRb suppression .....	59
3.2 The role of HPV E7 in pRb degradation by SB .....	61
3.3 pRb degradation induced by HDAC inhibitors is mediated by the 26S proteasome .....	64
3.4 Inhibitor of serine proteases, TLCK, modifies E7 proteins and inhibits its binding	
to pRb .....	65
3.4.1 Treatment of HPV16 E7-immortalised keratinocytes with TLCK also rescues	
pRb from its SB mediated degradation .....	68
3.5 HDAC inhibitors SB and TSA upregulate the cyclin dependent kinase inhibitor p21	
and induces G1 arrest in HeLa cells .....	70
3.5.1 Cell cycle analysis after HDAC inhibition .....	71
3.5.2 TLCK interferes with sodium butyrate induced G1 arrest .....	72
3.6 HDAC inhibitors SB and TSA induce apoptosis only in the presence of HPV .....	74
3.6.1 SB and TSA induce apoptosis in HPV positive cells via the proapoptotic	
gene p73 .....	77
3.6.2 TLCK treatment leads to a suppression of the HDAC inhibitor induce	
proapoptotic p73 transcription .....	78

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<b>IV. DISCUSSION</b> .....	<b>80</b>
4.1 HDACi mediated pRb degradation requires the presence of the HPV E7 .....	<b>80</b>
4.2 HDACi mediated pRb degradation increases the expression of the negatively regulated cyclin E .....	<b>84</b>
4.3 Physical association of pRb and E7 is important for the HDACi mediated degradation of pRb .....	<b>86</b>
4.4 Proteasomal degradation of pRb in HPV positive cells .....	<b>88</b>
4.5 HDACi mediated cell cycle arrest and induction of apoptosis in HPV positive cells .....	<b>90</b>
4.5.1 HDACi mediated cell cycle arrest .....	<b>90</b>
4.5.1 HDACi mediated apoptosis .....	<b>91</b>
 <b>Conclusion and future perspectives</b> .....	<b>95</b>
 <b>V. THANKS</b> .....	<b>97</b>
 <b>VI. REFERENCES</b> .....	<b>98</b>



**ABBREVIATIONS**

APS	Ammonium persulfat
ARF	Alternative reading frame protein, neighbour of p16
BSA	Bovine serum albumin
CDK	Cycline dependent kinases
cDNA	complementary DNA from RNA
CIA	Chloroform-Isoamylalcohol
CIN	cervical intraepitheliale neoplasia
CIP	Cyclin inhibitor protein
Cyc	Cyclin
dATP	deoxy-adenosine-triphosphat
dCTP	deoxy-cytidin-triphosphat
dGTP	deoxy-guanosin-triphosphat
dTTP	deoxy-thymidin-triphosphat
DEPC	Diethyl-pyrocarbonat
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E-64	N-N-(L-3-trans-carboxyoxirane-2-carboyl)-L-leucyl-aggmatine
ECL-Reagent	Enhanced chemiluminescence Reagent
EDTA	Ethlyendiaminetetraacetic acid
EGTA	Ethylen glycol-bis[ $\beta$ -aminoethylether]-N, N, N', N'-tetraacetic acid
FCS	Fetal Calf Serum
For	Forward
GAPDH	Glyceradehyde-3-phosphat dehydrogenase
HAT	Histone acetylase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPV	Human papillomavirus
HPV-E7	Human Papillomavirus oncoprotein E7
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
KIP	Kinase inhibitor protein, p21

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Kbp	Kilobasepair
KDa	Kilodalton
LB-Medium	Luria-Bertoni Medium
mM	Mili Molar
MG132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
Min	Minute
MOPS	3-[N-Morpholino] propanesulfonic acid
mRNA	Messenger RNA
OD	Optical density
PAGE	polyacrylamide gel electrophoresis
PARP	Poly (ADP-Ribose)-Polymerease
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pefabloc SC	4-(2-Aminoethyl)-benzolsulfonylfluorid
PVDF	Polyvinylidene fluoride
pRb	Retinoblastoma protein
Rev	Reverse
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolution per minute
RT	Reverse transcription
Sec	second
SDS	Sodium dodecyl sulphate
SB	Sodium butyrate
TEMED	N, N, N', N',-Tetramethylethylendiamine
TLCK	L-1 Chlor-3-(4-tosylamido)-7 amino-2-heptanonhydrochlorid
TPCK	L-1 Chlor-3-(4-tosylamido)-4- phenyl-2-butanon
t-RNA	Transference RNA
TNF- $\alpha$	Tumor necrosis factor – alfa
Tris	Tris (hydroxymethyl)-aminomethane
TSA	Trichostatin A
U	Units
% (v/v)	Volume percentage
% (w/v)	Weight percentage

## ABSTRACT

Human papillomaviruses have been identified as the major aetiological factor in cervical carcinogenesis. Constitutive expression of the high-risk HPV E6 and E7 oncoproteins are important for the malignant transformation during the course of infection. In particular, E6 binds to p53 and E7 binds to pRb to mediate their proteasomal degradation which is a crucial step for the HPV procured cell cycle progression. In order to analyse the biological outcomes in context of the HPV transformed cells, anti-tumour agents known as HDAC inhibitors (HDACi) were used.

In the work presented here, the interest was precisely focused to analyse the role of E7 in tumour suppressor protein pRb degradation following HDAC inhibition. Initial observation in HPV18 positive cervical carcinoma cells HeLa and SW756, demonstrated an E7 dependent degradation of pRb upon SB and TSA. This observation was confirmed further by using the HPV negative cervical cancer cells C33-A and HT-3. Furthermore, to prove whether tumorigenic phenotype was a critical determinant, the effect of HDACi on the Stanbridge cell system (non-tumorigenic 444 and the tumorigenic CGL-3 cells) was analysed. No discernible difference in pRb degradation was observed between the different cell segregates. Moreover the use of the individual E6 and E7 immortalised keratinocytes significantly demonstrate the functional link between E7 and pRb degradation.

The pRb protein is able to repress the transcription of the cell cycle regulatory proteins (cyclin E, DNA polymerase- $\alpha$ , dihydrofolate reductase (DHFR) etc.), involved in the G1 to S phase by binding the transcription factor E2F and recruiting the HDAC1 molecules to their promoters. To investigate whether pRb degradation was accompanied with the upregulation of the key molecules involved in G1 to S transition the protein level of cyclin E was examined. Western Blot analysis was performed in HeLa, 444, CGL3 cells and in E6- and E7- immortalised keratinocytes. Essentially, cyclin E upregulation was observed while the E2F protein level was unchanged in HPV 18 containing cells and in E7 immortalised keratinocytes. These results demonstrate that the presence of E7 oncoprotein is a prerequisite for pRb degradation upon HDAC inhibition. In order to establish more functional link between E7 and pRb degradation upon HDAC inhibition, two experimental approaches were used. At first, the E7 oncoprotein was knocked down by using siRNA in the HeLa cells. Secondly, E7 oncoprotein was introduced in TE-671 which are HPV negative, but pRb positive cells. In both systems, we observed that E7 has an indispensable role on pRb degradation upon HDACi treatment.

In order to dissect the role of the individual oncoprotein in HDACi induced pRb degradation, E6 and E7 immortalised keratinocytes were used. Treatment of the cells with proteasome-inhibitor MG132 (2  $\mu$ M, 16 h) shows that the degradation of pRb occurs via 26S proteasome pathway in E7 immortalised keratinocytes.

In order to accentuate the importance of the direct interaction between E7 and pRb, a serine protease inhibitor TLCK was used. In particular, modification of the E7 protein by using the TLCK, rescued pRb from HDACi induced degradation. Furthermore, cyclin E which is negatively regulated by pRb was downregulated again. These results emphasized the importance of the physical association between

E7 and pRb and the crucial role of the LXCXE motif of E7 protein. In addition, co-treatment of SB with TLCK interferes with the SB mediated G1 arrest, which was shown by the cell cycle distribution analysis and downregulation of the cyclin-dependent kinase inhibitor p21. Furthermore, p21 is activated in response of HDACi which is responsible for the inactivation of the cyclin/CDK complexes in G1 phase.

In order to analyse the HDACi induced apoptosis in the context of HPV, Hela cells were treated with SB and TSA and apoptotic cells were analysed by flow cytometry. Consistently, HeLa cells expressed apoptotic markers within at 24 hours while the HPV negative cervical cancer cells C33-A were not sensitive for apoptosis in response to HDACi treatment. Furthermore this confirmed the crucial role of the E7-pRb interaction for the HDACi mediated apoptosis. HDACi induced cell death mainly by activating the intrinsic apoptotic pathway. In order to address the target apoptotic gene expression in the HPV context and individual oncogenes, Hela cells and the E6- and E7-immortalised keratinocytes were used. After treatment with SB, RT-PCR (real-time) analyses were performed for p73 expression. The E2F mediated apoptotic protein p73 was upregulated in response to HDACi treatment. This effect was reversed by TLCK treatment in HeLa as well as in E7 immortalised keratinocytes. Thus p73 transcription was reduced in HeLa cells as well as in E7 immortalised keratinocytes. These data confirm the presence of HPV E7, which sensitizes the cells for SB mediated apoptosis, and which can be reversible by posttranslational modification of the HPV E7. Finally the HDAC inhibitor mediated apoptosis can be inhibited by TLCK. Subsequently this study bears new future aspects in understanding the role of serine proteases during HDAC inhibition.

## ZUSAMMENFASSUNG

In der Ätiologie der Zervixkarzinome spielt die Infektion mit „high-risk“ Typen humaner Papillomviren eine entscheidende Rolle (zur Hausen, 1996). Die konstitutive Expression der viralen Onkogene E6 und E7 und deren Interaktionen mit Regulatoren des Zellzyklus sind wichtig bei der malignen Transformation der Wirtszellen. Insbesondere die proteasomale Degradation des Tumorsuppressorproteins p53 durch E6 und die Degradation von pRb durch Interaktion mit E7, zeichnen sich als eine wichtige Stufe im Rahmen der HPV vermittelten Deregulation des Zellzyklus dar. Diese Gruppe von antitumor Agenzien zeichnen sich durch potenziellen Inhibierung der Zellzyklus- Progression, Differenzierung und/oder von Apoptose in Tumorzellen aus, während normale humane Zellen eher moderat beeinflusst werden.

In der vorliegenden Arbeit wurde im besonderem die Rolle von HPV E7 bei der HDAC inhibitoren induzierten Degradation des Tumorsuppressor Proteins pRb untersucht. Die ersten Ergebnisse mit den HPV18 positiven HeLa und SW756 Zellen zeigten, dass die Präsenz von HPVE7 eine Rolle bei der HDAC-Inhibitoren induzierten pRb Degradation spielt. In weiteren Untersuchungen zeigten die HPV negativen Zervixkarzinom Zellen C33-A und HT-3 keine pRb Degradation. Des weiterem wurde festgestellt, dass der tumorigene Phänotyp keine Rolle in diesem Kontext spielt, indem das Stanbridge System verwendet wurde, welches aus der Fusion von HeLa und humanem Fibroblasten, den 444 und deren tumorigene Subklonen CGL-3 besteht. Im besonderen zeigte die Behandlung von Keratinozyten, welche individuell mit E6 und mit E7 Onkogenen transfiziert worden sind, dass nur in E7 immortalisierten Keratinozyten eine pRb Degradation Zustande kam, während in E6 Keratinozyten nur hypophosphoryliertes pRb zu beobachten war. Das pRb ist unter anderem auch zuständig für die transkriptionelle Regulation der regulatorischen Proteine der G1 Phase durch direkte Bindung an den E2F Transkriptionsfaktor. Im Rahmen der HDACi Behandlung war es daher wichtig zu untersuchen, ob die von pRb negativ regulierten Proteine wie Cyclin E hochreguliert werden konnten, wenn pRb degradiert war. Western blot Analysen zeigten, dass dies in HPV 18 positiven und E7 immortalisierten Keratinozyten der Fall war, während die Proteinmenge von E2F unverändert blieb. Dies bestätigte des weiteren, dass die E7 Präsenz eine Voraussetzung für die HDAC inhibitoren vermittelte pRb Degradation ist. Weiterhin wurde durch Behandlung mit dem Proteasom-Inhibitor MG132 bewiesen, dass die Degradation von pRb bei der HDACi- Behandlung zum größten Teil über das 26S Proteasom System durchgeführt wird und das E7- Protein in diesem Zusammenhang eine direkte Funktion übernimmt. Um unsere Hypothese zu bekräftigen, wurden zwei Ansätze verfolgt. Zuerst wurden HeLa Zellen mit einer siRNA die gegen HPV18E7 gerichtet ist transfiziert. Obwohl dadurch keine bemerkenswerte Hochregulierung von pRb beobachtet wurde, konnte man feststellen dass eine hypophosphorylierte pRb Bande nach SB Behandlung entstand. Als nächstes wurde in HPV negativen aber pRb positiven Rhabdomyosarcoma Zellen (TE-671), HPV E7 eingeführt. Die Behandlung mit SB zeigte dass nach Einführen des E7 Onkoproteins eine gesteigerte Degradation von pRb zu beobachten war.

Um zu beweisen dass die direkte Interaktion zwischen E7 und pRb eine entscheidende Rolle für die beobachtete HDACi vermittelte Degradation ist, wurden HeLa Zellen zusammen mit dem Serineprotease Inhibitor (TLCK) mit SB behandelt. Die Ergebnisse zeigten, dass TLCK die SB vermittelte Degradation zum größten Teil aufheben konnte und das von pRb negativ regulierte Cyclin E abnahm. Zudem konnte auch gezeigt werden, dass TLCK mit dem von SB verursachten G1 Arrest interferieren kann, der ab 16 Stunden Behandlung zustande kam. Diese Beobachtung konnte durch Western Blot Analyse von p21 bestätigt werden, die eine Abnahme von p21 unter TLCK Behandlung zeigten. Der HDACi vermittelte G1 Arrest wird im wesentlichen dadurch verursacht, dass p21 auf der transkriptionellen Ebene aktiviert wird. Längere Behandlungen mit HDACi, beginnend ab 24 Stunden zeigten einen apoptotischen Phänotyp in HeLa Zellen, während die HPV negativen Zervixkarzinom Zellen C33-A sich nicht apoptotisch verhielten. Das bekräftigte die Voraussage, dass die Präsenz von HPV die Zellen zu HDACi induzierter Apoptose begünstigen. Insbesondere hebt dieses die wichtige Interaktion zwischen pRb und E7 hervor. Die von HDAC Inhibitoren verursachte Apoptose wird in den meisten Fällen durch den mitochondrialen Weg vermittelt. Um auf der molekularen Ebene festzustellen welche Gene die diesen Mechanismus einleiten können, auf Grund der aufgehobenen pRb Repression aktiviert werden, wurde die transkriptionelle Untersuchung des proapoptotischen proteins p73 nach Behandlung mit HDACi analysiert. Hierfür wurden HeLa und die mit den Onkogenen E6 und E7 immortalisierten Keratinozyten eingesetzt. Die Ergebnisse zeigten, dass p73 in HeLa und in E7 Keratinozyten induziert wurde, während in E6 Keratinozyten eine moderate Hochregulierung zu sehen war. Interessanterweise wurde diese Transkription nach der Inkubation zusammen mit TLCK reduziert. Diese Daten belegen, dass E7 die HPV positiven Zellen für HDAC Inhibitor vermittelte Apoptose sensibilisieren kann, welches durch posttranslationale Modifikation von E7 aufgehoben wird. Darüber hinaus zeigen diese Ergebnisse dass die Serinproteasen eine wichtige Rolle bei HDAC Inhibitor vermittelten Effekten haben können.

## I. INTRODUCTION

### 1.1 Human papillomavirus (HPV) infection can cause benign and malignant tumors on squamous epithelial cells

Human papillomviruses (HPVs) are small DNA tumor viruses that cause benign and malignant tumors of squamous epithelia. So far more than 100 HPV subtypes have been identified and all of them appear to be strictly epitheliotropic: They infect epithelial cells of the skin or of the anogenital and oropharyngeal mucosa (Aguilar-Lemarroy et al. 2002) zur Hausen, 1999 and 1996).

Approximately 40 HPV types that infect the genital tract fall into two discrete groups:

-*Low-risk*, non-oncogenic HPV types 6 and 11 and their relatives causes anogenital warts (condylomata accuminata) and are rarely detected in malignant disease (Boshart and zur Hausen 1986; zur Hausen, 2000).

-*High-risk*, oncogenic HPV (hrHPV) types 16, 18, 31, 33, 35, 45, 52, 56 plus about eight other minor types (zur Hausen, 2000 and 2002).

These can be detected in almost 100% of cervical cancer biopsies and more than 90% of the high-grade, cervical intra-epithelial neoplasia (CIN2/3) precursor lesions. HPV 16 is the most frequent detected HPV in cervical cancers (about 50–60%) with HPV 18 (10–12%) being the second most common (Stanley et al., 2004).

### 1.2 Cervical cancer is causally linked with HPV infection

Carcinomas of the anogenital tract, particularly cancer of the cervix, account for almost 12 % of all cancers in woman and represent the second most common gynaecological malignancy in the world. Almost all squamous cell carcinomas and a vast majority of adenocarcinomas of the cervix are HPV positive (Tjalma et al., 2005; zur Hausen 2002; Bosch et al., 2003a and 2003b).

The development of cervical cancer has been linked to persistent infection with high-risk HPVs and is generally preceded by a long latency period. However, only a minority of woman infected with HPVs develop cancer. These observations strongly suggest that accumulation of host genetic changes and predisposing host factors play a central role in malignant progression of high-risk HPV associated lesions (Stanley et al., 2004).

Infection with the high-risk HPV type -16 and -18, results initially in unobtrusive squamous intraepithelial lesions (SIL) in women. Most of the lesions are cleared 6-12 months after appearance, possibly due to the host immunological defence. A small percentage progresses to

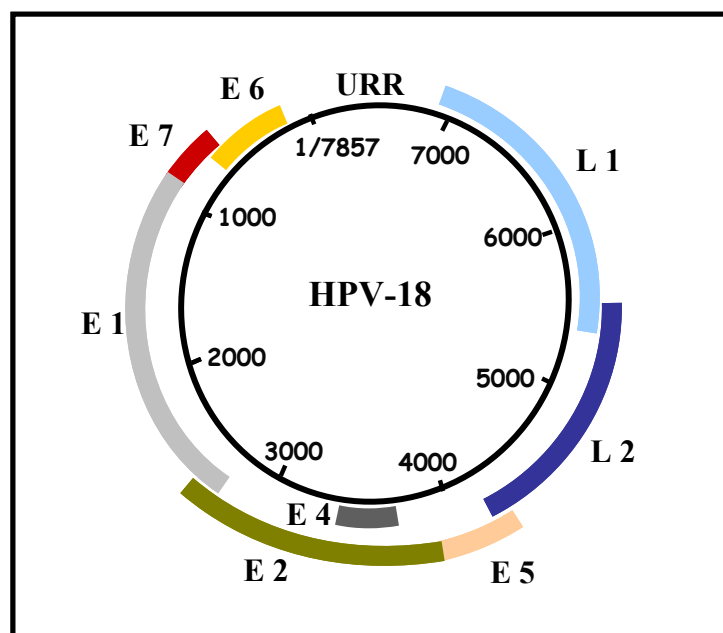
high-grade SIL (HSIL), carcinoma *in situ* and without surgical interference to squamous-cell carcinoma or adenocarcinoma of the cervix (zur Hausen 2000 and 2002).

### 1.3 Functional organization of the HPV Genome

Papillomaviruses are a group of small non-enveloped viruses with 55 nm diameter icosahedral capsids. The supercoiled, circular DNA of human papillomavirus (HPV) genome contains between 6800 and 8000 base pairs and can be divided into three major regions: early, late and long control region (LCR) (zur Hausen et al., 2002).

**Figure-1.1: The organization of circular HPV 18 DNA**

Schematic overview of genome organization of HPV-18. E1-E7 are coding for the ‘early’ (E) genes. L1 and L2 coding for the ‘late’ (L) genes. Viral transcripts, uniformly span the E6 and E7 region, and are often linked to flanking cellular sequences. A non-coding 800-bp fragment, called upstream regulatory region (URR) or long control region (LCR), is located upstream of E6 gene, and controls both viral transcription and DNA replication (Cole and Danos, 1987).



These three regions are separated by two polyadenylation (pA) sites: early pA (AE) and late pA (AL) sites (Zheng and Baker, 2006). The early region of the papillomavirus genome encodes six open reading frames (ORF), (E1, E2, E4, E5, E6 and E7), that translate individual proteins for ‘early’ (E) functions. The late region of all HPV genomes, encodes L1 and L2 ORFs, for translation of major (L1) and minor (L2) capsid protein. The LCR contains the origin of replication (ori) and numerous *cis* and *trans* elements that are necessary to regulate viral transcription and replication. A notable feature of all papillomavirus genes transcripts is that they are transcribed as a bicistronic or polycistronic mRNA containing two or more ORFs which are polyadenylated at either an early or late poly (A) site (Desaintes and Demeret, 1996; Zheng and Baker, 2006).



Papillomaviruses replicate and assemble exclusively in the nucleus. Papillomaviruses infect the basal keratinocytes basal layers of the stratified squamous epithelium. In the course of cancer development, the viral molecule frequently is integrated into host-cell DNA.

This integration is accompanied with disruption of the E2 coding region, resulting in an uncontrolled E6/E7 expression. The viral transcripts, which uniformly span the E6 and E7 region, and are often linked to flanking cellular sequences, are present and transcription might be modulated (enhanced) by flanking host-cell promoters (Zheng and Baker, 2006; Barksdale et al., 1993; zur Hausen, 2002).

#### **1.4 HPV oncoprotein and their transformation capacity**

The high-risk HPVs associated with malignant progression are capable of transforming a variety of cells in tissue culture, including primary human squamous epithelial cells.

A number of cell lines have been established from cervical carcinoma tissue samples. A high percentage of these cells have been shown to contain HPV16 or HPV18 DNA which is generally transcriptionally active (Werness et al., 1991; Howley et al., 1991a and 1991b).

One of the key events of HPV induced carcinogenesis is the integration of the HPV genome into a host chromosome. HPV genome integration occurs near common fragile sites of the human genome (Thorland et al., 2003). This integration leads to constitutive expression of E6 and E7 oncoprotein, whereas other portions of the viral DNA are deleted or their expression is disturbed. For example, loss of the HPVE2 transcriptional repressor is significant, and results in deregulated HPV E6 and E7 expression. Cells that express E6 and E7 from integrated HPV sequences have a selective growth advantage compared to cells with episomal HPV genomes (Jeon et al., 1995).

High-risk HPVs encode for at least three proteins with growth-stimulating and transforming properties: E5, E6, and E7 oncoprotein. E5 protein appears to be important in the early course of infection and is expressed in productive infections. The E5 region encodes a hydrophobic protein which is preferentially found in Golgi apparatus and plasma membrane. It stimulates cell growth by forming a complex with the epidermal-growth factor receptor, the platelet-derived growth-factor- $\beta$  receptor, and the colony-stimulating factor-1 receptor (DiMaio and Mattoon, 2001). In addition, it has been shown to prevent apoptosis due to DNA damage. After integration of the viral genome into the host cell chromosome the open reading frame coding for E5 is frequently deleted in cervical carcinoma cells. This demonstrates that E5 is not necessary to maintain the transformed phenotype (Genther et al., 2003; Münger et al., 2004).

A more significant role for malignant transformation can be assigned to E6 and E7 proteins (Münger and Howley, 2002). E6 and E7 are consistently expressed in malignant tissue culture and inhibiting their expression blocks the malignant phenotype of cervical cancer cells (zur Hausen, 2002; Münger et al., 2004). The oncogenic potential of human papillomaviruses (HPVs) is reflected *in vitro* by their ability to immortalize keratinocytes and to change their differentiation behaviour (Schlegel et al., 1988). They are independently able to immortalize various human cell types in tissue culture, but efficiency is increased when they are expressed together (Hawley-Nelson et al., 1989; Watanabe et al., 1989; Münger et al., 1989a; McDougall et al., 1994).

#### 1.4.1 The high-risk E6 oncoprotein and its biological activities

The full length HPV E6 genes encode an approximately 160 amino acid protein (18 kDa) and contain two domains consisting of Cys-XX-Cys-X<sub>29</sub>-Cys-XX-Cys, which are expected to be involved in zinc binding (Barbosa et al., 1989; Grossman and Laimins 1989). E6 is involved in the viral life cycle, blocks apoptosis, alters the transcription machinery disturb cell-cell interactions and increases life span of the cells (Münger and Howley, 2002). It binds physically to p53, CBP/p300, E6 associated protein (E6-AP), E6 binding protein (E6-BP), paxilin, Bak, Tyk 2 and interferon regulatory factor-3 (IRF-3) (Chakrabarti and Krishna, 2003).

Indeed, some of the prominent functions of the E6 protein originate from its interaction and degradation of p53 (Werness et al., 1990; Howley et al., 1991b) and the pro-apoptotic protein BAK (Jackson et al., 2000), which results in resistance to apoptosis and an increase in chromosomal instability. p53 is tumor suppressor protein (Vogelstein et al., 1990) which is induced in response to cellular stress such as UV-irradiation, hypoxia or viral infections while p53 expression can also be increased by posttranslational stabilization, leading to arrest in G1 (Kuerbitz et al., 1992). The p53-G1 block enables the repair of damaged DNA before proceeding in the cell cycle (Lane et al., 1992). p53 activation can stimulate the expression of the genes involved in cell cycle and apoptosis, such as of the cyclin-dependent kinase inhibitor p21<sup>WAF/CIP</sup>. E6 mediated degradation of p53 (Scheffner et al., 1990) involves the cellular protein E6-AP (Huibregste et al., 1993) which acts as an E3 ligase (Scheffner et al., 1993). Only in the presence of the E6 protein E6-AP can degrade p53; resulting in uncontrolled proliferation. p14<sup>ARF</sup>, an alternate gene product of p16<sup>INK4a</sup>, described as a tumor suppressor, induces p53-dependent cell cycle arrest causing elevated levels of p21 (Stott et al., 1998). E6 can overcome this arrest signal in a p53 dependent mechanism that involves the repression of the cyclin B and cdc2 promoters (Passalaris et al., 1999). E6 has been implicated to prevent apoptosis also in a p53 independent

pathway via binding the Bak protein, a proapoptotic member of the Bcl-2 family. The degradation of the Bak protein via the proteasome involves also the recruitment of the E6-AP (Thomas and Banks et al., 1998 and 1999).

HPV E6 is capable of altering the transcription of target promoters in a positive or negative manner such as the HPV early promoter in the upstream regulatory region (URR) (Gius et al., 1988). It can also bind to and inhibit the transcriptional co-activators like CREB-binding protein (CBP) and p300, thereby abrogating the transcriptional activation of p53 (Patel et al., 1999; Zimmermann et al., 1999). Furthermore E6 can stimulate the transcription of VEGF, (the most important inducer of angiogenesis) (Lopez-Ocejo et al., 2000) due to the positive impact on the activity of other transcription factors such as Sp-1 (Gille et al., 1997).

Furthermore the high risk HPVE6 contain a carboxyl terminal PDZ domain binding protein motive X-(S/T)-X-(V/I/L)-COOH and can bind a number of cellular PDZ domain containing proteins, such as hDLG (human homologue of the drosophila melanogaster tumor suppressor protein), MAGUK (member of membrane associated guanylate kinase) etc. (Kiyono et al., 1997; Nakagawa and Huibregste 2000) and promotes their degradation by E6-AP recruitment (Gardioli et al., 1999; Glaunsinger et al., 2000). PDZ containing proteins are involved in negatively regulating cellular proliferation. It is conceivable that interaction with PDZ proteins may contribute to the transforming activities of high-risk HPV E6 proteins.

In addition, the activation of telomerase and the postulated inhibition of degradation of Src-family kinases by the E6 oncoprotein seem to fulfil important functions in growth stimulation (Veldman et al., 2001; Oda et al. 1999). It is speculated that the stabilization of the activated forms of specific members of the SRC family of kinases could contribute to the HPV-transformed phenotype (Oda et al., 1999).

#### **1.4.2 The high-risk HPV E7 oncoprotein and its biological activities**

HPV E7 proteins are low-molecular-weight proteins of approximately 100 amino acids and predominantly localised in the nucleus (Greenfield 1991). Like other oncoproteins encoded by small DNA viruses, they associate and modify the function of cellular protein complexes.

The amino terminal domain of HPV E7 is composed of two conserved region: (CR1 and the CR2 which have sequence similarity to a small portion of adenovirus E1A and with SV40 T (Smian Virus-40 T antigen). The HPV E7 carboxyl terminus contains a zinc-binding domain consisting of two copies of a Cys-XX-Cys motif which are separated by a 29-amino-acid spacer acting as a dimerisation/multimerisation domain (Clemens et al., 1995) (Represented in result Figure 3.13).

HPV E7 proteins are phosphoproteins (Smotkin and Wettstein 1987) and contain a casein kinase II consensus phosphorylation site in their amino terminal site (Barbosa et al., 1990). An additional carboxyl terminal phosphorylation site has been described but the protein kinase that targets this site is unknown (Massimi and Banks, 2000).

The CR2 region contains a conserved LXCXE sequence which interacts with the retinoblastoma tumor suppressor protein pRb and the related “pocket proteins” p107 and p130 (Dyson et al., 1989 and 1992). These proteins interact with the transcription factor E2F, which is able to regulate cell cycle transition (Chellapan et al., 1991; Hiebert et al., 1992; Zhu et al., 1995; Cam et al., 2003). The association between E2F and pocket proteins prevents the binding of E2F to target promoter sequences and therefore represses genes involved in cell cycle progression and DNA replication (Macaluso et al., 2005).

This interaction between E7 and pRb is critical for the maintenance of the transformed phenotype while high-risk HPV E7 has a higher affinity to pRb than low-risk HPV E7 protein (Münger et al., 1989b; Gage et al., 1990). Several studies have demonstrated that disruption of the E7-pRb interaction is accompanied by a loss of cellular transformation (Barbosa et al., 1990; Heck et al., 1992; Sang and Barbosa, 1992).

In addition to pRb binding and degradation, E7 has other cellular target proteins, including transcription factors, cell cycle regulators, and metabolic enzymes. The interaction with these proteins need the carboxyl-terminal domain (Münger et al., 2001) which contributes to association with chromatin-modifying enzymes, such as histone deacetylases and histone acetyl transferases (Brehm et al., 1999a). E7 interacts with the transcriptional co-activators p300, CBP, and pCAF (Huang and McCance, 2002; Avvakumov et al., 2003; Bernat et al., 2003).

#### **1.4.2.1 Control of the cell cycle and its disruption by high-risk HPV E7**

Cell cycle control is regulated by the activity of cyclin-dependent kinases (CDKs) and their activating coenzymes, cyclins. The activity of CDKs (CDK1, CDK2, CDK4, CDK6) is regulated by the abundance of their activating partner cyclins (Cyclins A, B, D, E), phosphorylation by various kinases and interaction with CDK inhibitory proteins (CDKIs) (Sherr et al., 1995a; Beijersbergen and Bernards 1996). Two classes of mammalian cyclin-dependent kinase inhibitors have been described: The CIP/KIP family, comprised p21, p27, and p57, and the INK4 family, comprised of p15, p16, p18, and p19 (Sherr and Roberts, 1995b). Generally CDKs, cyclins, and CDK inhibitors function within several pathways, including the *p16<sup>INK4A</sup>-cyclin D1-CDK4/6-pRb-E2F*, *p21<sup>WAF1</sup>-p27<sup>KIP1</sup>-cyclin E-CDK2*, and *p14<sup>ARF</sup>-MDM2-p53* pathways (Kim and Zhao, 2005). The INK4 molecules specifically inhibit cyclin D complexes by interaction with

CDK4 and CDK6 components. The KIP family is affecting cyclin E, cyclin A/CDK2, and cyclin B/CDK1 by binding both the cyclin and CDK subunit. Alteration in CDKs, CDKIs, and cyclins can lead to uncontrolled proliferation and might contribute to malignant of the uterine cervix (Sherr and Roberts, 1995b).

In many cancers and cancer cell lines the mechanisms which control the growth and differentiation mechanism are disrupted or at least impaired (Sherr et al., 1996). Considering that HPV infected and HPV E6/E7 oncogene expressing cervical cancer cells display aberrant regulation of the cell cycle, due to interaction with cell cycle regulatory proteins, such as cyclin A, cyclin E, p16, p21 and p27 is a characteristic feature (Kim and Zhao, 2005; Tommassino et al., 1995).

In a dividing cell, mitogenic stimulation leads to synthesis and assembly of cyclin D/CDK4 complexes, which contribute to the phosphorylation and consequent inactivation of pRb, increased expression of cyclin E, sequestration of cdk2 inhibitors of the Cip/Kip family. Cyclin E/CDK2 continues to inactivate Rb and also phosphorylates substrates important for DNA synthesis and S phase entry. CyclinA-CDK2 is assembled during S phase and remains active through G2 phase (Sherr et al., 1993; Sherr and Roberts, 1999).

E7 affects these cyclin-CDK complexes by deregulating transcriptional levels of cyclin E due to loss of E2F mediated repression; but also by affecting the posttranscriptional levels of cyclin E (Zerfass et al., 1995; Botz et al., 1996; Martin et al., 1998). E7 protein has been shown to bind to cyclin A-cdk2 in a cell cycle dependent manner, with maximal activity in the S and G2 phases (Tommassino et al., 1993).

The cyclin A-CDK2 interacts and phosphorylates E2F, leading to its loss of DNA binding capability. Hence interaction of E7 with cyclin A/CDK2 prevents the inactivation of E2F and thereby permitting the cell to bypass normal checkpoints with consequent loss of DNA replication fidelity, explaining increased chromosomal abnormalities (White et al., 1994).

HPVE7 can override growth-inhibitory activities of cyclin dependent kinase inhibitors, including p21<sup>CIP1/WAF1</sup> and p27<sup>KIP1</sup> (Jones et. al., 1997; Zerfass-Thome et al., 1996). Since these proteins are critical regulators of cell cycle arrest during keratinocyte differentiation (Missero et al., 1996), their inhibition by E7 may also contribute to the maintenance of a replication-competent cellular milieu in differentiated host epithelial cells (Cheng et al., 1995). A carboxyl-terminal E7 domain that does not contribute to pRb binding and/or degradation is necessary for the ability of E7 to override p21-mediated growth arrest (Helt et al., 2002). The association with p21 abrogates its activity to inhibit cyclin/CDK activity as well as PCNA-dependent DNA replication (Funk et al., 1997; Jones et al., 1997). Since p27 has been implicated as a mediator of cellular growth

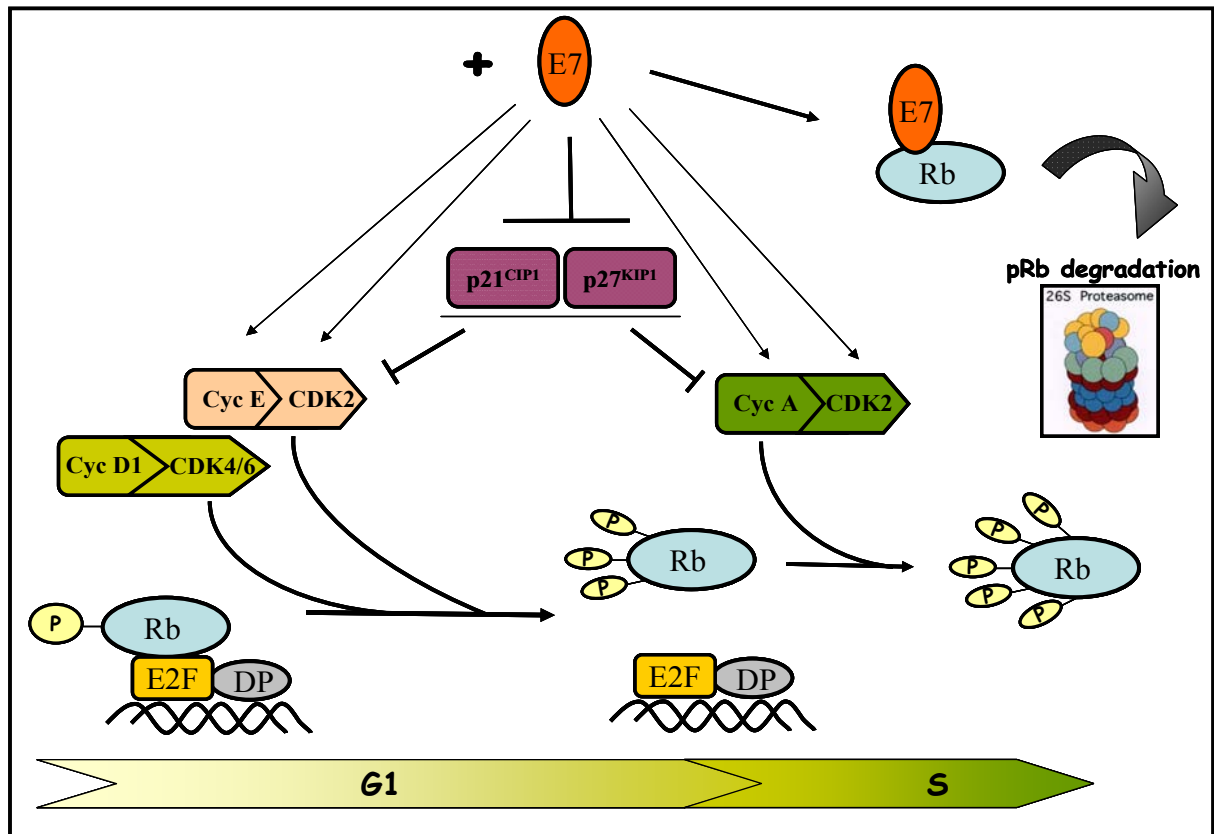
inhibition by TGF- $\beta$  in keratinocytes, this activity may contribute to the ability of cellular growth inhibition by TGF- $\beta$ -associated growth arrest (Pietenpol et al., 1990).

E7 oncoprotein mediated degradation of pRb releases the transcription factor E2F from pRb inhibition and upregulates p16<sup>INK4</sup> (Dyson et al., 1989; Kiyono et al., 1998). The resulting high E2F activity might lead to apoptosis in E7-expressing cells. The upregulation of p16<sup>INK4</sup> counteracts the E6-mediated growth stimulatory function by inactivation of the D-type CDK activity (Khleif et al., 1996). Due to direct activation of cyclin A and cyclin E it can bypass this inactivation (Zerfass et al., 1995).

#### **1.4.2.2 Binding of High-risk HPVE7 to tumor suppressor protein pRb abrogates the control for transition from G1 to S phase during the cell cycle**

The tumor suppressor protein pRb is differently phosphorylated during the cell cycle. It is present in a hypophosphorylated form during the cell cycle phase G0/G1, at the G1/S boundary it is hyperphosphorylated and during late G1, S, G2 and during M phase (Cooper et al., 2001). pRb is reconverted to the hypophosphorylated state by specific phosphatases (Ludlow et al., 1993; Tamrakar et al., 2000). In-vivo experiments have been shown that hyperphosphorylation of pRb occurs on 13 of 16 potential CDK phosphorylation site (Mittnacht et al., 1994; Ezhevsky et al., 1997 and 2001). Hypophosphorylated pRb represents the active form and inhibits S-phase entry. The HPVE7 preferentially forms complexes with hypophosphorylated pRb. This leads to inactivation of pRb and permit cells to enter into S phase (Cobrinik et al., 1992).

The growth suppressive activity of pRb and other pocket proteins (p107 and p130) is mediated by members of the E2F family of transcription factors (Cobrinik, 2005). In G0/G1 hypophosphorylated pRb is bound to E2F (Adams et al., 1995; Du and Pogoriler, 2006). As a transcriptional repressor protein pRb bind to E2F via its transcriptional repressor domain (A/B and C Pocket, represented in Figure 1.4). Upon phosphorylation by cyclin dependent kinases in G1 phase, pRb/E2F complexes dissociate and E2F acts as a transcriptional activator of the cell cycle responsive genes (Sherr et al., 1996).



**Figure-1.2: The HPV E7 abrogates pRb mediated regulation of the G1/S transition of the cell cycle** Upon cell cycle progression pRb is sequentially phosphorylated by cyclin D/CDK4, cyclin E/CDK2 and cyclin A/CDK2. Hyperphosphorylated pRb in G1 phase does no longer interact with E2F and free E2F can induce genes which play role in cell cycle progression such as DNA polymerase alpha, dihydrofolate reductase, thymidine kinase. The E7 oncoprotein counteracts the pRb mediated G1-arrest by direct activation of the cyclin/CDK complexes and inhibition of the CDK inhibitors p21 and p27 and by proteasomal degradation of pRb (adapted from Christian Kuntzen, DKFZ).

## **1.5 The tumor suppressor protein “Retinoblastoma Protein”**

### **1.5.1 The tumor suppressor protein pRb and Cancer**

pRb was the first tumor suppressor identified through human genetic studies. The retinoblastoma gene is mutated in many human cancers, including retinoblastoma, osteosarcoma and small cell lung cancer (Weinberg et al., 1995a).

Mouse genetic studies revealed that pRb functions are essential for embryonic development. *RB*<sup>-/-</sup> null embryos die at 13.5 day gestation with prominent defects in the central nervous and hematopoietic system (Zhu et al., 2005; Jacks et al., 1992; Lee et al., 1992). To this day *RB1* remains the only single gene in which mutation is necessary and sufficient to cause human cancer.

Furthermore, pRb protein can be inactivated by phosphorylation through cyclin-dependent kinases (in particular CyclinD/ CDK4) (Du and Pogoriler, 2006). Overexpression of cyclin D1, activating mutations in CDK4/6, and inactivating mutations in the cyclin D/CDK4 inhibitor p16<sup>INK4a</sup> are frequent events in various human cancers (Kim and Zhao et al., 2005). Since most, if not all, human cancers have one or more of these events it has been proposed that disruption of pRb function is a general feature of cancer cells (Sherr et al., 1996). Some immunohistochemical studies of pRb in human cancer and in normal tissues, including uterine cervix, have shown that pRb is expressed in mature and in differentiated cells (Kim and Zhao et al., 2005).

### **1.5.2 pRb belongs to the pocket protein family and exerts different function**

The Rb protein is a member of a family of three closely related mammalian proteins that includes p107 and p130. Together these are often referred to as the ‘pocket proteins’ because their main sequence similarity resides in a domain, which mediates interactions with viral and cellular proteins, namely the pocket domain (Classon and Dyson, 2001). Although pRb is the best characterised family member (Harbour and Dean, 2000a). The 105 kDa retinoblastoma protein (pRb) has been implicated in many cellular processes, such as regulation of the cell cycle, DNA-damage responses, DNA-repair, DNA-replication, protection against apoptosis and differentiation (Classon and Harlow, 2002).

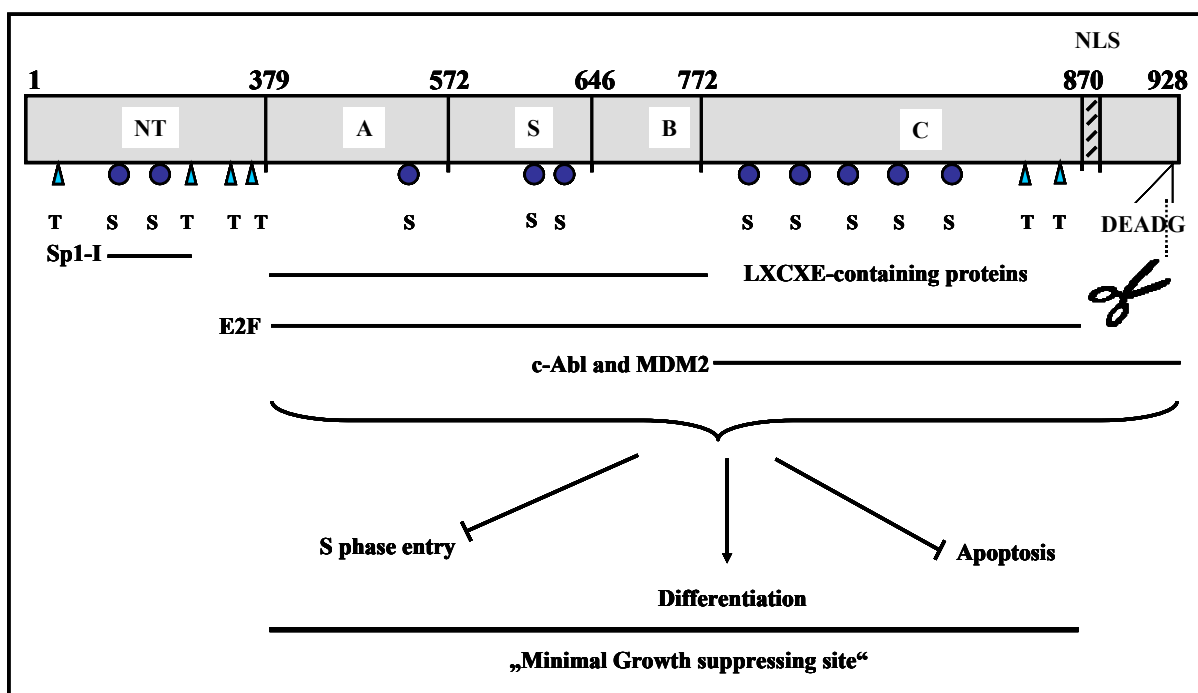
The most important function of pRb as a tumor suppressor is growth suppression which is mediated via interaction with other proteins (Qin et al., 1992; Chow and Dean 1996; Lee et al., 1998; Morris and Dyson 2001). pRb keeps cells in G1 by repressing the transcription of genes



required for cell cycle transition into S phase, and those genes that encode other cell-cycle regulators or enzymes required for DNA synthesis.

The pRb protein is known to bind to more than 100 cellular proteins ranging from transcriptional regulators to enzymes involved in signal transduction and chromatin remodelling systems (Lee et al., 1998; Rubin et al., 1998; Morris and Dyson, 2001).

The pRb contains several functional domains. The N-terminal region of pRb amino-acids 1-378, has been shown to be important for Sp-1 transactivation as it relieves repression by the inhibitor Sp1-I (Kim et al., 1992). Amino acids 379-772 are considered the small pocket domain, and are further subdivided by spacer region into A- (amino acids 394-571) and B- (amino acids 649-773) domains, while the large pocket includes A, B, and C domains.



**Figure-1.3 Structure of pRb:** Numbers at the top of the bar show the amino acids comprising the amino terminus (NT), A-pocket (A), spacer region (S), B-pocket (B), C-pocket (C) as well as nuclear localization signal (NLS). The consensus cdk phosphorylation sites for serine (S) and for threonine (T) are indicated. The solid lines denote the regions of pRb involved in the binding of an inhibitor of Sp1 (Sp1-I), LXCXE-proteins, transcription factor E2F, c-Abl and MDM2. The LXCXE, E2F, c-Abl and MDM2 binding site comprises the minimal growth suppression domain. The consensus cleavage site for the Ced-3/ICE family of proteases (between aa 886D and 887G) is outside the minimal growth suppression domain (obtained kindly from Wang et al., 1997).

Domain A is required for an active conformation (Cho et al., 1997) while domain B binds to LXCXE motifs in interacting binding proteins like histone deacetylase (HDAC)-1 and HDAC-2, ATPase, BRG1, from the SDWI/SNF nucleosome remodelling complex (Brehm et al., 1998; Luo

et al., 1998; Magnaghi-Jaulin et al., 1998), D-type cyclins (Weinberg et al., 1995b), the transcription factors UBF (Cavanaugh et al., 1995), Elf-1 (Wang et al., 1993) and the phosphatase PP1 a type 1 serine/threonine protein phosphatase (Durfee et al., 1993).

The large pocket of pRb is composed of the A/B region together with the C-pocket amino acids 773-870 which is most noted for its ability to bind to a class of transcription factors known as E2F (Chellappan et al., 1991; Lees et al., 1993). The strongest growth suppressive effects are mediated through this interaction.

The C pocket of pRb is located in the carboxy-terminal region. This region contains binding sites for the c-Abl tyrosine kinase MDM2 which are distinct from the E2F site in the carboxy-terminal region (Welch and Wang, 1993; Xiao et al., 1995; Hsieh et al., 1999).

Several of the reported pRb binding proteins have a pro-apoptotic function. For example the nuclear c-Abl tyrosine kinase can induce apoptosis due to its kinase activity (Welch and Wang 1993), which is inhibited by the interaction with pRb (Wang et al., 2000). Furthermore it can regulate the stability and apoptotic function of p53 via binding to MDM2 (Hsieh et al., 1999). pRb contains several consensus sites for caspase cleavage (Tan et al., 1998; Fattman et al., 2001 and 1997) on its carboxyl terminus. Degradation of pRb due to caspase function observed in response to cell death signals and executing cell killing (Tan et al., 1997).

### **1.5.3 Regulation of E2F transcription factors by pRb**

The pRb lacks the DNA binding domain and is tethered to promoter sequences as a consequence of its interaction with other sequence specific transcription factors, such as E2F family proteins. Three transcription factors of the E2F family (E2F1, E2F2, E2F3) are the most extensively studied targets during the G1 phase. E2F binds to DNA upon heterodimerization with members of the differentiation-regulated transcription factor polypeptide (DP) family (Dyson et al., 1998; Helin et al., 1998a). The E2F family comprises five members (E2F1-E2F5) all contain an acidic activation domain at their C-terminus, required for binding to pRb and transcriptional activation (Helin et al., 1993a and 1993b).

pRb can negatively regulate E2F responsive genes by at least two mechanisms:

- I. Binding to the E2F transactivation domain and directly block E2F activity.
- II. Suppressing transcription while tethered to promoters by E2F through recruitment of HDACs (Brehm et al., 1998) and the ATP-dependent SWI/SNF complex (Zhang *et al.*, 2000).

Binding of the pRb protein to the promoter through E2F turns off E2F-responsive gene transcription. Many of them are essential and rate limiting for the cell to enter into S phase whilst during G1 phase they act as negative elements by recruiting the E2F/pRb complex (Zwicker et al., 1997).

Before the cell proceeds to S phase, pRb is phosphorylated at multiple sites by cyclin dependent protein kinases (CDKs) resulting in loss of its transcriptional repression activity through the dissociation of the E2F-pRb complex, which allows S-phase-specific genes to be transcribed (Buchkovich et al., 1989; Classon and Harlow 2002). The importance of the E2F-pRb repressor complex for cell-cycle control is highlighted by the fact that pRb mutants isolated from human cancer cells fail to bind to E2F (Dyson et al., 1998; Nevins et al., 1992).

#### **1.5.4 Transcriptional repression by the pRb/HDAC/E2F complex**

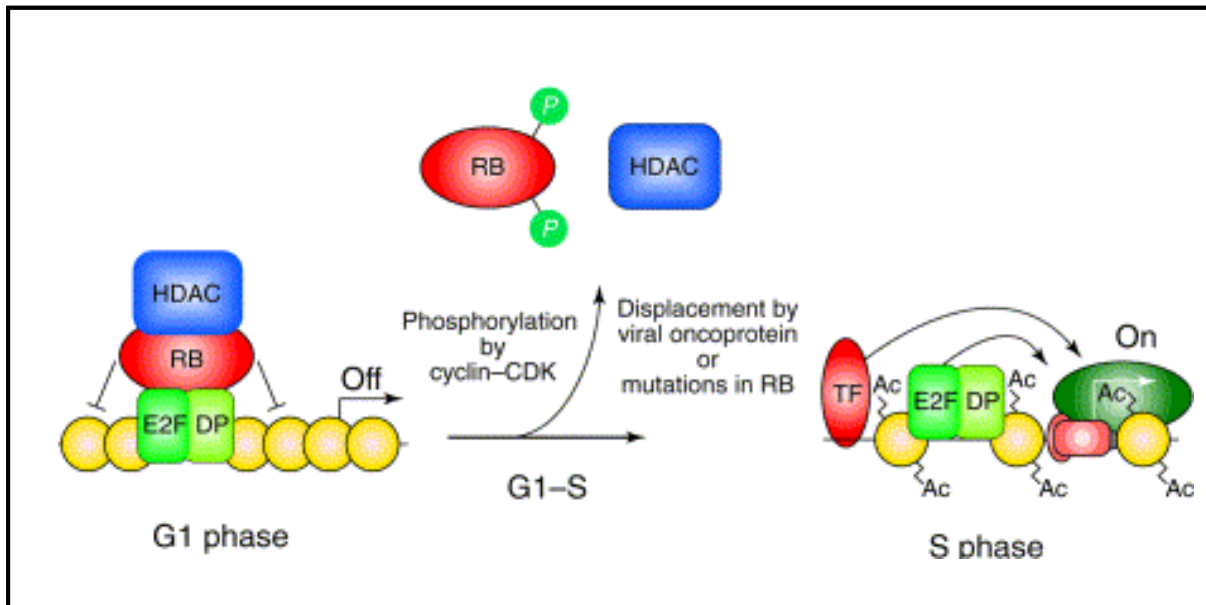
Modification of the DNA structure is critical for regulating gene transcription. One manner in which chromatin structure can be altered is by acetylation/deacetylation of histone molecules at the nucleosomes. It has been shown, that histone acetyltransferases (HAT) act as transcriptional coactivators, by altering chromatin structure, thereby allowing transcription factors to access the promoter (Grunstein et al., 1997). In contrast, HDACs have been associated with transcriptional inhibition and are found in corepressor complexes.

Studies on the mechanism of transcriptional repression by pRb revealed, an interaction with HDAC1, HDAC 2 and HDAC3 (Brehm et al., 1998; Chen and Wang et al., 2000; Dahiya et al., 2000; Magnaghi-Jaulin et al., 1998). The recruitment of HDACs by pRb can reverse histone acetylation at promoters and correlates with repression of transcription (Luo et al., 1998).

E2Fs does not contain LXCXE sequence and bind pRb at a distinct site from the HDACs, allowing recruitment of an HDAC-pRb-E2F repressor complex at promoters of cell cycle genes (Harbour and Dean 2000a and 2000b). Genes regulated by pRb-E2F repressor and activated near G1/S transition appear to require HDAC activity for their repression during mid –G1, suggesting that functional HDACs are critical for pRb's ability to induce G1 arrest (Zhang et al., 2000). Furthermore, inhibition of HDACs with trichostatin-A prevents pRb mediated repression of a set of cellular genes including cyclin E, suggesting an important role of HDAC activity in pRb function (Zhang et al., 2000; Luo et al., 1998).

The transcriptional repression functions of pRb reside in the pocket domain (residues 379-792) and so far all mutations identified in cancer patients, map this region. Several studies with pRb mutants containing amino-acid substitutions in the LXCXE-binding site showed no effect on the ability of the pRb mutants to inhibit transcriptional activity of E2F, but they were unable to

induce growth arrest (Dahiya et al., 2000; Brehm et al., 1998; Magnaghi-Jaulin et al 1998; Dick et al, 2000). Furthermore, the pRb-HDAC interaction is targeted by viral oncoproteins such as E1A, SV40-T antigen and HPVE7 (Lee et al., 1998; Dick et al., 2000 and 2002). This indicates that the pRb-HDAC interaction, mutation of the pocket or oncoprotein action correlates with progression into S-Phase.



**Figure-1.4: pRb-mediated repression of chromatin during the cell cycle.**

Prior to the G1-S transition, phosphorylation of pRb by CDKs, or displacement of HDACs by viral oncoproteins, such as E7, leads to dissociation of the E2F-pRb-HDAC repressor complex. E2F is then free to activate transcription by contacting basal factors or by contacting histone acetyltransferases, such as CBP, that can alter chromatin structure (Trouche et al., 1996; Trouche and Kouzarides 1996). HDAC: histone deacetylases; DP: differentiation-regulated transcription factor polypeptide, CDK: cyclin-dependent kinases, TFs: Transcription factors. 'Ac' denotes acetylated lysine residues (obtained kindly from Brehm et al., 1998).

A repressor complex containing hypophosphorylated pRb and HDACs forms early in G1 phase. This complex interacts with E2F that is bound to S-phase-specific promoters. HDAC then deacetylates the nucleosomes surrounding the promoter and thereby induces a change in chromatin conformation. The now-repressive chromatin denies transcription factors access to their binding sites, and transcription is shut off.

## **1.6 Chromatin regulation and the role of HDAC inhibitors**

### **1.6.1 Chromatin regulation by HATs and HDACs**

In eukaryotic cells DNA is condensed around nucleosomes. Nucleosomes are the regularly repeating units of chromatin in which 146 base pairs of DNA are tightly wrapped around the core histone octamer (Luger et al., 1997). Each nucleosome contains two units of core histone H2A, H2B, H3 and H4. The nucleosomes are also connected to their neighbour by a short segment of linker DNA (approximately 10 to 80 base pair) which is bound and stabilized by histone H1 protein.

Posttranslational covalent modifications of the N-terminal histone tails of nucleosomes, acetylation/deacetylation of lysines, methylation of lysines or arginines, phosphorylation of serines and threonines, ubiquitylation and sumoylation of lysines, and ADP-ribosylation of glutamic acids (Felsenfeld et al., 2003; Khorasanizadeh et al., 2004) all regulates gene expression.

These modifications determine the status of the chromatin (Jenuwein and Allis 2001; Agalioti et al., 2002; Richards and Elgin et al., 2002) and regulate the access of the transcription-regulatory complexes to the DNA (Jenuwein and Allis 2001; Turner et al., 2003).

Histone acetyltransferases (HATs) and histone deacetylases (HDAC) are the two classes of enzymes that mediate the acetylation and deacetylation of histones (Marks et al., 2004). In general, increased histone acetylation is associated with open and active chromatin status which leads to enhanced transcription while deacetylated histones are associated with condensed chromatin and transcriptional repression. HATs and HDACs are also recruited to gene promoters by multiprotein transcriptional complexes, where they regulate transcription through chromatin modification without directly binding the DNA (Cress and Seto, 2000; Lindemann et al., 2004). Additional HDACs exist also in large multimeric complexes that are recruited to the gene promoters by co-repressors, which are associated with transcription factors (Feinberg et al., 2004). Importantly HATs and HDACs have also been shown to affect the acetylation status of lysines in transcription factors. (e.g p53, E2F, GATA1, relA, YY1, Mad/Max, TFII and TFIIF, and hormone receptors), which may affect their DNA binding and transcriptional activity (Bolden et al., 2006; Minucci and Pelicci, 2006). In addition it has been shown that non-transcriptional factors such as cytoskeleton protein  $\alpha$ -tubulin, nuclear import protein importin- $\alpha$ -7, DNA repair protein Ku70 and heat shock protein 90 can be deacetylated by HDAC molecules (Bannister et al., 2000; Blander, 2002; Haggarty et al., 2003; Cohen et al., 2004).

HATs can be divided into subfamilies based on the presence of highly conserved structural motifs. These include the GNAT, MYST, and the p300/CBP subfamilies (Johnstone et al., 2003). The p300/CBP family does not bind directly to DNA but is recruited to promoters by DNA-bound transcription factors (Roth et al., 2001).

The HDAC family is divided into Zn-dependent (Class I and Class II, Class IV) and Zn-independent NAD-dependent (Class III) enzymes (Cheng et al., 2003). The class I HDACs (1, 2, 3, and 8) are ubiquitously expressed and located in the nucleus (De Ruijter et al., 2003) while class IIa HDACs (4, 5, 6, 7, 9, and 10) can shuttle between the nucleus and the cytoplasm. The class IIb HDACs, HDAC6 and HDAC10, are found in the cytoplasm and contain two deacetylase domains. HDAC6 has a unique substrate specificity conferred by with  $\alpha$ -tubulin deacetylase domain (Bolden et al., 2006). HDAC 11 is the sole member of the class IV HDACs, which shares sequence similarity with the catalytic core regions of both class I and II enzymes (Gao et al., 2002).

### 1.6.2 Inhibitors of HDAC molecules

Structurally diverse classes of HDACi were classified as either products isolated and purified from natural sources or synthetically produced compounds. Based on their chemical structure HDAC inhibitors can be divided into six classes. These include the hydroxamic acid derivatives (TSA, SAHA, Oxamflatin), short-chain fatty acids (sodium butyrate, valproic acid, 4-phenylbutyrate), Synthetic pyridyl carbamate derivative (MS-275), Synthetic benzamide derivatives (CI-994 (N-acetyldinaline)), electrophilic ketones (trifluoromethyl ketone) and cyclic peptides (trapoxin, depsipeptide). These compounds have a polar end and can block the catalytic activity of the HDAC pocket structure by chelating zinc ion or other part produce effect through block passage of active site (Finnin et al., 1999; Marks et al., 2004).

The various HDAC inhibitors studied so far have been shown to inhibit class I (HDACs 1, 2, 3, and 8) and class II a (HDACs 4, 5, 6, 9, and 10), although only the hydroxymic acid analog HDACi like TSA, have been shown to inhibit class II b (HDACs 6 and 10). Class III HDACs (SIR T1, 2, 3, 4, 5, 6, and 7) known as sirtuins, require NAD<sup>+</sup> rather than zinc as a co-factor for their activity, and are not inhibited by commonly used HDAC inhibitors.

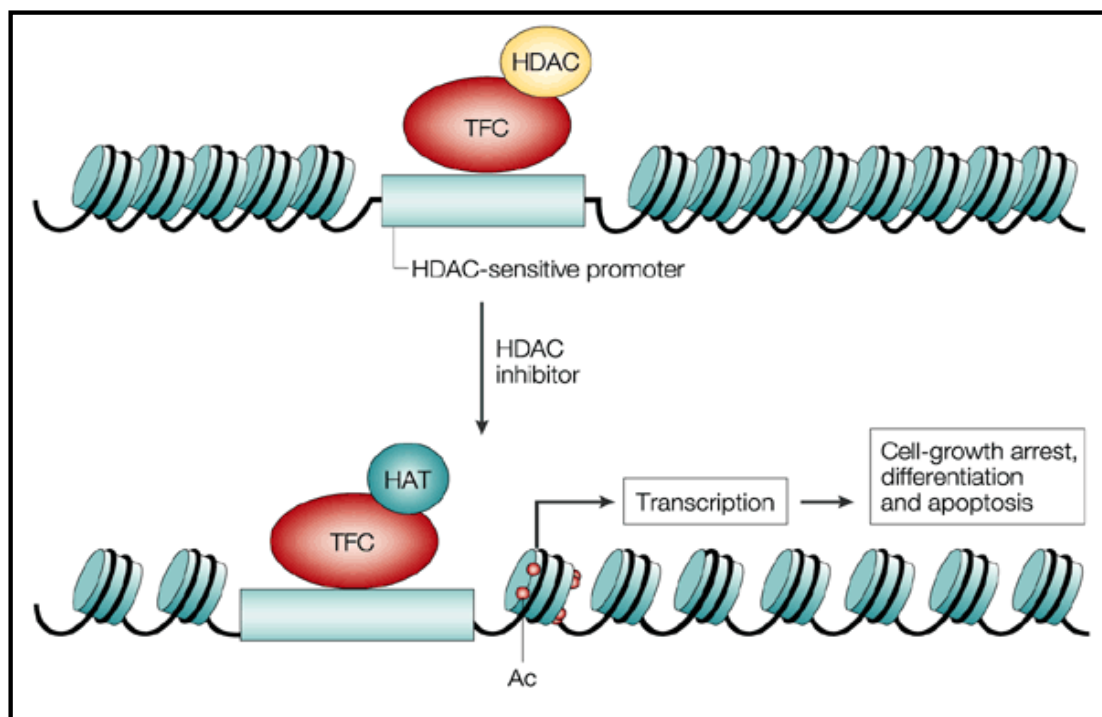
### 1.6.3 HDAC inhibitor induced cellular effects

HDAC inhibitors (HDACi) can mediate a diverse range of intrinsic effects on cell growth, differentiation or apoptosis and survival *in-vivo* and *in-vitro*. These effects are dependent of the

cell type and concentration used. In some cases, growth arrest is induced at low doses, and apoptosis is induced at higher doses; in other cases growth arrest precede apoptosis. The molecular mechanisms behind these biological effects are not completely understood.

### 1.6.3.1 Influence of HDAC inhibition on gene transcription

The mechanism of the antiproliferative effects of HDAC inhibitors involves, either by directly affecting chromatin structure or the activity of transcription factors. Recent cDNA microarray studies have shown that treatment with HDACi modulates the expression of a selective subset of genes consisting of approximately 20 % of all genes in different cell types (Glaser et al., 2003; Mitsiades et al., 2004; Peart et al., 2005). A significant number of apoptotic (i.e., CD95, CD95 ligand, gelsolin, Bak, Bcl-X<sub>L</sub>) and cell cycle regulatory genes (i.e., cyclin D, cyclin E, cyclin A, p21<sup>WAF/CIP1</sup>), have been identified (Huang et al., 2000; Mariadason et al., 2000; Chen et al., 2002; Johnstone et al., 2002; Henderson et al., 2003) to be activated or repressed by different HDACi. Importantly, activation of some genes by HDACi involves the Sp1 site in the promoter region, which recruits the HDAC containing transcription complex. Therefore treatment with HDACi induced the acetylation of these genes which are involved in growth arrest, differentiation and/or apoptosis (Huang et al., 2000; Takakura et al., 2001).



**Figure-1.5: Proposed mechanism of action of histone deacetylase inhibitors.**

Histone deacetylases: HDAC Transcription factor complex (TFC), Ac: acetyl group, HAT: histone acetyltransferase.

### 1.6.3.2 HDAC inhibitor and cell cycle

In general, treatment with HDAC inhibitors induces cell cycle growth arrest in G1 or G2/M, differentiation and/or apoptosis of transformed cells (Bhalla 2005). All HDACi studied to date, with exception of tubacin, can induce cell cycle arrest at G2/S boundary mediated by the retinoblastoma protein pRb, p107 and p130 (Haggarty et al., 2003). The HDACi mediated induction of p21 correlates with an increase in the acetylation of histones associated with p21 promoter region (Richon et al., 2000; Lagger et al., 2002; Gui et al., 2004). Activation of p21 occurs in a p53-independent manner which promotes hypophosphorylation of pRb (Richon et al., 2000).

Additionally the repression of cyclin D and cyclin A contributes to the loss of CDK2 and CDK4 kinase activities and hypophosphorylation of pRb (Kim et al., 1999; Qiu et al., 2000; Sandor et al., 2000). Surprisingly, HDACi often transactivate cyclin E, although this is not associated with increased CDK2 activity, most likely due to the concomitant increase in p21 levels (Kim et al 1999; Sambucetti et al., 1999; Sandor et al., 2000). HDACi induced transcriptionally repression of genes involves the CTP synthase and thymidylate synthetase, which play role in DNA synthesis and by progression of S phases (Glaser et al., 2003).

Since most of the tumor cells have a defective G2-phase checkpoint, HDACi can mediate G2/M phase arrest by activating a G2-phase checkpoint. Although this is a much rarer event than HDACi mediated G1 arrest (Qiu et al., 2000; Burgess et al., 2001).

### 1.6.3.3 HDAC inhibitor induced apoptosis

The therapeutic potential of HDACi stems from their capacity to selectively induce apoptosis (Marks et al., 2005). The clinical trials and preclinical animal experiments clearly show that HDACi can have potent anticancer activities at concentrations that are minimally toxic to the host (Kelly et al., 2005; Drummond et al., 2005; Dokmanovic et al., 2005).

Treatment with HDAC inhibitors triggers both the intrinsic, and the mitochondria-initiated signalling for apoptosis (Johnstone et al., 2003; Lindemann et al., 2004).

A large number of proapoptotic genes (Bak, Bax, CD95, CD95 ligand, gelsolin, p53, caspases-9, caspase-8, Apaf-1, p53, caspase-3, Bim, Bid, Bad), and repression of anti-apoptotic genes (Bcl-2, Bcl-X<sub>L</sub>, c-FLIP, surviving, XIAP, NFκB; Mcl-1) was demonstrated after treatment with HDAC inhibitors (Johnstone et al., 2003; Chen et al., 2002; Henderson et al., 2003; Zhang et al., 2003).



A large number of independent studies strongly support a role for the mitochondrial pathway in HDACi-mediated tumor cell death via induction of BCL2 or BCL-X<sub>L</sub>, which block the intrinsic apoptotic pathway (Ruefli et al., 2001; Peart et al., 2003; Johnstone et al., 2002; Mitsiades et al., 2003; Henderson et al., 2003). Several types of HDACi especially the hydroxamic acid analogs, have been shown to participate in mitochondrial permeability transition. They release pro-apoptotic molecules such as cytochrome c, Smac, and Omi into the cytosol, thereby triggering the activity of the Apaf-1 "apoptosome", which leads to the processing and activation of caspase-9 and -3 (Marks et al., 2003; Fuino et al., 2003; Guo et al., 2004).

The proapoptotic Bcl-2 (BH3 only proteins) family member Bid is also cleaved and activated in response to various HDACi (Ruefli et al., 2001). Nevertheless HDACi triggered intrinsic apoptotic cascade is a major question that remains to be fully answered. One possibility is that HDACi induce global changes in gene expression that alter the balance of expression of pro- and anti-apoptotic genes in favour of a pro-apoptotic biological response.

#### 1.6.4 HDAC inhibitors and treatment of cancer

Inhibitors of HDAC are promising as single-agent anti-cancer drugs, which are highly effective in up-regulating tumor suppressor gene expression, reducing tumor growth and inducing programmed cell death *in vitro* and in cancer patients in phase I and II clinical trials (Liu et al., 2006).

HDAC inhibitors such as Trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), and sodium butyrate (SB), can inhibit cancer cell growth *in vitro* (Yoshida et al., 1990; Wharton et al., 2000) and *in vivo* (Vigushin et al., 2001; Yoshida et al., 1990), reverse oncogene-transformed cell morphology (Kwon et al., 1998; Marks et al., 2000), and enhance cell differentiation (Kosugi et al., 1999; Zhou et al., 2000).

Sodium Butyrate is the first confirmed HDAC inhibitor and belongs to the natural short-chain fatty acid; produced by bacteria in colon (Newmark et al., 1994). It has been demonstrated to induce growth arrest *in vitro* and differentiation *in vivo* and *in vitro* in several cancer cells like human myeloid leukaemia cells (Rosato et al., 2001; Chen et al., 1994), MCF-7 breast carcinoma cells (Heerdt 1999) and colon carcinoma cells (Hodin et al., 1996; Litvak et al., 1998). SB induced growth inhibition and cell death both *in vitro* and *in vivo* in prostate cancer cells (Maier et al., 2000; Kuefer et al., 2004). Furthermore, it induced apoptosis in human myeloma cells (Lavelle et al., 2001), and growth arrest and apoptosis in colon cancer cells (Archer et al., 1998; Mahyar-Roemer et al., 2001). The mechanism by which n-butyrate causes cell cycle arrest and apoptosis are not fully understood but involve at least in part the p21<sup>WAF</sup>-Rb-E2F pathway.

Another frequent HDAC inhibitor is TSA, derived from the metabolic product of streptomycete (Chen et al., 2004). TSA belongs to the group of the hydroxamic acid derivatives which contain a functional group that interacts with the critical zinc atom at the base of the catalytic pocket of the class I and II HDACs (Marks et al., 2000 and 2001).

Exposing A2780 ovarian cancer cells with TSA produced a marked change in cellular morphology, proliferation, and differentiation (Strait et al., 2002). TSA and SB blocked two colon cancer cell lines (SW1116 and Colo-320) mainly in the G1 phase. In these human cancer cell lines, increased acetylation of the gene-associated histones, and a G1 cell cycle arrest was also observed (Chen et al., 2004).

Clinical trials show that HDAC inhibitors are well tolerated and can inhibit HDAC activity in peripheral mononuclear cells and tumors. More importantly, they display clinical activity with objective tumor regression. HDACi induce equivalent histone hyperacetylation and induction of p21 in both normal and tumor cells while normal cells undergo cell cycle arrest at the G1/S checkpoint in response to HDACi, they are largely insensitive to HDACi-induced apoptosis (Qiu et al., 1999; Zhang et al., 2003). How HDAC inhibitor are selectively cytotoxic against tumor cells, whilst leaving normal cells apparently unharmed remains to be determined.

Several HDAC inhibitors are currently in clinical trials including butyrates, valproic acid, SAHA, pyroxamide, depsipeptide, MS-275 and CI-994. These HDAC inhibitors are in phase I and II evaluation as monotherapy, as well as in combination with other differentiation therapies and cytotoxins. Warrel *et al.*, (1998) first used HDAC inhibitor for treatment of acute promyelocytic leukaemia patients. After solo all-trans retinoic acid treatment (ATRA); sodium phenylbutyrate and ATRA were applied for combined chemotherapy, next bone marrow report showed leukaemia cells were cleared and reached complete remission ten days later (He et al., 2001).

HDACi have been shown to function synergistically with structurally and functionally diverse chemical compounds such as Hsp90 inhibitor geldanamycin (17-AAG) (Rahmani et al., 2003; George et al., 2005; Rahmani et al., 2005), proteasome inhibitor bortezomib (Pei et al., 2004; Hideshima et al., 2005; Nawrocki et al., 2006) and biologically active polypeptides (Lindemann et al., 2004; Bhalla, 2005), but the mechanistic basis for this synergy has not been fully elucidated.

An important finding in predicting the potential utility of HDAC inhibitors in the clinic is their activity in cell-lines that are resistant to existing chemotherapeutics. For example Gleevec-resistant Bcr/Abl human chronic myelogenous leukaemia (CML) cells are sensitised to Gleevec upon co-treatment with SAHA (Yu et al., 2003).

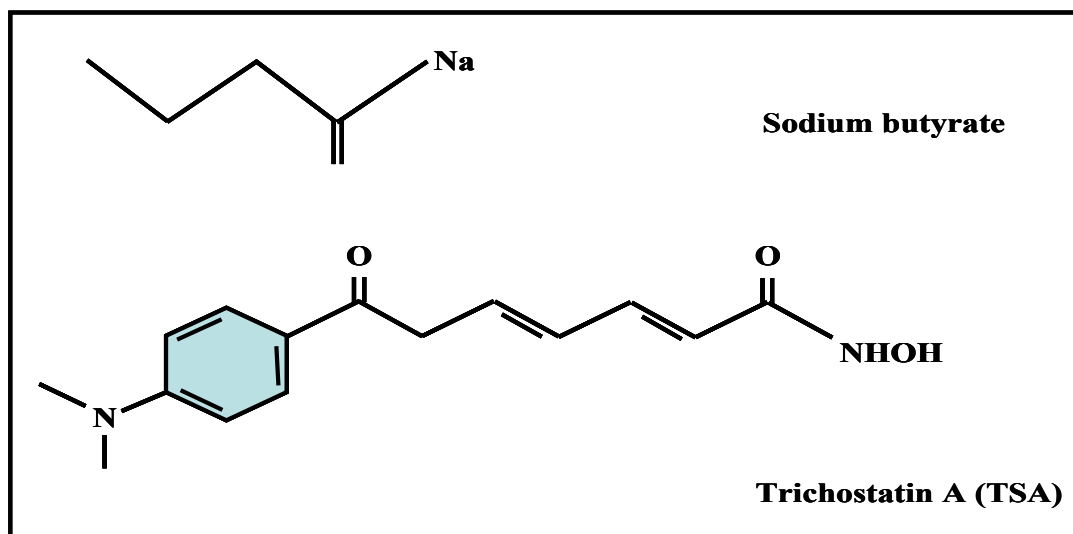


Figure-1.6: Structure of HDAC inhibitors sodium butyric acid and Trichostatin A

**Aim of this study:**

Our Group has maintained a long standing interest in analysing the biologic significance of HDACi in HPV transformed cells. Initial observations with SB and TSA on HPV16/18 positive cervical carcinoma cells demonstrated growth arrest at the G1 phase of cell cycle progression (Finzer et al., 2003). Concomitant with G1 arrest, an increase of the cyclin-dependent kinase inhibitors p21 and p27 was also observed, whilst CDK2 activity was suppressed (Finzer et al., 2001). Moreover longer treatment with these agents sensitized the cells to apoptosis. Among these, one of the interesting observations was the enhanced degradation of the pRb while its interaction partners HPV E7 and E2F were not affected.

Hence, the aim of this study was to address the following questions:

1. What is the role of HPV E7 in HDAC inhibitor mediated pRb degradation in cervical cancer cells?
2. Does the tumorigenic phenotype of the target cell plays a role in HDAC inhibitor induced pRb degradation?
3. What is the importance of the direct interaction between HPV E7 and pRb during HDAC inhibition?
4. Which mechanisms play a role in the pRb degradation upon HDAC inhibition?
5. What is the role of pRb degradation in cell cycle control and apoptosis upon HDAC inhibition?

## II. MATERIALS & METHODS

### 2.1 MATERIALS

#### 2.1.1 Chemicals and Reagents

[ $\alpha^{32}\text{P}$ ] dCTP 10 mCi/ml	Amersham, Life Science
Acetic acid glacial	Merck, Darmstadt
Acrylamide/ bis acrylamide (29:1), 30%	Sigma, Deisenhofen
Active charcoal	Serva, Heidelberg
Agarose	Invitrogen, Karlsruhe
Amonium acetate	Fluka BioChemika, Deisenhofen
Ampicillin	Roche Diagnostics, Mannheim
Amonium persulfate	Sigma, Deisenhofen
Bacto-Agar	Gibco Life Technologies
Bacto-Tryptone	Difco, Detroit
BenchMark (protein marker)	Gibco Life Technologies
Biogel P30	BioRad, München
Boric acid	Sigma, Deisenhofen
Bovine Serum Albumin	Sigma, Deisenhofen
Bradford Reagent (Bio-Rad Protein Assay)	BioRad, München
Bromophenol Blue	Sigma, Deisenhofen
Calcium chloride (dihydrate)	Sigma, Deisenhofen
Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132)	Calbiochem, Bad Soden
Chloride acid	Merck, Darmstadt
Cycloheximide	Sigma, Deisenhofen
Diethylpyrocarbonat (DEPC)	Sigma, Deisenhofen
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Dithiothreitol (DTT)	Sigma, Deisenhofen
DNA marker Gene Ruler 1kb DNA ladder	MBI Fermentas, Vilnius
DNA 6X loading buffer	MBI Fermentas, Vilnius
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco, Eggenstein
E-64	Roche Dignostics, Mannheim
ECL-Reagent	NEN Lifescience Products Inc
EDTA	Roche Dignostics, Mannheim
EGTA	Sigma, Deisenhofen

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Ethanol absolute	Merck, Darmstadt
Fetal calf serum	Gibco Life Technologies
Formaldehyd	Merck, Darmstadt
Formamide	Fluka BioChemika, Deisenhofen
[ $\gamma^{32}\text{P}$ ] dATP 10 mCi/ml	Amersham, Life Science
Geneticin (G-418 Sulfate)	Calbiochem, Bad Soden
Glycerin	Merck, Darmstadt
Glycine	Sigma, Deisenhofen
HEPES	Gerbü, Gaiberg
Hexanucleotide	Amersham-Pharmacia, Freiburg
Hydrochloric acid	Merck, Darmstadt
Isoamylalcohol	Merck, Darmstadt
Klenow Fragment (DNA polymerase I, 5000U/ml)	New England Biolabs, Frankfurt
LB-Medium	Gibco Life Technologies
Magnesium chloride	Merck, Darmstadt
2-Mercaptoethanol	Sigma, Deisenhofen
Methanol	Merck, Darmstadt
Milk powder	Roth, Karlsruhe
MOPS	Gerbü, Gaiberg
Nonidet-P40	Roche Dagnostics, Mannheim
Nucleotides (dNTPs for PCR)	Roche Dagnostics, Mannheim
PBS	Gibco, Eggenstein
Pefablock SC	Roche Dagnostics, Mannheim
Penicillin [10000U/ml]/Streptomycin [10000 $\mu\text{g}$ /ml]	Gibco, Life Technologies
Potassium acetate	Merck, Darmstadt
Potassium chloride	Merck, Darmstadt
Potassium hydrogen phosphate	Roth, Karlsruhe
Potassium hydroxide	Merck, Darmstadt
2-Propanol, Isopropanol	Merck, Darmstadt
Random primers	Roche Dagnostics, Mannheim
Restriction enzymes	Amersham-Pharmacia, Freiburg
RNAse A	Serva, Heidelberg
Roti-Phenol	Roth, Karlsruhe
Sodium azide	Sigma, Deisenhofen
Sodium butyrate	Sigma, Deisenhofen

Sodium citrate	Sigma, Deisenhofen
Sodium chloride	Merck, Darmstadt
Sodium dihydrogen phosphate (monohydrate)	Merck, Darmstadt
Sodium dodecyl sulphate	Sigma, Deisenhofen
Sodium fluoride	Merck, Darmstadt
Sodium ortho-vanadate	Sigma, Deisenhofen
SuperScript II (RNase H (-) 200U/μl)	Gibco Life Technologies
Taq platinum DNA polymerase (5U/μl)	Invitrogen, Karlsruhe
TEMED	Gibco Life Technologies
tosyl-L-lysine chloromethyl ketone (TLCK)	Roche Diagnostics, Mannheim
t-RNA	Sigma, Deisenhofen
Trichostatin A (TSA)	Sigma, Deisenhofen
Triton X-100	Sigma, Deisenhofen
Triton X-100 cell lysis buffer (3X)	BD PharMingen, Heidelberg
Trizma-Base	Sigma, Deisenhofen
Trypan blue	Sigma, Deisenhofen
Trypsin/EDTA	Gibco Life Technologies
Tumor Necrosis Factor- $\alpha$ (TNF- $\alpha$ )	Strahtmann Biotech, Hannover
Tween 20	Sigma, Deisenhofen
Urea	Merck, Darmstadt

### 2.1.2 Laboratory equipment

Analytic Scale AE 160	Mettler, Gießen
Analytic Scale basic	Sartorius, Göttingen
Centrifuge 2 K15	Sigma, Deisenhofen
Centrifuge Biofuge, Varifuge RF	Heraus, Hanau
Developer machine Curix 60	AGFA
Electrophoresis camera	Renner GmbH, Dannstadt
Geiger counter LB 1210B	Berthold, Wildbad
Gel drier 483	Bio-Rad, München
Hybridization Oven, Hybrid Mini	Bachofer, Reutlingen
Incubator (for cells) B5061 EC/CO <sub>2</sub> , Forma Scientific	Heraeus, Hanau
Mini-PROTEAN II (minigels western)	BioRad, München

Neubauer count chamber	Bender and Hobein, Bruchsal
PCR Thermo Cycler MultiCycler PTC-2000	M.J. Research
pH-Meter Calimatic 765	Knick
Photometer Ultraspec Casette	Amersham-Pharmacia, Freiburg
Pipette boy acu	Integra Biosciences
Pipettes p2.5, p10, p20, p200, p1000	Eppendorf, Hamburg
Power supply EPS 600	Amersham-Pharmacia, Freiburg
Scale 1216 MP	Sartorius, Göttingen
Sonifier 250	Branson/Heinemann, Schwaebisch Gmünd
Sterile hood (BioGard Hood)	Baker Company, Sandford
Thermomixer 5436	Eppendorf, Hamburg
Trans-illuminator 254-366 nm	Vetter, Wiesloch
Vortex	Heidolf, Rust
Water Bath	Julabo, Seelbach
Western blot transfer camera Hoefer, Semiphor	Amersham-Pharmacia, Freiburg

### 2.1.3 Solutions and Buffers

<b><i>Ampicillin</i></b>	[50 mg/ml] in water, store at -20°C
<b><i>Biogel suspension</i></b>	1 X TE buffer 5 mM sodium chloride 0.2 % (w/v) SDS 50 % (w/v) Biogel
<b><i>Blocking buffer (Western Blot)</i></b>	1 X TBS pH 7.6 5 % (w/v) Milk powder 0.1 % (v/v) Tween 20 Store at 4°C
<b><i>BSA</i></b>	[1 µg/µl] in water for protein quantification curve Store at -20°C
<b><i>Buffer A (Cytoplasmic protein extraction)</i></b>	10 mM HEPES pH 7.9 10 mM Potassium chloride 0.1 mM EDTA pH 8.0 0.1 mM EGTA pH 7.9 Store at -20°C, Before use add protease and phosphatase inhibitors



<b><i>Buffer C (Nuclear protein extraction)</i></b>	20 mM HEPES pH 7.5 400 mM Sodium chloride 1 mM EDTA pH 8.0 1 mM EGTA pH 7.9  Store at -20°C, Before use add protease and phosphatase inhibitors
<b><i>CaCl<sub>2</sub> (2M)</i></b>	147.02 g/mol CaCl <sub>2</sub> 14.7 g CaCl <sub>2</sub> resolved in 50 ml H <sub>2</sub> O Sterile filtration
<b><i>Chloroform/ Isoamylalcohol (24:1)</i></b>	24 parts chloroform 1 part isoamylalcohol Store at 4°C protected from light
<b><i>Denaturing solution (Southern bot)</i></b>	0.5 N sodium hydroxide 1.5 M sodium chloride
<b><i>Denhardt's Solution</i></b>	5 % (w/v) BSA 5 % (w/v) Ficoll type 400 5 % (w/v) Polyvinylpyrrolidon 25
<b><i>DEPC Water</i></b>	0.1 % (v/v) DEPC 1 L water
<b><i>DMEM complete</i></b>	500 ml DMEM 10 % Fetal Calf Serum 100 U/ml Penicillin 100 µg/ml Streptomycin
<b><i>DNA lysis buffer (3X)</i></b>	1.5 % (w/v) SDS 150 mM Tris/HCl pH 7.8 150 mM EDTA
<b><i>DTT</i></b>	0.1 M stock solution prepared freshly
<b><i>EDTA</i></b>	0.5 M EDTA pH 8.0
<b><i>E64</i></b>	2.5 mg/ml Stock solution in 50 % ethanol, 50% bidest water Store at -20°C
<b><i>EGTA</i></b>	0.25 M EGTA pH 7.9
<b><i>Freezing medium (cell culture)</i></b>	10 % (v/v) DMSO 30 % (v/v) FCS 60 % (v/v) Medium (DMEM) Store at -20°C

<b><i>G418 (Geneticin sulphate)</i></b>	[375 mg/ml] stock solution in DMEM complete Store at -20°C
<b><i>2X HBS Buffer, pH 7.05</i></b>	3.2 g NaCl 0.148 g KCl 0,054 g Na <sub>2</sub> HPO <sub>4</sub> x 2H <sub>2</sub> O 0.4 g Dextrose (D (+) Glucose) 2 g HEPES
<b><i>Hybridization solution (Northern and Southern blot)</i></b>	50 % Formamide 10 U/ml t-RNA 5 X SSC 0.1 % Denhardt's solution 50 mM sodium phosphate buffer pH 6.5 1 % (w/v) SDS
<b><i>Hybridization washing buffer (Northern and Southern blot)</i></b>	2 X SSC 0.1 % SDS
<b><i>Laemmli buffer SDS-PAGE (10X)</i></b>	0.25 M Tris Base 1.9 M Glycine 1 % (w/v) SDS
<b><i>LB-Medium</i></b>	10 g sodium chloride 10 g Bacto-Trypton 5 g yeast extract add water to 1 liter, pH 7.2
<b><i>LB-Ampicillin plates</i></b>	LB-Medium 2 % (w/v) Bacto-Agar Add 50 mg ampicillin per liter
<b><i>MOPS (20 X)</i></b>	400 mM MOPS 100 mM sodium acetate 20 mM EDTA pH 7.0 (DEPC water) Store protected from light
<b><i>Neutralization solution (Southern Blot)</i></b>	1 M Tris pH 7.4 1.5 M sodium chloride
<b><i>PBS</i></b>	123 mM sodium chloride 17.6 mM di-sodium hydrogenphosphatedihydrate 10 mM potassium dihydrogenphosphate pH 7.2-7.8
<b><i>Pefabloc SC</i></b>	[23.8 mg/ml] stock solution Store at -20°C

<b><i>RNA loading buffer (2X)</i></b>	50 % Formamide 2.2 M Formaldehyde 1 % (w/v) Ficoll (type 400) 0.02 % (w/v) Bromophenol blue 1 X MOPS buffer pH 7.0 (DEPC water), Store at -20°C
<b><i>RNase A</i></b>	10 mg/ml RNase A 10 mM Tris pH 7.5 15 mM Sodium chloride 80°C /20 min, store at -20°C
<b><i>SDS</i></b>	10 % (w/v) in water
<b><i>SDS total protein extraction buffer</i></b>	50 mM Tris pH 6.8 10 % Glycerin 2 % (w/v) SDS 0.74 M 2-Mercaptoethanol
<b><i>SOC medium</i></b>	2 % (w/v) Bacto-Trypton 0.5 % (w/v) yeast extract 10 mM NaCl 2.5 mM KCl 10 mM MgCl <sub>2</sub> 10 mM MgSO <sub>4</sub> 20 mM Glucose
<b><i>Sodium floride</i></b>	500 mM Sodium floride Store at -20°C
<b><i>Sodium ortho-vanadate</i></b>	10 mM Sodium ortho-vanadate pH 10 Store at -20°C
<b><i>Sodium Phosphate buffer</i></b>	1 M sodium dihydrogenphosphate (3 parts) 1 M di-sodiumhydrogenphosphate (2 parts) ph 7.0
<b><i>SSC (20 X)</i></b>	3 M sodium chloride 0,3 M tri-sodium citrate dihydrate ph 7.0
<b><i>TAE (50 X)</i></b>	2 M Tris/HCl 0,25 M sodium acetate 0.05 M EDTA ph 8.0
<b><i>TBE (10 X)</i></b>	0,9 M Tris base 0,9 M Boric acid 0,02 M EDTA ph 8.0

<b>TBS (1 X)</b>	10 mM Tris/HCl pH 7,5 100 mM sodium chloride
<b>TBST</b>	1 X TBS pH 7.6 0,1 % (v/v) Tween 20
<b>TE (1 X)</b>	10 mM Tris-HCl 1 mM EDTA pH 8.0
<b>TNE (1 X)</b>	10 mM Tris/HCl pH 8.00 1 mM EDTA 100 mM sodium chloride
<b>TNF<math>\alpha</math></b>	100 U/ $\mu$ l Stock solution 10 $\mu$ g in 200 $\mu$ l bidest water add DMEM complete to 10 ml Store at - 70C
<b>Towbin (1 X)</b>	25 mM Tris Base 192 mM Glycine 0.1% (w/v) SDS 15 % (v/v) Methanol
<b>Trypan blue</b>	0.25 % in PBS
<b>Western blot loading buffer (5X)</b>	10 % SDS 5 mg Bromophenol blue 12.5 % (v/v) 2- Mercaptoethanol 5 mM EDTA pH 8.0 50 % Glycerin 300 mM Tris pH 6.8 Store at -20°C

#### 2.1.4 Cell Lines

All cell lines are from human origin and described in detail as follows:

<b>HeLa:</b>	Tissue:	adenocarcinoma; cervix
	Morphology:	epithelial
	Age stage:	31 years
	Ethnicity:	Black
	Growth properties:	adherent
	HPV presence:	Type 18 (about 50 copies per cell)
	Reference:	(Boshart <i>et al.</i> 1984; Schwarz <i>et al.</i> 1985)

<b>SW756:</b>	Tissue:	squamous cell carcinoma; cervix
	Morphology:	epithelial
	Age stage:	46 years
	Ethnicity:	Caucasian
	Growth properties:	adherent
	HPV presence:	Type 18 (12-20 copies per cell)
	Reference:	(Freedman <i>et al.</i> , 1982)
<b>C33 A:</b>	Tissue:	carcinoma; cervix
	Morphology:	epithelial
	Age stage:	66 years
	Ethnicity:	Caucasian
	Growth properties:	adherent
	HPV presence:	negative
	Reference:	Yee <i>et al.</i> 1985.
<b>HT-3:</b>	Tissue:	adenocarcinoma; cervix
	Morphology:	epithelial
	Age stage:	31 years
	Ethnicity:	Black
	Growth properties:	adherent
	HPV presence:	Type 18 (about 50 copies per cell)
	Reference:	Boshart <i>et al.</i> 1984; Schwarz <i>et al.</i> 1985)
<b>TE-671:</b>	Tissue:	adenocarcinoma; cervix
	Morphology:	epithelial
	Age stage:	31 years
	Ethnicity:	Black
	Growth properties:	adherent
	HPV presence:	Type 18 (about 50 copies per cell)
	Reference:	Boshart <i>et al.</i> 1984; Schwarz <i>et al.</i> 1985)

**444 and CGL-3:** Non-tumorigenic and tumorigenic hybrids made between HeLa and human fibroblasts, respectively. These were kindly provided by Eric Stanbridge.

**HPV E6 and E7 immortalised human keratinocytes:** Primary human keratinocytes derived from neonatal foreskins were immortalized with E6, E7 or both HPV-16 oncogenes after infection with amphotropic retroviral vectors (Halbert *et al.*, 1991). All of these cell lines were kindly provided from Dr. Noel Whitaker (DKFZ-Heidelberg).

### 2.1.5 Antibodies

primary Antibodies	Company	Epitope	Dilution
$\beta$ -Actin, (Clone 4) 691001	ICN Biotech.	Chicken gizzard actin as immunogen	1:100000
pRb, Mouse IgG <sub>1</sub> Cat # 554136 (Clone G3-245)	BD, PharMingen	Epitope between amino acid residues 332-344 of human Rb	1:1000
HPV18E7, Goat-IgG Cat# sc-1590 (N-19)	Santa Cruz Biotechnology	N-Terminus of Human papilloma virus type 18 early E7	1:1000
HPV16E7, Mouse (NM2 $\alpha$ -16E7)	Obtained from Martin Müller, DKFZ	Hybridoma supernatant	1:4
Cyclin E, Mouse-IgG <sub>2b</sub> Cat# sc-247 (HE12)	Santa Cruz Biotechnology	Recombinant Human cyclin E (doublet bands at 50 kDa and single band of 42 kDa)	1:1000
E2F, Mouse-IgG <sub>2a</sub> Cat# sc-193 (C-20)	Santa Cruz Biotechnology	Carboxy -terminus of human E2F-1 (p60)	1:1000
p21, CIP1/WAF1 Mouse-IgG <sub>2a</sub> (Clone 70) Cat# 610233	BD Transduction Laboratories	N-terminus of human CIP1	1:500
PARP-1, Mouse IgG <sub>2a</sub> Cat# sc-8007 (F-2)	Santa Cruz Biotechnology	Epitope mapping the aa 764-1014 of C-terminus of human origin Molecular Weight: 116/85 kDa	1:1000
p73, Rabbit polyclonal IgG, referred: p73SAM	Purified serum was kindly obtained from A. Emre Sayan, 2005, Leicester, UK	Epitope mapping the SAM domain (amino acids 487-554) of human p73	1:2500

Secondary Antibodies	Company	Epitope	Dilution
Rabbit -IgG-HRP Cat# W401B	Promega	conjugated to horse radish peroxidase	1:10000
Mouse-IgG-HRP Cat# W4021	Promega	conjugated to horse radish peroxidase	1:10000
Goat-IgG-HRP Cat# sc-2020	Santa Cruz Biotech.	conjugated to horse radish peroxidase	1:10000
Mouse isotopic specific IgG <sub>1</sub> -HRP, cat# sc-2060	Santa Cruz Biotech.	conjugated to horse radish peroxidase	1:10000
Mouse isotopic specific IgG <sub>2a</sub> -HRP, cat# sc-2060	Santa Cruz Biotech.	conjugated to horse radish peroxidase	1:10000
Mouse isotopic specific IgG <sub>2b</sub> -HRP, cat# sc-2060	Santa Cruz Biotech.	conjugated to horse radish peroxidase	1:10000

### 2.1.6 PCR Primers

Gene	Sequence	Annealing Temperature	# of cycles
<b>RB1</b>	<b>For 5'-GGAAGCAACCCTCCTAAACC-3'</b> <b>Rev 5'-TTTCTGCTTTTGCATTCGTG-3'</b>	<b>60°C</b>	<b>35</b>
<b>TAp73</b> (E. Sayan)	<b>For 5'-AACCAGACAGCACCTACTTCGACC-3'</b> <b>Rev 5'-GCGACATGGTGTCTGAAGGTGGAGC-3'</b>	<b>62°C</b>	<b>35</b>
<b>TAp73</b> (R. Schmidt)	<b>For 5'-TGATGGGGGGCACCACGTTTGAG-3'</b> <b>Rev 5'-TGTAGTCGTGGCCCTGCTTCA-3'</b>	<b>56°C</b>	<b>35</b>
<b>GAPDH</b>	<b>For 5'-TGGATATTGTTGCCATCAATGACC-3'</b> <b>Rev 5'-GATGGCATGGACTGTGGTCATG-3'</b>	<b>50°C</b>	<b>30</b>

### 2.1.7 Plasmids

Plasmid	Insert/ target mRNA Sequence	References
<b>pcDNA3.1(-/+) empty</b> (5,446 kb)	Contains CMV, T7 Sp6, SV40 promoter; ORF for Amp and Neomycin; origin for SV40, ColE1; Poly A site for SV40 and BGH and multiple cloning site (MCS).	M. Müller, (Intvitrogen)
<b>pcDNA3.1/16E7 kozak</b> (5,717 kb)	Contains the HPV16 E7 sequence and an additional “Kozak” consensus translation initiation site.	M. Müller (DKFZ, Heidelberg)
<b>pSUPER-18E6/E7</b> (siRNA)	<b>5'-CCACAACGUCACACAAUGU-3'</b> (HPV18 nt 755–773)	Kuner et al., 2007 (DKFZ, Heidelberg)
<b>pSUPER/luciferase</b> (siRNA)	<b>5'-CAUCACGUACGCGGAAUAC-3'</b> variant:GL2- <i>Photinus pyralis</i>	Butz et al., 2003 (DKFZ, Heidelberg)
<b>pSUPER/Empty</b>	without any insert	Brummelkamp et al., 2002
<b>pEGFP</b> (4,7 kb)	enhanced green fluorescent protein gene (used as a transfection marker)	Clontech, Laboratories

### 2.1.8 Oligonucleotides for Northern Blot analyses

Gene	Insert /Plasmid	Origin
HPV18E7	entire HPV18-Genome/pRB322	Boshart et al., 1984
$\beta$ -Actin	nearly complete $\beta$ -Actin-gen/pHFA1	Gunning et al., 1983; obtained from Dr. L. Kedes (Medical Center, Palo Alto, USA)

### 2.1.9 Size markers for DNA /RNA electrophoresis

#### DNA Analysis:

- 1Kb plus DNA Ladder cat# 10488-085
- 100 bp DNA Ladder cat# 10488-058

#### Company:

Invitrogen

Invitrogen

#### RNA Analysis:

The ribosomal RNA (28S, 18S, 5,85S and 5S bands), which was visible in ethidium bromide stained gels was used as a standard.

28 S	4718 bp
18S	1874 bp
5,85S	160 bp
5S	120 bp (in eukaryonts)

### 2.1.10 Kits

Absolutely RNA <sup>TM</sup> RT-PCR Miniprep Kit	Stratagene
Effectene <sup>TM</sup> transfection Reagent	Qiagen, Hilden
<i>Polyfectamine<sup>TM</sup> Transfection Kit</i>	<i>Qiagen, Hilden</i>
Hexalabel Plus <sup>TM</sup> DNA Labeling Kit	MBI Fermentas, Vilnius
High Sensitivity $\beta$ -galactosidase Assay Kit	Stratagene
One-Shot® TOP10	Invitrogen
Plasmid Purification Kit	Qiagen, Hilden
Qiaquick Gel Extraction Kit	Qiagen, Hilden



**2.1.11 Others**

Autoradiography cassettes (Kodak)	Sigma, Deisenhofen
Cell culture flasks	Costar
Cell culture plates	Greiner, Nürtingen
Cryo-tubes	Greiner, Nürtingen
Eppendorf Tubes	Eppendorf, Hamburg
Films (Hyperfilm <sup>TM</sup> ECL)	Amersham-Pharmacia, Freiburg
Glass pipettes	Renner, Darmstadt
Glass wool	Serva, Heidelberg
Nylon membranes (GeneScreen Plus <sup>TM</sup> )	NEN Lifescience Products Inc
Paperfilters	Schleicher and Schüll, Dassel
Pipet Tips (Sterile)	Becton and Dickinson, Heidelberg
Pipet Tips	Greiner, Nürtingen
Photometer plastic Cuvettes	Greiner, Nürtingen
Polypropilene conical tubes	Falcon, Becton-Dickinson
PVDF-membranes (Immobilon P)	Millipore, Eschborn
Sterile filters	Millipore, Eschborn
X-ray films	Fuji
Whatmann 3MM paper filter	Schleicher and Schüll, Dassel

## 2.2 METHODS

### 2.2.1 Cell culture techniques

#### 2.2.1.1 Cell culture

Cervical carcinoma cell line HeLa, SW756, human fibroblast and HeLa hybrid cells 444 and CGL-3, and the stably transfected TE-671 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FCS, 100 U/ml penicillin and 100 µg/ml streptomycin.

The E6- and E7- immortalised keratinocytes were sustained in Keratinocyte Growth Medium (Serum –Free Keratinocyte Medium supplemented with Bovine Pituitary Extract (BPE; 20-30 µg/ml).

In order to keep the selection pressure the clones were grown in a media containing 750 µg/ml to 1 mg/ml G418. The concentration of G418 was increased to achieve a better selection. Incubation was carried out at 37°C with 5% CO<sub>2</sub> and 95% humidity. Cell cultures that reached sub-confluent growth (80-90% confluence) were washed once with PBS and treated with Trypsin/EDTA at 37°C until the cells detached from the plate. The trypsinization was stopped by the addition of complete DMEM and the cells were splitted between 1/5 and 1/30.

#### 2.2.1.2 Thawing and freezing of eukaryotic cells

For thawing, the cryo-vials were warmed up to 37°C, once unfrozen the cell-containing medium was transfer to a 15 ml falcon tube and diluted with DMEM complete medium in the ratio of 1:10. The cells were sedimented by centrifugation at 2000 rpm and 4°C, for 5 min (Varifuge RF, Heraeus). Then the pellet was resuspended in fresh medium and transferred to a culture flask or plate for incubation at 37°C with 5% CO<sub>2</sub> and 95% humidity.

For freezing sub-confluent culture plates (80-90% confluence) were trypsinized as mentioned in 2.3.1.1. The cells were centrifuged for 5 min at 2000 rpm (Varifuge RF, Heraeus), 4°C and the pellet was resuspended in freezing medium. The cell suspension was transferred to cryo-vials (~ 1ml = 10<sup>6</sup> cells/ml/vial) and cooled down to -70°C. Later the cryo-vials were transferred to a storage tank with liquid nitrogen for long term conservation.

#### 2.2.1.3 Cell Counting

In order to determine the cell concentration (cells/ml), trypan blue staining was used which allows visualising of living cells. After trypsinization the cell suspension was transferred to a 15 ml blue tube (Falcon). A certain volume of cell suspension was mixed with trypan blue dye solution in a ratio of 1:1. Living cells were counted in a Neubauer chamber.

The number of cells was calculated as follows:

$$\text{Cells / ml} = \frac{\text{number of cells counted} \times 10^4 \times \text{Dilution factor}}{\text{Number of squares counted}}$$

#### **2.2.1.4 Cell treatment with Sodium Butyrate, MG-132, TLCK**

- i) Confluent cells were treated with 6 mM SB (0,66 mg/ml) dissolved in DMEM for 16 or 24 h. The solution was always prepared fresh.
- ii) Confluent cells were treated with 50  $\mu$ M, 100  $\mu$ M, 125  $\mu$ M and 250  $\mu$ M TLCK for 16 hours with or without SB. The stock solution for TLCK was prepared as 50 mM dissolved in bi-distilled water. The control treatment was implemented using DMEM.
- iii) Confluent cells were treated with 2  $\mu$ M of MG132 for 16 h. The stock solution was prepared at 20 mM and stored at -20°C. The final concentration of the solvent DMSO in the culture media was 0.1% (v/v). The control cultures received media containing 0.1% (v/v) DMSO alone and were incubated under the corresponding conditions.

### **2.2.2 Preparation of DNA probes**

#### **2.2.2.1 Transformation of Competent bacteria (Dagert and Ehrlich, 1979)**

Commercially available One-shot® TOP 10 competent cells were thawed on ice, 2 ng of DNA were added, mixed and incubated for 30 minutes on ice. Later the cells were incubated at 42°C for 30 seconds and then chilled on ice for 2 minutes. The cells were diluted 1:5 in pre-warmed SOC-Medium and incubated for 1 hr at 37 °C under constant shaking. Twenty and two hundred microliters of the transformed cells were plated on LB-ampicillin plates and incubated at 37°C overnight. Next day, an individual colony was picked, transferred to 10 ml ampicillin-containing LB-medium and incubated for about 8 h at 37°C.

#### **2.2.2.2 Plasmid-DNA restriction analysis**

See section 2.3.4.3 DNA restriction analysis and 2.3.4.4 DNA electrophoresis and Southern blot.

#### **2.2.2.3 DNA extraction from agarose gel (QIAquick gel extraction kit)**

DNA fragments were extracted from agarose gels using the QIAquick gel extraction kit (Qiagen). The protocol allows the extraction of DNA fragments in size ranges between 100 bp to 10 Kbp. After agarose gel electrophoresis the DNA fragment was cut out from the gel, transferred into an eppendorf tube and treated (subsequently) as described in the attached instruction manual.

### 2.2.3. Protein analysis

#### 2.2.3.1 Preparation of nuclear and cytoplasmic proteins (Schreiber et al., 1989)

Nuclear extracts were prepared using the method of Schreiber et al. with the only modification that phosphatase inhibitors and protease inhibitors were included in the buffers A and C at concentrations suggested by the manufacturer.

Stock:	Activity:	Final concentration:
0.1 M DTT		1 mM
23.8 mg/ml (in H <sub>2</sub> O) Pefabloc SC	Serine proteases inhibitor	0.5 mg/ml
2.5 µg/ml (in H <sub>2</sub> O) E-64	Cysteine-protease inhibitor	5 µg/ml
0.5 M NaF	Phosphatase inhibitor	1 mM
10 mM Na <sub>3</sub> VO <sub>4</sub>	Phosphatase inhibitor	0.2 mM

For the nuclear-cytoplasm protein extraction a 60 cm<sup>2</sup>-culture plate with 90-100% confluence (app. 3x10<sup>6</sup> cells) was washed twice with isotonic PBS. Then 1.6 ml of the hypotonic buffer A was added and the cells were scrapped. The cell suspension was placed into an eppendorf tube and cooled on ice for 15min to allow swelling of the cells. Subsequently, 100 µl of a 10% Nonidet P-40 solution, a non-ionic detergent were added to the cell suspension and the eppendorf tube was vortexed for 10sec for plasma membrane lysis. After centrifugation at 14.000 rpm, 1 min., 4°C the supernatant contains the cytoplasmic proteins and the pellet the intact nuclei, which kept the nuclear proteins inside.

The cytoplasm protein-containing extract was aliquoted and stored at -70°C. In this extracts the protein concentration was determined by the Biorad DC (Detergent Compatible) protein assay based on a modified Lowry method (Lowry, 1951). For each sample 2-4 µl of cytoplasmic protein extract was diluted in 200 µl of bi-distilled water, next 100 µl of the reagent A\* (1ml Reagent A plus 20 µl Reagent C) and 800 µl of Reagent B were added, mixed and incubated for 15min at room temperature. Finally, the absorbance of the samples was measured photometrically at 750 nm and the concentration was determined using a standard BSA curve as reference.

The nuclei-containing pellet was resuspended in 200 µl of ice cold buffer C and placed on ice for 15 min mixing it every 2 min. Finally, the extract was centrifuged at 14.000 rpm, 5 min, 4°C and the nuclear protein containing-supernatant was aliquoted and stored at- 70°C. The protein concentration of the nuclear extracts was determined by Bio-Rad Bradford-Method (Bradford, 1976). Two to five µl of the nuclear protein preparation were diluted in 800 µl of bi-distilled water and 200µl of the Biorad reagent was added. After gentle mixing the absorbance was

measured at 595 nm and the protein concentration was determined using defined amounts of BSA as standard.

#### **2.2.3.2 SDS-total protein extracts (Harlow and Lane, 1988)**

To prepare protein extracts, cells were washed twice with cold PBS and were lysed with 500 µl of RIPA buffer supplemented with freshly added proteases inhibitor cocktail (Complete™). The lysates were scrapped and transferred to an eppendorf tube. Afterwards, the lysates were sonified three times for 15 sec (50% power capacity, position 5) at 4°C and incubated for 30 min on ice. Following centrifugation for 5 min at 14000 rpm and at 4°C, the supernatant was divided in 200 µl aliquots and stored at -70°C. Protein concentration was determined by the Bradford method (Bradford, 1976). 2 µl of cell extract were diluted in 798 µl of bidistilled water, and 200 µl of the Bio-Rad reagent were added, mixed gently and quantified at 595 nm. The concentration was determined automatically by the spectrophotometer using BSA as reference.

#### **2.2.3.3 SDS-Polyacrylamide gel electrophoresis (Laemmli, 1970; Hames and Rickwood, 1990)**

This system is based on a discontinuous gel in which the stacking and separating gel layers differ in salt concentration, pH and acrylamide concentration. Depending on the size of the protein 8% to 15% acrylamide gels were used, the separating gel contains 0.4M Tris pH 8.8, 0.1%SDS, 8-15 % Acrylamide/Bis-acrylamide (29:1), 0.5% ammonium persulfate and 0.07% TEMED. After 1 h of polymerisation at room temperature, a 3% stacking gel solution was added, the stacking gel contains 0.125M Tris pH 6.8, 0.1% SDS, 3% Acrylamide/Bis-acrylamide (29:1), 0.5% ammonium persulfate and 0.16% TEMED. Between 25-50 µg of total protein were mixed with 2x Western loading buffer, heated at 95°C for 5 Min and loaded on the gel. The run was performed in 1x Laemmli's running buffer and the running time was approximately 2 h (15 mA until dye front reached separating gel, then 30 mA).

#### **2.2.3.4 Western Blot "Semidry" (Gallagher et al., 1997)**

The proteins were transferred from the gel to polyvinylidene fluoride PVDF-membranes using a semidry blotting system. After electrophoresis the transfer stack was assembled: on the semidry blotter 9 sheets of whatman 3MM filter paper were placed cut to the gel dimensions and soaked with 1x Towbin buffer, followed by the pre-activated membrane and on top of the gel another 9 sheets of whatman 3MM paper soaked with Towbin buffer. The transfer proceeded at 0.8-1.2 mA/cm<sup>2</sup> at 4°C for 70 min, depending on the protein size.

Then PVDF-membrane was incubated for 1 hour at room temperature in blocking solution, and subsequently incubated overnight at 4°C with the first antibody (diluted in blocking solution or BSA following the manufacturer recommendations). Next day, the membrane was washed five times with TBST 5 min each, followed by the incubation with the secondary antibody in blocking solution for 1h at room temperature. Finally, the membrane was washed again as described before, incubated for 2 min with ECL reagent and exposed to Hyperfilm™ ECL films. In order to use the same membrane for incubation with additional antibodies, membranes were “stripped” in 200mM NaOH for 5 min and washed with water and TBS. Then they were ready for blocking and further antibody incubation.

## **2.2.4 Nucleic acid analysis**

### **2.2.4.1 RNA extraction (Absolutely RNA™ RT-PCR Miniprep kit)**

RNA was isolated with the Absolutely RNA™ RT-PCR miniprep kit (Stratagene) according to manufacturer's instructions. For the RNA extraction a 60 cm<sup>2</sup>-culture plate with 90-100% confluence (appr.  $3 \times 10^6$  cells) was washed twice with isotonic PBS, 500 µl of PBS were added and the cells were scrapped and transferred to an eppendorf tube. Cells were sedimented at 3000 rpm (Varifuge RF, Heraeus), room temperature, 5 min. The cell pellets were re-suspended in 600 µl of Lysis buffer supplemented with 4.2 µl of 2-mercaptoethanol and vortexed repeatedly to ensure that the viscosity of the lysate was low. Afterwards, the lysate was filtered and mixed with 70% DEPC-ethanol. The filtrate was transferred to a Fiber-Matrix Spin Cup in order to allow the binding of the nucleic acids to the matrix. The matrix was washed once with low salt washing buffer and then the DNase treatment was proceeded. Next, the matrix was washed once with high salt buffer and twice with low salt buffer and the pure RNA was eluted from the matrix with TE pH 7.5 at 60°C. The concentration of the RNA was determined photometrically by measuring the optical density (OD) at 260/280 nm. 1 OD unit corresponds to 40 mg RNA/ml. To check RNA quality, 5 µg of RNA were separated on a 1% agarose /1X MOPS gel in the presence of ethidium bromide under non-denaturing conditions (section 2.2.4.5.)

### **2.2.4.2 DNA extraction from eukaryotic cells**

Cells were washed twice with PBS, incubated with 3 ml “DNA lysis buffer” for 10 min at room temperature, scrapped and transferred into 15 ml falcon tubes. Proteinase K was added to a final concentration of 200 ng/ml and incubated at 56°C for 1 hour. Nucleic acid was purified using phenol/ chloroform extraction. After centrifugation, the upper DNA-containing aqueous phase was transferred into a new tube where DNA was precipitated after addition of ammonium acetate (2 M final concentration) and 0.8 volumes of isopropanol. The mixture was centrifuge and the

pellet was washed twice with 70% ethanol, air-dried and resuspended in TE buffer. The concentration of DNA was determined photometrically by measuring the optical density (OD) at 260/280 nm. 1 OD unit corresponds to 50 mg DNA/ml. DNA was treated with RNase A (Ribonuclease A, 10µg/ml) for 10 minutes at 37 °C.

#### **2.2.4.3 DNA restriction analysis**

For restriction analysis, 1 µg of DNA was supplemented with 2U of restriction enzyme with the corresponding buffer as recommended by the manufacturer. After 2 hours at 37°C, DNA was loaded on agarose gels.

#### **2.2.4.4 DNA electrophoresis and Southern Blot**

Agarose gel electrophoresis is a standard method to separate, identify and purify nucleic acid fragments. The concentration of the agarose used for the gel depends on the size of the DNA fragments to be analysed. By using the fluorescent intercalating dye ethidium bromide at a final concentration of 0.5 µg/ml, the visualization of the nucleic acids within the gel is possible.

The required amount of agarose was added to the appropriate amount of 1X TAE buffer and the mixture was heated to allow the agarose to dissolve. The solution was cooled down and ethidium bromide was added to a final concentration of 0.5 µg/ml. The gel was poured and allowed to solidify. The gel was transferred to an electrophoresis chamber filled with TAE 1X buffer and the samples were loaded after being mixed with agarose loading buffer (6:1). The electrophoresis proceeded between 2-8 V/cm. Later the gel was placed on an UV transilluminator and photographed.

After DNA restriction and electrophoresis, the agarose/1X TAE gel was incubated for 45 min in denaturing solution.

For blotting, a pack of high absorbent paper was used and 4 pieces of 3MM paper were placed on it (first 2 papers dry and then 2 papers wet after soaking in non-denaturing solution). Gene Screen nylon membrane, previously incubated for 1min in bidistilled water and 15 min in denaturing-solution, was placed in the 3MM wet paper. Finally, the agarose gel was laid on the nylon membrane (see Figure 2.1). The whole assembly was then covered with plastic paper and left overnight at room temperature. Next day, the nylon membrane was washed for 15 min in neutralization solution and air-dried over 3MM paper. The membrane was then ready for hybridization.

#### **2.2.4.5 RNA electrophoresis and Northern Blot (Alwine et al., 1977)**

RNA was separated in 1% agarose/ 1X MOPS/ ethidium bromide gels under non-denaturing conditions. One volume of RNA-loading buffer was added to each probe, heated 10 min at 65°C and cooled on ice for 3 min before loading. The gel was run at 60 V for 2 h and then photographed.

After electrophoresis the gel was incubated for 15 min in 20X SSC buffer. For blotting, a pack of high absorbent paper was used and 4 pieces 3MM paper were placed on it (first 2 papers dry and then 2 papers wet by soaking in 20X SSC buffer). Gene Screen nylon membrane previously incubated for 1 min in bi-distilled water and 15min in 20X SSC was laid on the 3MM paper. Finally, the gel was deposited over the nylon membrane and the whole assembly was wrapped with plastic paper (see also Figure-2.1). RNA transfer proceeded over night at room temperature. Next day, the nylon membrane was washed for 5 min in 2X SSC and air-dried over 3MM paper. After this step, the membrane was ready for hybridization.

#### **2.2.4.6 Probe labelling, “Random Priming” (Feinberg and Vogelstein, 1983) and hybridization (Southern, 1975)**

The radioactive probes for hybridization analysis were obtained by random priming, in which a mixture of random hexamers is used to prime DNA synthesis *in vitro* from a linear denaturated DNA template. For this purpose the “Hexalabel™ DNA labelling kit” was used. Briefly, 100ng DNA were diluted in 1X reaction buffer, boiled 10min and cooled down on ice. Later, the appropriated nucleotides mix (-dCTP), 25 µCi/µl  $\alpha^{32}\text{P}$ -dCTP and 5U of Klenow-polymerase were added. The reaction was incubated for 30 min at 37°C, then a complete dNTP mix was added and the incubation was prolonged 5 min. Subsequently, the unincorporated label was removed by filtration of the TNE-diluted labelling reaction through a Biogel-suspension column. Blot pre-hybridization was performed in hybridization buffer for at least 1 h at 42°C, after that denaturated radioactive probe was added to the pre-hybridized membrane and hybridization proceeded over night. Next day, filters were washed two to three times depending on the radioactive signal, with hybridization washing buffer at 68°C and exposed on Hyperfilm™ ECL films at -70°C.

#### **2.2.4.7 Reverse transcription and Polymerase Chain Reaction (PCR): Semi-quantitative RT-PCR**

cDNA was obtained from one to five micrograms of RNA by using oligo(dT)22 or gene specific primers (as template-primer) and SuperScript II reverse transcriptase following the manufacturer's instructions. The Oligo(dT)22 (500µg/ml) or gene-specific primer (GSP) (2



pmol) and the RNA were heated at 70°C for 10 min. and chilled on ice. Then the mixture was supplemented with 1X reverse transcription (RT) buffer (50mM Tris-HCL pH 8.3, 75mM KCl, 3mM MgCl<sub>2</sub>), 10mM DTT, 500μM dNTPs and incubated at 42 °C for 2 min for the annealing step. After annealing, 100 U of reverse transcriptase SuperScript II were added and the reaction was incubated for 50 min at 42°C for the cDNA first-strand synthesis. Finally, the cDNA was heated to 70°C for 15 min and chilled on ice.

RT-PCR reactions were prepared with the Oligo(dT)<sub>22</sub> primer in order to analyse the transcriptional level of pRb, GAPDH and p73 in HeLa cells while the reverse primer sequence from the p73 (Sayan et al., 2001) was deployed as a gene-specific primer (GSP) for the RT reaction in E6 and E7 keratinocytes in order to analyse the p73 transcription.

PCRs were performed in 50μl final volume containing 10mM Tris-HCl pH 8.3, 200μM dNTPs mix, 500nM of each primer, 50mM MgCl<sub>2</sub>, 5U of Taq platinum polymerase and 1-5 μl of cDNA. Except of some individual cases all PCR reactions were performed for 35 cycles consisting of the following steps:

1. Step: Denaturation at 95°C for 5 min.
2. Step: Denaturation at 95°C for 30-45 sec.
3. Step: Annealing with corresponding temperature for 30 sec (1 min. for p73 PCR with the primers from Rainer Schmidt)
4. Step: Extension at 72°C for 1min (2 min. for p73 PCR with the primers from Rainer Schmidt)
5. Step: repeat of the steps from 2 to 4 for 34 times
6. Step: Final extension at 72°C for 10 min.

The amount of cDNA and the number of cycles were carefully standardized in order to reach a semi-quantitative indication of the RNA content. The PCR products were analysed in 0,5-2% agarose gels.

Notably, for the transcriptional analysis of p73 two different primer pair was used. Thus for HeLa cells the primers designed from Rainer Schmidt, while for the E6 and E7 keratinocytes the primer sequences from Emre Sayan (MRC Toxicology Unit, University of Leicester, UK) (Sayan et al., 2001) were applied.

## 2.2.5 Transfection analysis

### 2.2.5.1 Stable transfection protocol “Effectene”

The stable transfections of the TE-671 cells (section 3.2) were done according to the Effectene protocol. Thus  $2.5 \times 10^6$  cells were plated in 60-cm<sup>2</sup> dishes the day before transfection. Next day, 2μg of the plasmid DNA (pcDNA3.1/16E7-Kozak and pcDNA3.1/(-)) were mixed with 300 μl of DNA-condensation buffer, 16μl of enhancer and 60μl of effectene, following the incubation

time recommended to allow the complexes formation, 3 ml of complete DMEM were added and the transfection complexes were poured onto the cells. Cells were incubated overnight in the presence of the pDNA-transfection-complexes. Next day cells were washed with PBS and new DMEM medium was added. After the cells started to grow exponentially, DMEM medium was supplemented with the selection marker Geneticin (G418). The final concentration of the selection marker was 1mg/ml. Approximately 3 months after transfection cells were plated on 96-well plates to select single clone cells. Approx. after 6 months single cell clones, which were growing exponentially were plated on 60 cm<sup>2</sup> dishes later on 100- cm<sup>2</sup>. In order to check the presence of the introduced plasmid protein western blot analysis was performed.

The transfection efficiency in TE-671 cells was higher than 30% as determined by transfection with expression vector carrying the enhanced green fluorescence protein (EGFP).

#### **2.2.5.2 Transient transfection by Calcium-Phosphate co-Precipitation (Chen and Okayama, 1987).**

In this transfection protocol, calcium phosphate forms complexes with DNA gradually in the medium during incubation with cells and precipitates on the cells. The crucial factors for obtaining efficient transformation are the pH (6.95) of the buffer used for the calcium phosphate precipitation, the CO<sub>2</sub> level (3%) during the incubation of the DNA with the cells, and the amount (20 to 30 micrograms) and the form (circular) of DNA.

Thus, for the experiment in section 3.2 confluent grown HeLa cells were transiently transfected in 100-cm<sup>2</sup> Petri dishes with 20 µg of the respective pSUPER-derived vector constructs. For each transfection the following mixture was prepared:

420µl H<sub>2</sub>O were mixed with 20µg of the corresponding plasmid DNA. After addition of 500µl 2X HBS were mixed again. Immediately after adding 62µl of 2M CaCl<sub>2</sub> the samples were vortexed vigorously. The final volume of 1ml was added to the cell medium. After incubation at room temperature for 30 min the solution was mixed gently by pipetting up and down. The mixture was added to the cells dropwise.

After 12 hours cells were washed once and incubated for additional 24 hours in supplemented DMEM medium. Afterwards, cells were treated with 6 mM SB for 24 hours or the medium was changed. Finally, cells were harvested for protein and for RNA extraction (Figure-19 A and B). As control, HeLa cells were transfected with pSUPER-vector alone and pSUPER-vector expressing luciferase gene which should not interfere with any mammalian transcript.

The transfection efficiency in HeLa cells was higher than 80% as determined by transfection with expression vector carrying the enhanced green fluorescence protein.

### **2.2.6 DNA staining with DAPI and SR 101 for flow cytometry and cell cycle analysis**

As indicated in section 3.5 and 3.6 cells were analysed for their cell cycle distribution and for their sub-G1 fraction. Therefore  $4 \times 10^4$  cells per  $60\text{-cm}^2$  dish were seeded. After incubation for the corresponding treatment, the old DMEM medium (5 ml) has been transferred in a 15ml falcon tube. Cells were washed with 3 ml 1x PBS and this part was also added to the collection tube. Afterwards, cells were harvested with 2ml trypsin. The trypsinated cells were also added into the same tube. Cells were gently centrifuged at 1500 rpm for 5min. Next cells were washed three times with 1x PBS. Finally, cells were resuspended in 1ml 1x PBS and were fixed with ethanol (above 75%) for a minimum of 12 hours.

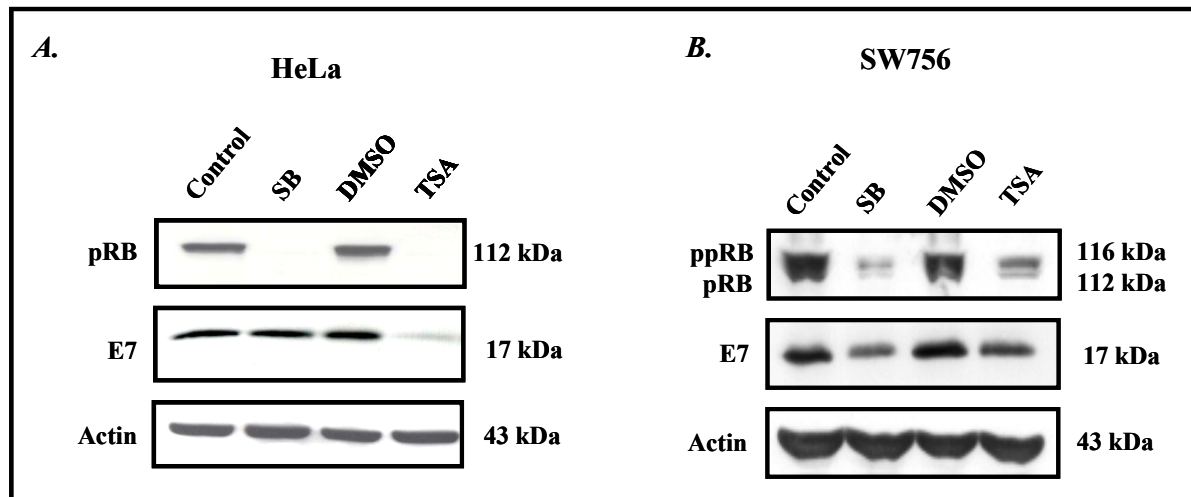
After mild centrifugation at 100 g for 15 min. the cell pellet was resuspended in a fluorochrome mixture of TRIS buffer (0.18M, pH 7.5) containing DAPI (Diamidinophenylindole from Serva  $5 \times 10^{-6}$  M) for DNA staining and SR 101 (sulforhodamine 101 from Eastman Kodak,  $5 \times 10^{-5}$  M) as a protein counter stain, exactly following a protocol published by Stoehr et al. (1978). Flow cytometry was performed using the UV lines of an argon ion laser to excite both the blue and red fluorescence of DAPI and SR101, respectively, which could easily be separated by simple optical filtering. Blue fluorescence of DAPI has been collected above 450 nm. Analysis of the cellular DNA frequency distributions for apoptosis and cell cycle distribution was carried out with computer assistance according to Dean and Jett (1974) and Stoehr et al. (1976 and 1991).

### III. RESULTS

#### 3.1 Analysis of HPV- positive cell lines after HDAC inhibition

##### 3.1.1 HDAC inhibitors induce pRb degradation only in the presence of HPV oncogenes

To analyse the degradation of pRb protein in the presence of viral E7 oncoprotein following administration of HDAC inhibitors (HDACi), HPV18 transformed HeLa cells were initially used. For preliminary analysis, HDAC inhibitor concentrations of SB (6 mM) and TSA (330 nM) were chosen. The cells were treated for 24 hours and total protein extracts were prepared. Figure-3.1 A, shows pRb degradation upon HDAC inhibition while the level of E7 oncoprotein was not affected by SB. To determine whether HDACi induced pRb degradation was cell line specific, another HPV18 positive cell line was tested. SW756 is a cervical cancer cell line containing 10-to 50 copies of HPV18 DNA integrated at a single site on chromosome 12 (Schwarz et al., 1985; Popescu et al., 1987a and b; Mincheva et al., 1987). Figure-3.1 B also demonstrates pRb degradation at the same HDAC inhibition conditions previously used for HeLa cells. However, the expression levels of E7 were moderately affected.



**Figure-3.1: Treatment of HPV 18 positive HeLa and SW756 cells with HDAC Inhibitors SB and TSA induce pRb degradation**

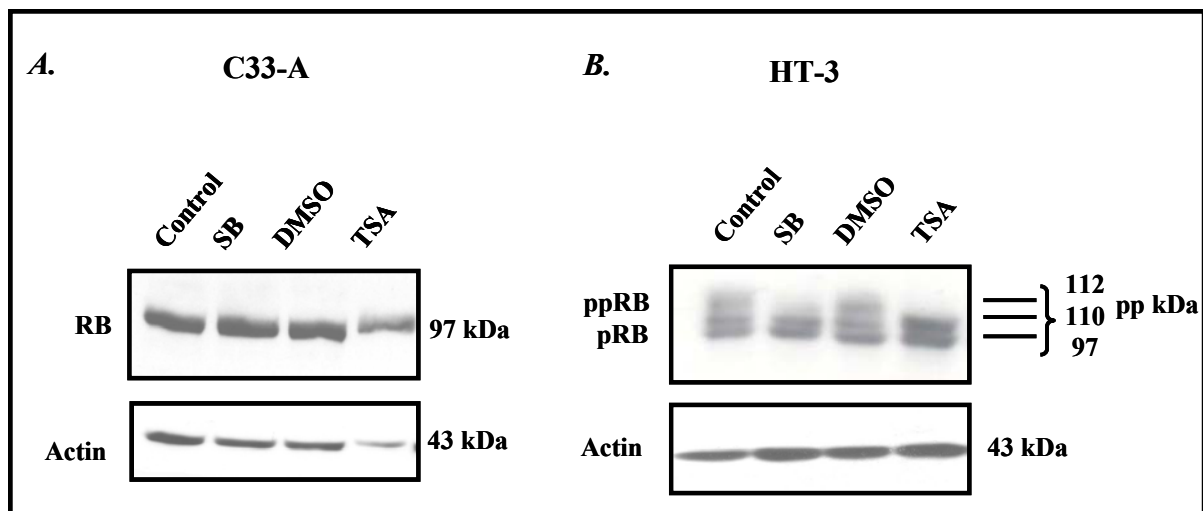
Western Blot analysis of cellular extracts obtained from (A) HeLa and (B) SW756 cells. Proteins were separated in 8% (pRb) and 12% SDS-PAGE. After electrotransfer, the filters were incubated with anti- pRb, HPV18E7. Control: untreated cells, DMSO: Cells treated with 0.1 % DMSO. Treatments for SB (6 mM) and TSA (330 nM) were done for 24 h. The protein loading was confirmed by using anti-actin antibody.

The multiple bands of pRb represent different phosphorylation states of the protein. In HeLa cells, only TSA down-regulated E7 protein levels, whilst in SW756 only a moderate repression

of the oncogene was visible. This might be due to the fact that TSA is an HDAC inhibitor with a broader spectrum than SB (Marks et al., 2001).

These first results, suggested there were two potential perspectives of how the observed degradation of pRb may occur: One possibility is the tumorigenic phenotype and secondly the presence of the viral oncogene E7. To discriminate between these two options, the HPV negative cervical cancer cells C33-A and HT-3 (Heilman et al., 1980) were analysed.

As demonstrated in Figure-3.2, HDAC inhibition SB and TSA resulted in an unaltered level of pRb protein. Notably, in each of the HPV-negative cell lines, HDAC inhibitors only altered pRb mobility. The faster migrating form of pRb, appeared as a 97 kDa band in both cell lines, indicates the presence of the hypophosphorylated form of pRb. In the case of HT-3 cells, levels of hyperphosphorylated forms of pRb protein could also be detected as described by Scheffner et al., 1991.



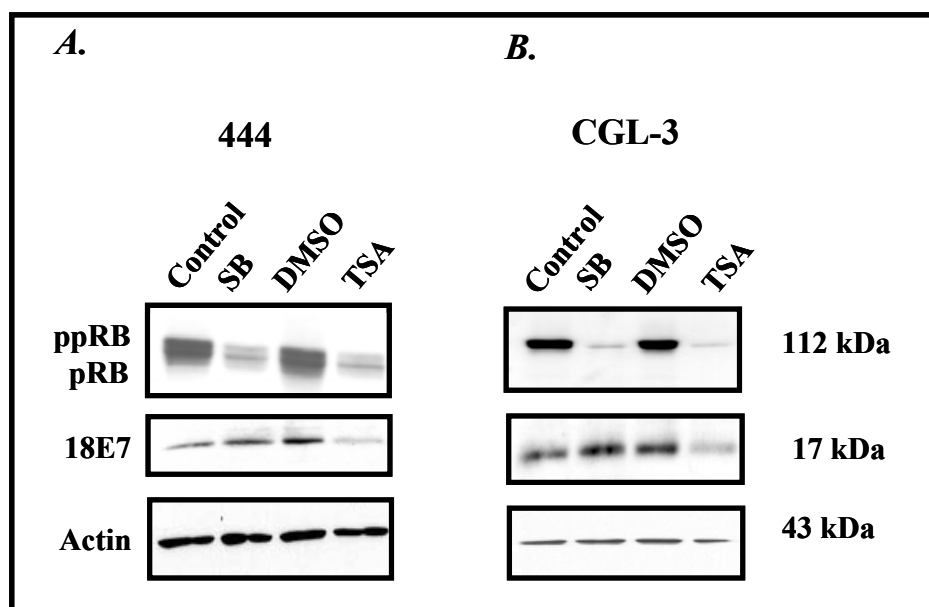
**Figure-3.2: Immunoblot Analysis of HPV negative cells, C33-A and HT-3**

Analysis of pRb in C33-A (A) and HT-3 cells (B). 75 µg of cellular extracts were separated on a 8% SDS-PAGE gel (pRb). After electrotransfer, the filters were incubated with anti-pRb and anti-actin antibodies. Control: untreated cells, DMSO: Cells treated with 0,1% DMSO. Treatments for SB and TSA were done for 24 h. Position of the pRb protein in hypophosphorylated (indicated here as pRb) or hyperphosphorylated (ppRb) forms.

From these results one can conclude that the presence of viral oncogenes, especially E7, is obviously a prerequisite for the observed pRb degradation in the presence of HDAC inhibitors. To prove this hypothesis and to exclude that the tumorigenic phenotype is a critical determinant, somatic cell hybrids were used (Stanbridge et al., 1976). This system is based on cell fusion between HeLa and non-tumorigenic fibroblast, resulting in non-tumorigenic cells (444). Long term *in vitro* propagation results in tumorigenic segregants (CGL-3).

The advantage of this model system is that these cells retain the integration and transcription pattern of HPV18 sequences of the parental HeLa cells whilst their *in vivo* properties are different. Inoculation of CGL-3 cells into athymic nude mice leads to a formation of progressive tumors, while “444” cells do not (Stanbridge et al., 1982) and are therefore considered to be non-tumorigenic.

‘444’ and the ‘CGL-3’ cells were treated with 6 mM SB or with 330 nM TSA for 24 hours. Figure-3.3 shows that, similar to HeLa cells, pRb was degraded. Hence, pRb degradation upon SB and TSA was occurred independent from the *in vivo* phenotype.



**Figure-3.3: Treatment of somatic cell hybrids with HDAC Inhibitors**

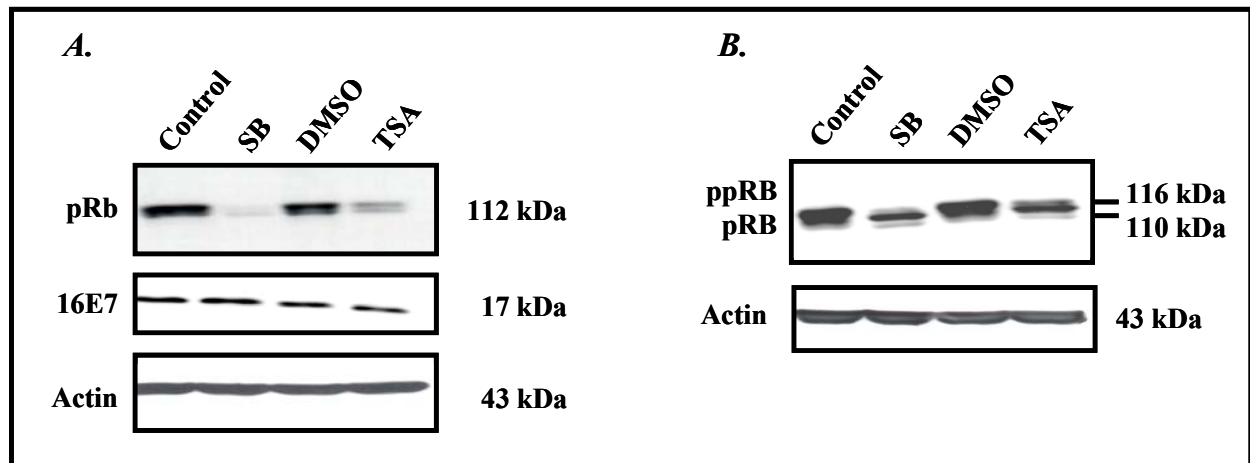
Western Blot analysis of cellular extracts (75 µg) obtained from (A) 444, (B) CGL3 cells after 24 h treatment. Total protein lysates were separated on 8% (pRb) or 12% (HPVE7) SDS-PAGE gels. After electrotransfer, the membranes were incubated with anti- pRb, HPV18E7 and anti-actin antibodies. Control: untreated cells, DMSO: Cells treated with 0.1 % DMSO. SB: (6 mM), TSA: (330 nM)

In summary, these data show that apparently the presence of the viral oncogenes is the major determinant for pRb degradation.

### 3.1.2 HPVE7 is selectively responsible for the degradation of pRb in the presence of HDAC inhibitors

In order to analyse which of the two viral oncogenes is responsible for the pRb degradation upon HDAC inhibition, human keratinocytes were used, which were individually immortalised with amphotropic retroviruses carrying either HPV16E6- or E7 (Aguilar-Lemarroy et al., 2002).

As demonstrated in Figure-3.4 A by Westernblot analysis, only E7 immortalised keratinocytes showed pRb degradation with SB and TSA, whereas in E6 immortalised cells, a hypophosphorylated form of pRb can be detected (Figure-3.4 B). This result suggests that pRb degradation is dependent on the presence of E7.



**Figure-3.4: Western Blot analysis of pRb and viral proteins of (A) E7-, (B) E6- immortalised keratinocytes after SB and TSA treatment.**

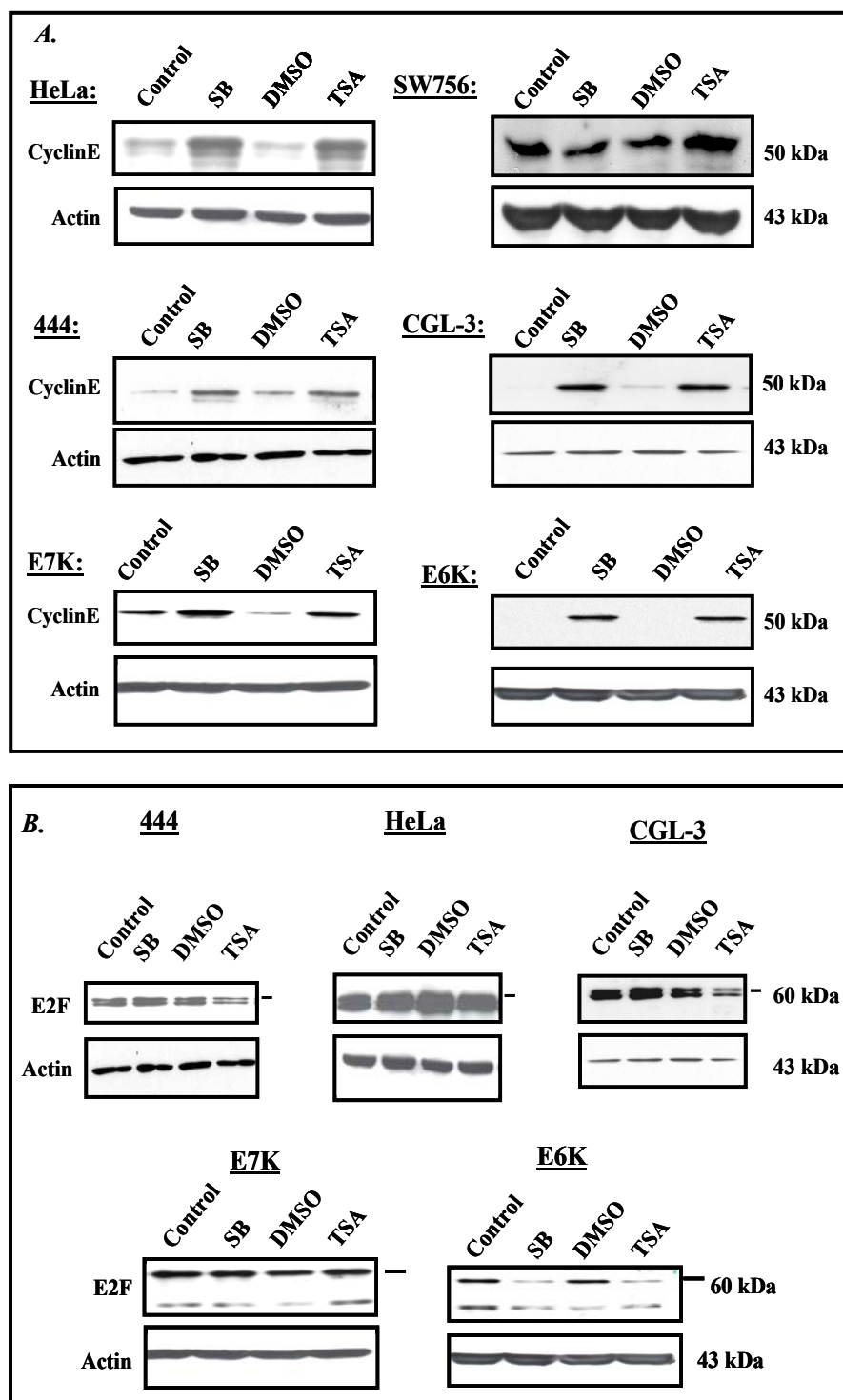
Western Blot analysis of cellular extracts obtained from (A) E7- and (B), E6-immortalised keratinocytes were separated on 8% (pRb) and 12% (E7) SDS-PAGE. After electrotransfer, the membranes were incubated with anti-pRb, and with anti-HPV16-E7 antibodies. Equal protein loading was confirmed by incubation of the membranes with an anti-actin antibody. Control: untreated cells, DMSO: Cells treated with 0.1 % DMSO, SB (6 mM), and TSA (330 nM). Treatments were done for 16 h.

### 3.1.3 Degradation releases negatively regulated target genes from pRb suppression

One of the main functions of pRb is the transcriptional regulation of genes involved in the G1 to S phase transition such as cyclin E, DNA polymerase- $\alpha$ , dihydrofolate reductase (DHFR), thymidine kinase (TK) (Weinberg et al., 1995b; Muller et al., 2001). The pRb protein is able to repress the transcription of the cell cycle regulatory proteins by binding to E2F, a transcription factor playing the major role during cell cycle progression and by recruiting the HDAC1 molecules to their promoters (Brehm et al., 1998; Harbour and Dean 2000a; Zhang and Dean, 2001). Therefore one might predict that a decrease in pRb levels could lead to increased release of E2F, which in turn can bind to target sequences in the respective genes and induce their transcription. Indeed, in tumorigenic HPV positive cells and keratinocytes expressing either E6 or E7, a clear increase of cyclin E expression was observed (Figure-3.5A). The E2F levels

remained unaffected in HPV positive cells and in E7 keratinocytes while a slight downregulation was observed in E6 keratinocytes (Figure-3.5 B).

Nevertheless, these results indicate that degradation of pRb was accompanied by an increased expression of genes, negatively regulated by pRb.



**Figure-3.5: Enhanced degradation of pRb upon HDAC inhibition activates E2F: Upregulation of cyclin E expression in HPV18 positive cells and in immortalised E6- and E7 keratinocytes**

Western Blot analysis of cellular extracts (75 µg) obtained from HeLa, SW756, 444 and CGL-3 cells and from HPV16E7- or E6 immortalised keratinocytes. Total protein lysates were separated on 12% SDS-PAGE.

After electrotransfer, the membranes were incubated with anti-cyclin E (A) and anti-E2F (The upper band corresponds to the 60 kDa E2F protein) (B). For loading control same membranes were incubated with anti-actin antibodies. Control: untreated cells, DMSO: Cells treated with 0.1 % DMSO. Treatments for SB (6 mM) or TSA (330 nM) were applied for 24 h (HeLa, SW756, 444, CGL-3) and for 16 h (E7- and E6 keratinocytes)

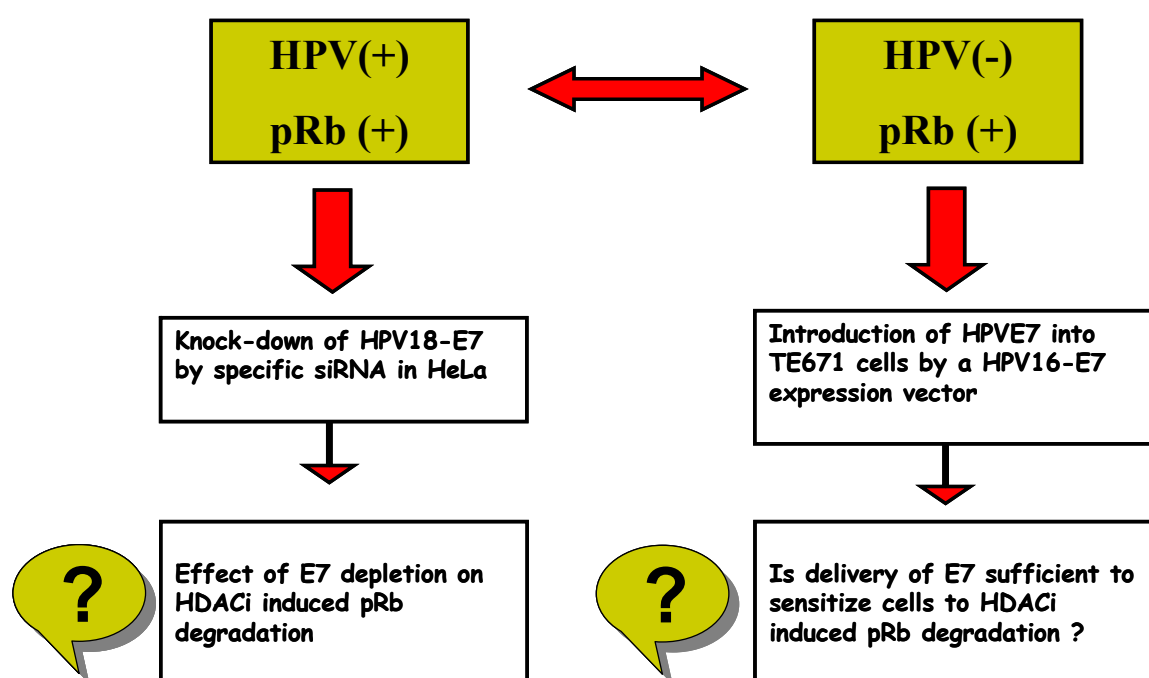


### 3.2 The role of HPV E7 in pRb degradation by SB

Based on the previous results, a functional link between pRb degradation after HDAC inhibition and the presence of HPVE7 oncoprotein was predicted.

Therefore, the following experimental strategy was carried out (Figure-3.6):

- Monitoring the effect of HDAC inhibitors on pRb in HeLa cells following HPV18-E7 knock down by siRNA.
- Ectopic expression of HPV16-E7 in HPV negative, but pRb positive Rhabdomyosarcoma cells (TE-671).



**Figure-3.6:** Scheme of the experimental strategy to study the role of HPV in HDAC inhibitor SB induced pRb degradation.

To test the first strategy in HeLa cells, a pSUPER vector containing a specific siRNA (19 nt, Figure-3.7 b) designed for the knock down of E7 transcripts (Figure-3.7 a) (Kuner et al., 2007) was used. The Figure-3.7 c shows its predicted secondary structure.

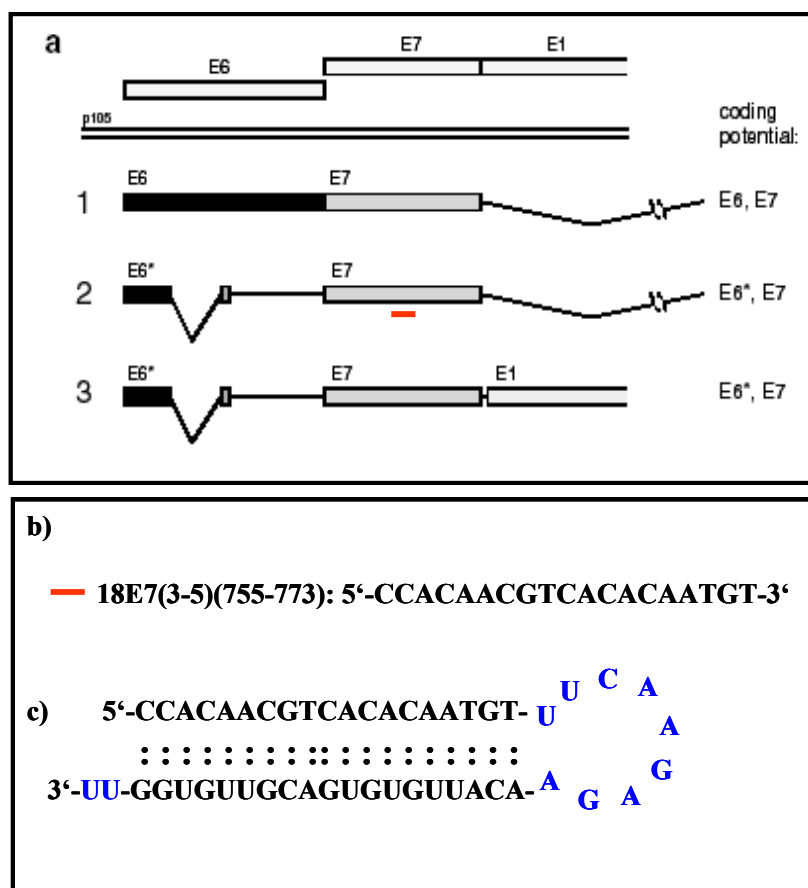
Exponentially growing HeLa cells were transfected with pSUPER-18E6/E7 while controls were transfected with empty pSUPER- or pSUPER-luciferase vector. After 48 h of transfection, cells were treated with 6 mM SB for 24 hours. Northern blot analysis was performed to monitor the specific transcriptional downregulation of E7. As seen in Figure-3.8 A (lanes 4 and 8), a marked

reduction of HPV transcripts was detected, while control transfected cells were not affected. The equal loading and RNA quality was monitored by ethidium bromide staining of the ribosomal RNAs 28S (4,9 Kb) and 18S (1,9 Kb). Transcriptional suppression of E7 oncogene was also analysed on protein level (Figure-3.8 B), where the amount of E7 was reduced after specific siRNA expression.

**Figure-3.7: Schematic presentation of the HPV18 siRNA plasmids (Vogt et al., 2007)**

(a) Schematic overview of the HPV18 transcripts in HeLa cells,. The sequence targeted by pSUPER-18-E6/E7 is schematically indicated by bold red colour line for pSUPER-18(3-5). (b) The target sequence of pSUPER18 E6/E7 (19 nt). Synthetic double stranded oligonucleotide derived from the HPV18-E7 gene was cloned into pSUPER vector resulting in pSUPER-18E6/E7 construct.

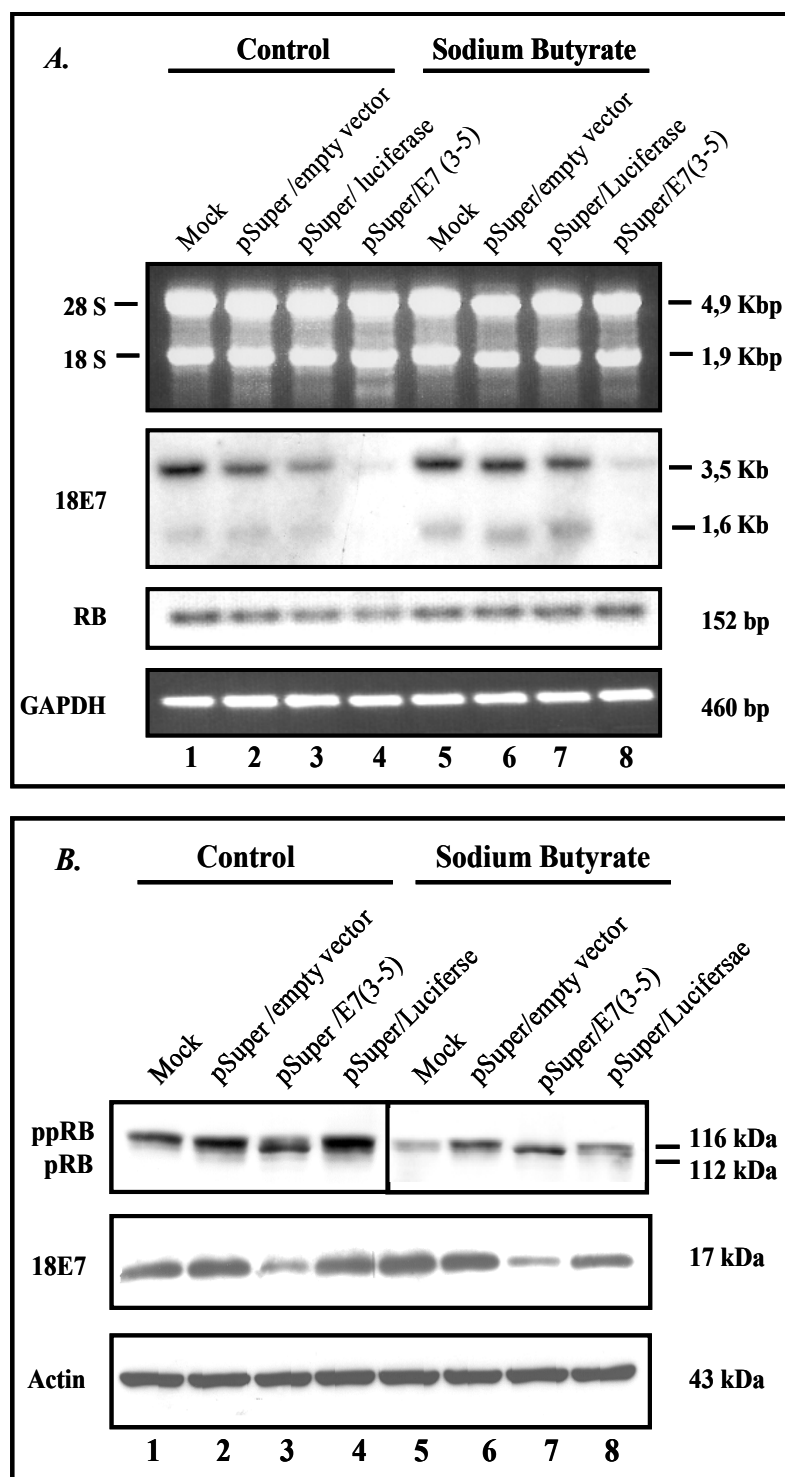
(c) Predicted secondary structure of the RNA encoded by pSUPER-18E6/E7-(3-5) (Kindly obtained from Felix-Hoppe -Seyler).



Previous results demonstrated that HDAC inhibitor induced degradation of pRb was due to posttranslational events, because the steady state level of pRb mRNA was maintained upon treatments with HDAC inhibitor SB (Finzer et al., 2001). Here, it was analysed whether transcription of pRb was influenced directly by downregulation of HPVE7 or whether siRNA per se could downregulate pRb transcription. Figure-3.8 A shows no effect on pRb mRNA in the absence of E7.

Since the metabolic half-life of pRb is inherently controlled by E7 oncoprotein (Boyer et al., 1996), one might expect that suppression of HPVE7 increases the posttranslational levels of pRb. Surprisingly, no pRb increase was observed either in control and in SB treated condition while E7 expression was suppressed as shown in Figure-3.8 B (Lane 3 and 7). This could explain that residual E7 is sufficient to degrade pRb. Nevertheless the only effect of the E7 siRNA transfection on pRb protein, was the presence of the hypophosphorylated pRb (Dyson et al., 1992; Muenger and Phelps 1993) which was maintained also after treatment with SB, whereas at

the control transfected cells both forms (hyper- and hypophosphorylated) of pRb was present (Weinberg et al., 1995b; Beijersbergen et al., 1996). Interestingly, the pRb from control transfected cells (Figure-3.8 B, Lanes 2 and 4) was still degraded by SB treatment (Lanes 6 and 8), suggesting the transcription of pRb was not be influenced by siRNA transfection (Figure-3.8 A).



**Figure-3.8: SB treatment of transiently transfected HeLa cells expressing HPV18-E6/E7 siRNA**

Exponentially growing HeLa cells were transfected with 20 µg of the respective pSUPER-derived vector using calcium phosphate co-precipitation (Chen and Okayama, 1987). siRNA-encoding vector pSUPER-HPV18-E6/E7 siRNA specifically reduces HPV18 E6/E7. As mRNA controls mock transfected HeLa cells or HeLa cells transfected with pSUPER-vector expressing siRNA against Luciferase or pSUPER empty/vector were used. Cells were treated for 24 h with Sodium Butyrate, 48 h after transfection. **(A) RNA analysis:** siRNA mediated suppression of HPV18-E7 transcription was detected by Northern blot. After transfer, the filter was hybridized with a radioactive  $^{32}\text{P}$ -labelled probe of HPV18. 18S and 28S ribosomal RNA are indicated. The transcription level of pRb was analyzed by semi-quantitative RT-PCR. As an internal control GAPDH-PCR was performed. **(B) Western blot analysis** was performed using anti-pRb1, anti-HPV18-E7 specific antibodies. Equal protein loading was confirmed using anti-actin antibody.

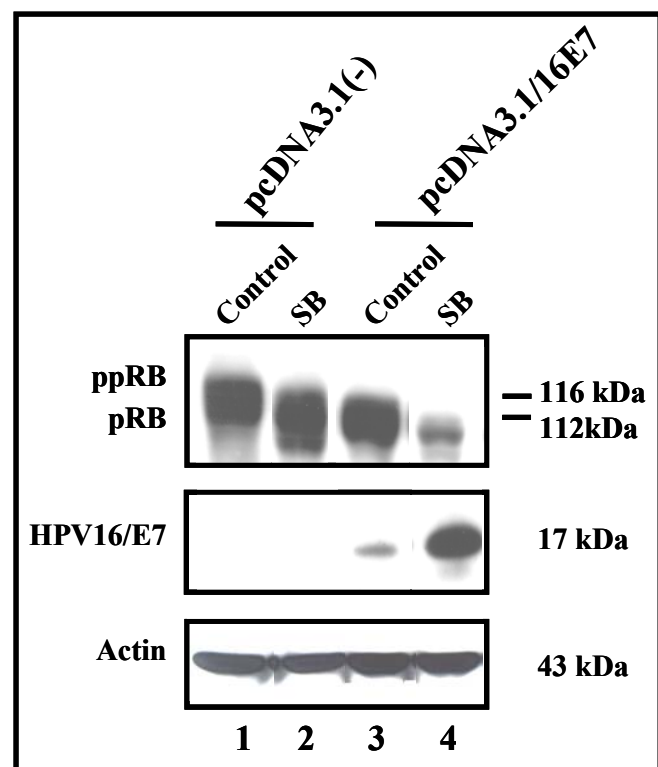
Although application of E7 specific siRNA did not increase pRb levels per se, but it led to the maintenance of the hypophosphorylated pRb upon SB treatment, which consequently could bind to its partner E7 to induce the degradation process.

Since the E7 knock-down experiment failed to abrogate pRb degradation, rhabdomyosarcoma cells, harbouring wild type pRb were stably transfected with E7 of HPV16.

As seen in Figure-3.9 (Lanes 3, 4) HPV16-E7 expression increased after SB supplementation. This was paralleled by a down regulation of pRb protein, while in control transfected cells pRb expression remained unaffected (Lane 1 and 2). Significantly, both hypo and hyperphosphorylated forms of pRb, were decreased in E7 positive TE-671 cells by SB treatment, supporting the crucial role of the E7 on enhanced pRb degradation in HDAC inhibition (Lane 4). Notably, a concomitant increase of E7 protein is observed with SB (Lane 4), which could induce the degradation of pRb per se. However, the disappearance of the hyperphosphorylated form of pRb by SB strongly recommends for HDACi mediated effect on pRb degradation that plays the major role in this process (Lane 4).

**Figure-3.9: Ectopic expression of E7 in TE-671 cells**

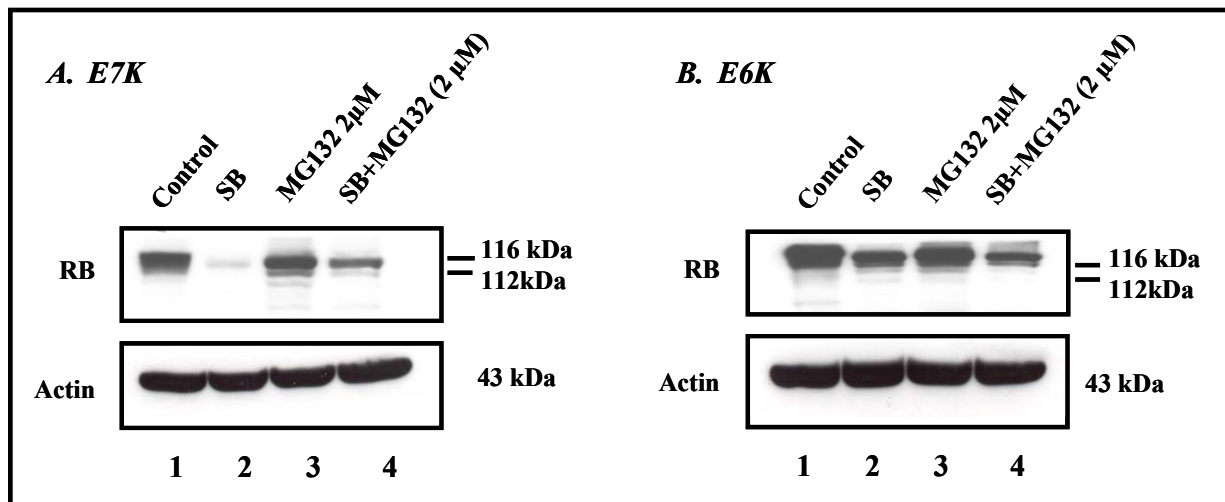
Stable transfection of TE-671 cells. Expression of HPV16-E7 was analysed by Western blot. Cells were incubated for 24 hours with SB. Total protein extract 75 µg were separated in (8 %) for pRb and in 12% SDS-PAGE for 16E7 analysis. After blotting membranes were incubated with E7 and pRb antibodies. Control of protein loading was done using an anti-actin antibody.



### 3.3 pRb degradation induced by HDAC inhibitors is mediated by the 26S proteasome

In order to dissect the role of the individual oncoproteins in HDAC inhibitor induced pRb degradation via 26 S proteasome, E6- and E7-immortalised keratinocytes were used.

The keratinocytes were treated with SB (6 mM) either in the absence or presence of MG132 (2  $\mu$ M), an inhibitor of the proteasome (Rock et al., 1994). Cells were incubated for 8 hours with SB and after MG132 addition the incubation proceeded another 16 hours. As shown in Figure-3.10, HDACi induced pRb degradation in E7-expressing cells was prevented by MG132. Interestingly, HDAC inhibitor induces hypophosphorylated form of pRb in E6-immortalised keratinocytes, whereas MG132 co-treatment reduced the amount of hypophosphorylated pRb. These results confirm that the pRb degradation in the presence of E7 upon HDAC inhibition is controlled by the 26S proteasome.



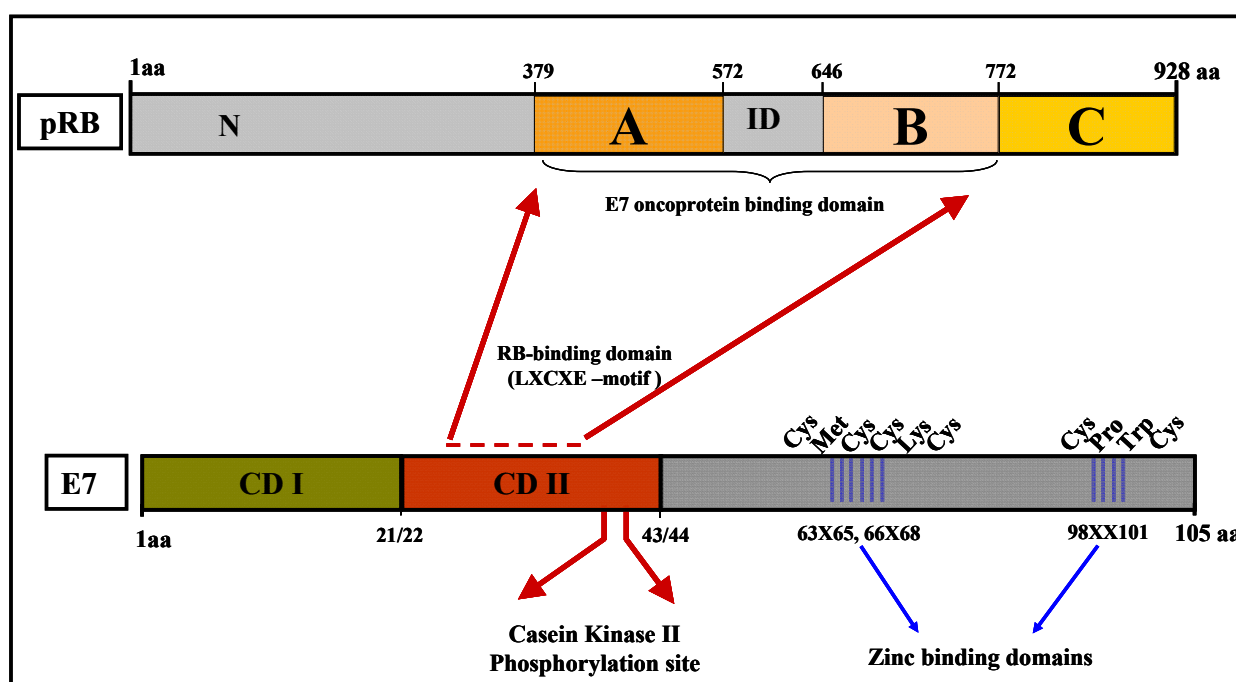
**Figure-3.10: pRb degradation is proteasome dependent in E7-immortalised keratinocytes.** Western blot analysis was performed for (A) E7- and (B) E6- immortalised keratinocytes. The cells were treated with SB for 24 hours for 16 hours MG132 alone or pretreated with SB for 8 hours, then MG132 (2  $\mu$ M), was added for additional 16 hours, incubation was continued for 16 hours (lanes 4). Lanes 3 for MG132.

### 3.4 Inhibitor of serine proteases, TLCK, modifies E7 proteins and inhibits its binding to pRb

Taken together, the results from HPV18 positive HeLa cells and HPV16 immortalised keratinocytes, show that HDAC inhibitors mediate pRb degradation in an E7-dependent effect. To clarify whether the LXCXE binding motif of E7 is directly involved in pRb degradation, cells were incubated with tosyl-L-lysine chloromethyl ketone (TLCK), a serine protease inhibitor which alkylates the histidine side chain in the active center of trypsin (Schoellmann and Shaw 1963; Shaw 1970).

Moreover, Stöppler et al., (1996a) reported that the HPV-E7 Rb-binding core resembles the active center of trypsin serine protease, which marks E7 protein as a potential target for TLCK.

Structurally the E7 protein consists of two conserved domains (CD-1 and CD-2). The CD1 and CD2 domain are important for host-cell transformation. Induction of DNA synthesis is contributed to additional elements in CD1 while CD2 contains the direct binding sequences for pRb and therefore enables the dissociation of E2F and pRb (Phelps et al., 1992; Brokaw et al., 1994; Demers et al., 1996). The HPV E7 protein interacts with pRb through a conserved LXCXE (aa 22- 26) sequence within the CD-2 domain (Banks et al., 1990; Dyson et al., 1992; Dahiya et al., 2000). These residues bind to the pocket region (A/B) of pRb which is placed between aa 379-772. The scheme in Figure-3.11 shows the interaction domains between pRb (pocket region) and HPV E7 (LXCXE motif).



**Figure-3.11: (Upper part): The structure of the Rb protein.** The predicted viral oncoprotein (E7, E1A and SV40 large T-Antigen) binding site including the A and B pocket regions (379 aa-772 aa), (ID): interdomain (C): C-pocket **(Lower part): Diagram of the wild type HPV18-E7 protein.** The scheme represents the 105 aa at the HPV18-E7 protein. The position of the Rb-binding core, the casein kinase II phosphorylation sites, and the zinc-binding domains are indicated. CDI and CDII is the conserved domain 1 and 2 respectively.

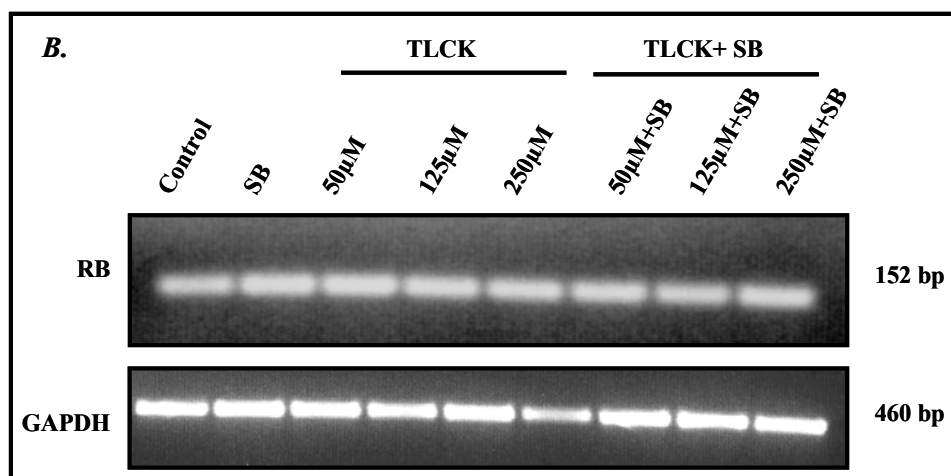
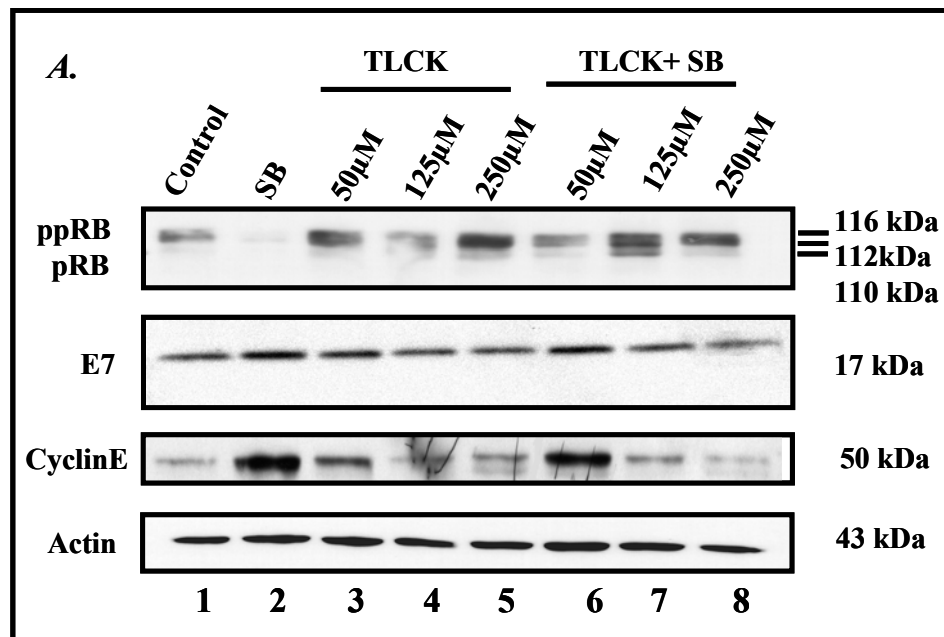
Since TLCK targets E7 by abrogating the binding to pRb, it raised the question whether pRb degradation by HDAC inhibitors could be prevented when cells were co-treated with TLCK. Consequently, HeLa cells were incubated with TLCK at different concentrations (50, 125, 250  $\mu$ M) with or without co-treatment with SB (6 mM) for 16 hours respectively.

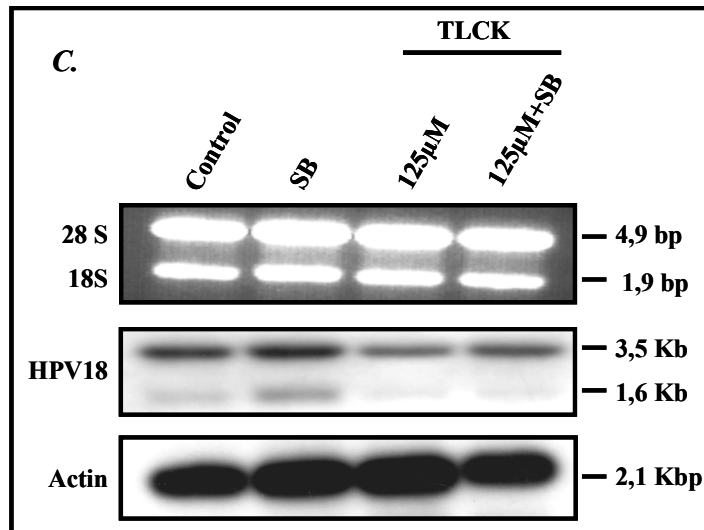
As outlined in Figure-3.12 A, TLCK treatment alone stabilized pRb protein levels in a concentration dependent manner (Lanes 3, 4, 5). It has been seen clearly, that pRb proportionally increases with TLCK concentrations (50  $\mu$ M versus 250  $\mu$ M). Low concentrations of TLCK

reduced SB mediated pRb degradation, whereas higher TLCK concentrations completely blocked this process (Lanes 6, 8). In the meantime the expression of the E7 protein was not altered by TLCK alone and in combination with SB (Figure-3.12 A).

To analyse whether TLCK could negatively regulate the transcription of pRb, the level of pRb was analysed by RT-PCR. Figure-3.12 B shows that TLCK treatment had no influence on the transcription level of pRb, indicating that the reappearance of pRb was on post-transcriptional level. Equally, the transcription of HPV18 E7 remains also unaffected even a slight suppression was observed in the presence of TLCK (Figure-3.12 C).

In order to test the functionality of the rescued pRb by co-treatment with TLCK and SB, the negatively controlled gene of pRb, cyclin E was analysed. Notably, the expression level of cyclin E was down-regulated in response to TLCK co-treatment (Figure-3.12. A), which is correlating with the rescued level of the pRb.





**Figure-3.12: TLCK rescues pRb from SB mediated degradation in HeLa cells.**

(A) Western blot analysis of HeLa cells after treatment with TLCK (50 µM, 125µM, 250 µM), SB (6 mM) without or in combination with TLCK for 16 h. Total cellular extracts (75 µg) were separated to analyse the posttranslational levels of pRb, HPV18E7, and cyclin E by Western blot. Protein loading was controlled using anti-actin antibody. (B) RT-PCR of Rb gene. The respective band for Rb (152 bp) is indicated. As an internal control

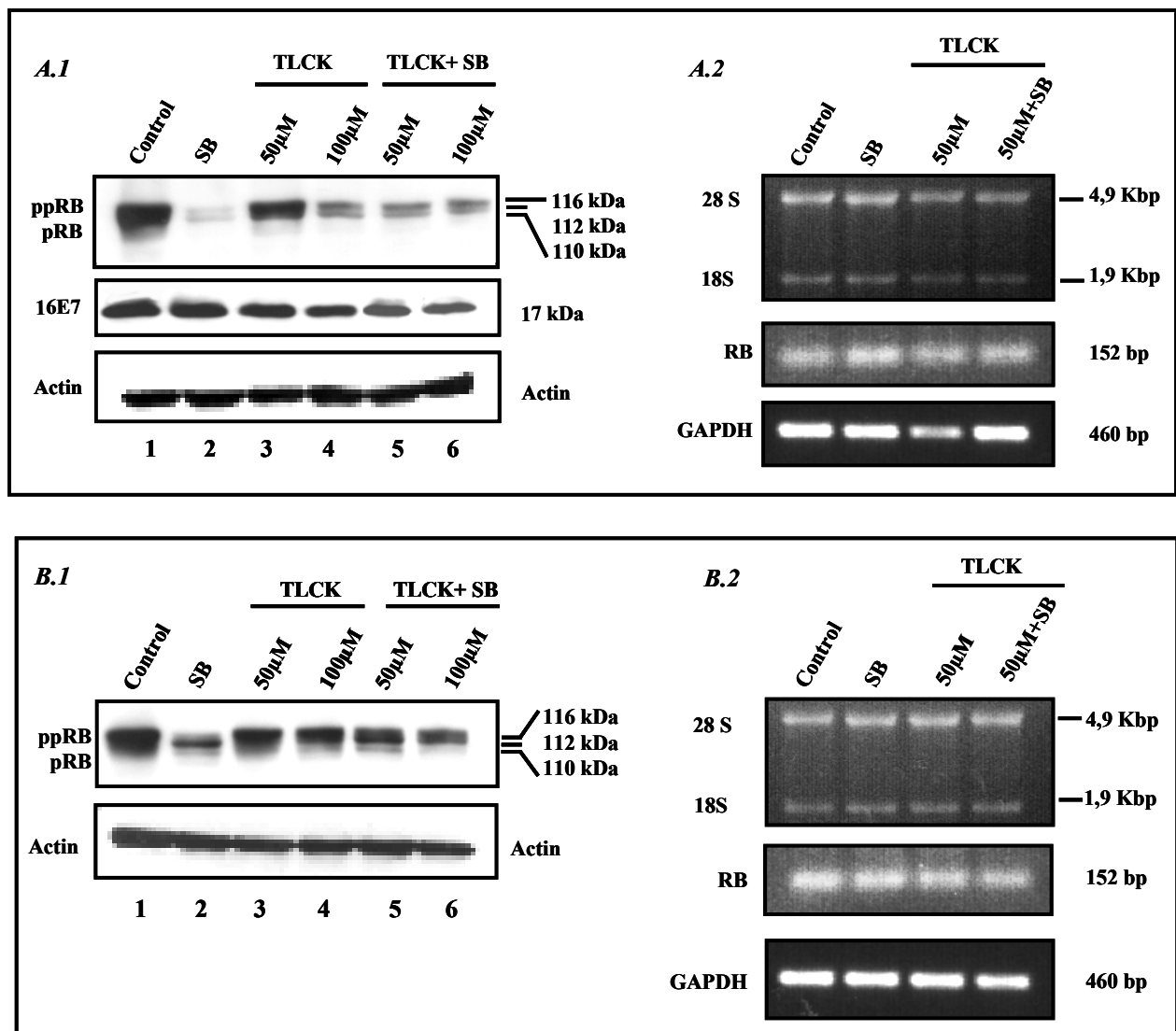
GAPDH- PCR was performed. (C) Transcriptional level of HPV18-mRNA was monitored by Northern blot analysis, RNA quality is shown in the RNA gel (1%) and equal loading was monitored by actin hybridization.

These results emphasize the importance of the direct E7-Rb interaction for the SB mediated degradation of pRb. Moreover, it indicates that rescue of pRb from its degradation, down-regulates the negatively controlled gene cyclin E.

### 3.4.1 Treatment of HPV16 E7-immortalised keratinocytes with TLCK also rescues pRb from its SB mediated degradation

To analyse whether TLCK also prevents pRb degradation in HPV16 E7 positive cells, immortalised keratinocytes HPV16 E7- and E6 were included into the subsequent experiments. In the presence of E7, TLCK treatment alone reduced pRb protein in a concentration dependent manner (Figure-3.13 A, A1 Lanes 3, 4). Nevertheless, higher protein levels of pRb were observed after TLCK incubation (Lanes 3, 4), compared to SB treatment alone (Lane 2). Co-treatment with SB and TLCK blocked pRb degradation (Figure-3.13 A, A1). However, already the lower concentration (50 µM) was able to block degradation (comparing Lane 5 with Lane 3), while the higher concentration of 100 µM was more effective (comparing Lane 6 with Lane 4). In contrast to HeLa cells and E7 keratinocytes, TLCK did not alter the steady state level of pRb in E6 keratinocytes. The combination of SB and TLCK diminished the hypophosphorylated pRb (Figure-3.13 B, B1). Considering the mRNA levels for pRb, no alteration was observed in both E6 and E7 immortalised keratinocytes (Figure-3.13 A2 and B2).





**Figure-3.13: TLCK rescues pRb from SB mediated degradation in HPV16-E7 immortalised keratinocytes**

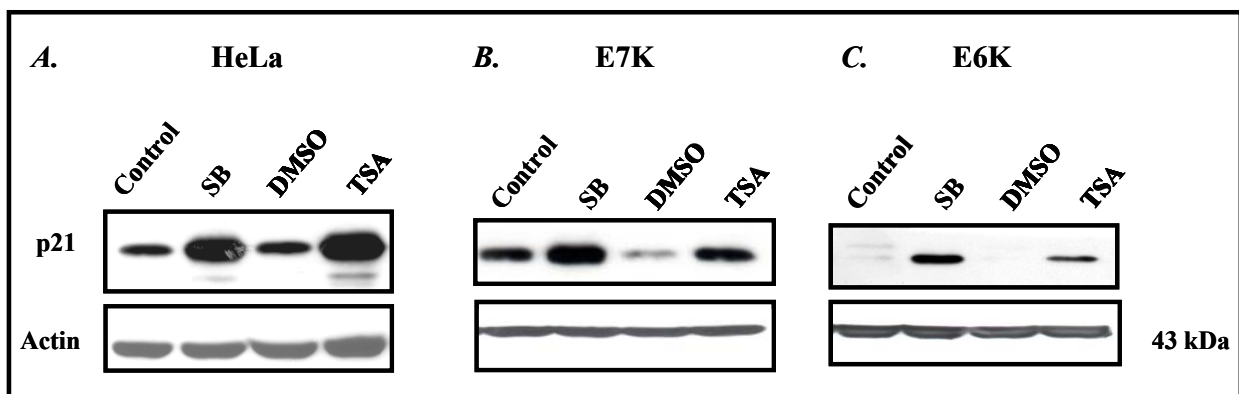
Protein extracts were performed from (A.1) E7- and (B.1) E6-immortalised keratinocytes. 75  $\mu$ g of protein extracts were loaded per lane in 8 % (for Rb) or 12 % (for HPV16-E7) SDS-PAGE. Equal loading and transfer was monitored by incubation of the membranes with anti-actin antibody. (A.2 and B.2) RT-PCR was prepared for p73. GAPDH was used as an internal control and 18S- and 28 S-RNA indicated the quality and equivalent loading on the RNA gel.

TLCK treatment of cells induces E7 modifications which in turn prevent E7 binding to pRb. This disruption between E7 and pRb binding prevents the pRb degradation upon HDAC inhibitor treatment and confirms its direct participation in enhanced degradation of pRb upon HDAC inhibition.

### 3.5 HDAC inhibitors SB and TSA upregulate the cyclin dependent kinase inhibitor p21 and induces G1 arrest in HeLa cells

The previous results have shown an E7 dependent degradation of pRb in the presence of HDAC inhibitors. One main function of pRb is its participation in cell cycle regulation. The pRb repression on E2F-mediated transcription is regulated by phosphorylation of pRb by cyclin-dependent kinases (CDKs) during the cell cycle. This phosphorylation disrupts the association between E2Fs and pRb (Buchkovich et al., 1989; Cooper et al., 2001).

HDAC inhibitor induced cell cycle arrest at the G1/S boundary is associated with induction of the CDKN1A (encoding p21<sup>WAF1/CIP1</sup>), which promotes hypophosphorylation of pRb (Vrana et al., 1999; Richon et al., 2000). It has been hypothesized that transcriptional induction of p21<sup>WAF1/CIP1</sup> is accompanied by an increase in histone acetylation within the p21 promoter region, indicating that treatment with HDACi could change expression levels of p21. To verify if HDACi treatment influences p21 expression by the promoter, we analysed the expression level of p21 in our model systems.



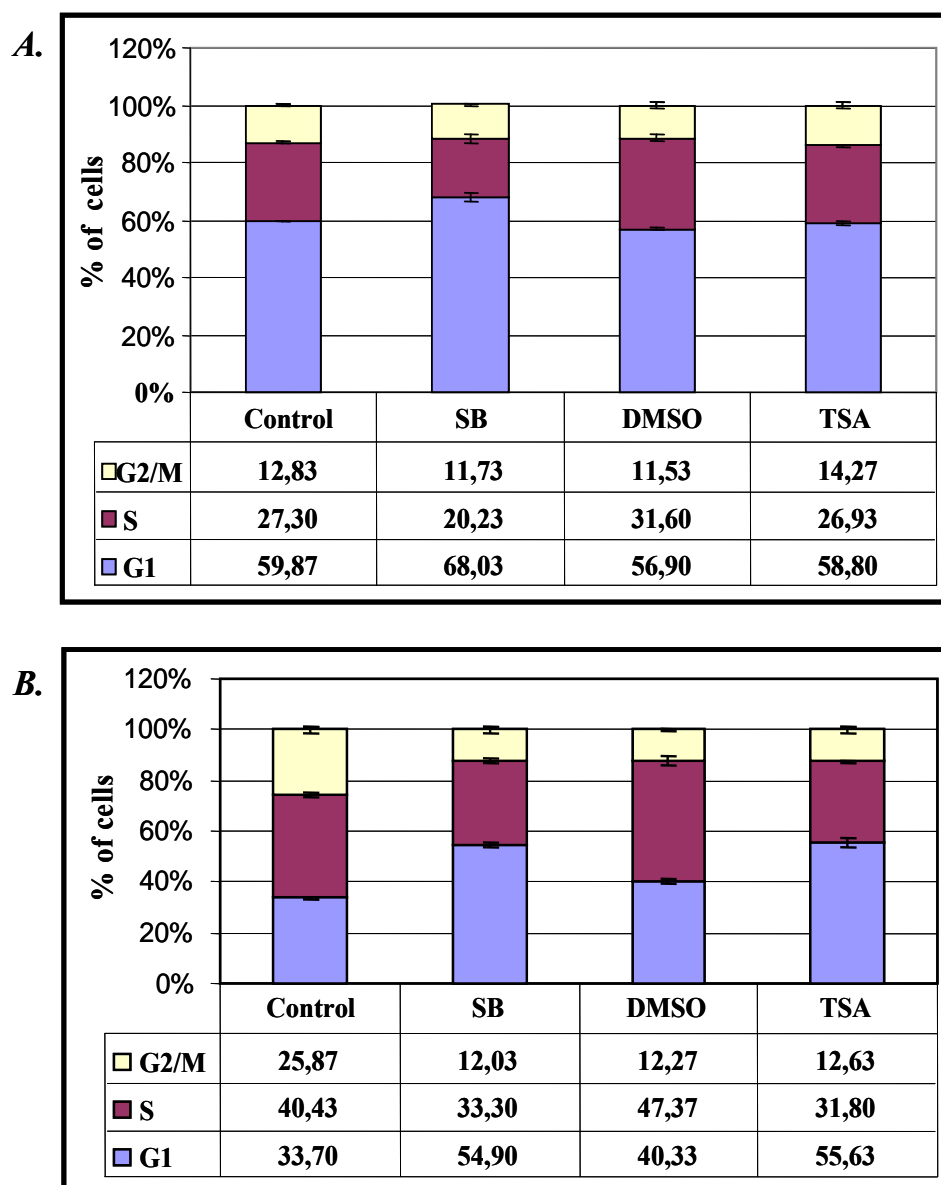
**Figure-3.14: SB and TSA induce upregulation of p21 in (A) HeLa, (B) E7- and (C) E6- immortalised keratinocytes**

Western Blot analysis of cellular extracts obtained from (A) HeLa, (B) E7- and (C), E6-immortalised keratinocytes were separated on 12% SDS-PAGE gels. After electrotransfer, the filters were incubated with p21 antibody. Equal protein loading was confirmed by incubation of the filters with an anti-actin antibody. Incubation of HeLa cells were done for 24 h and for immortalised keratinocytes for 16 h. Control: untreated cells, DMSO: Cells treated with 0.1 % DMSO, SB (16 h), and TSA (16 h).

As seen in Figure-3.14, p21 is upregulated upon HDAC inhibition in HeLa as well as in E7- and E6- immortalised keratinocytes.

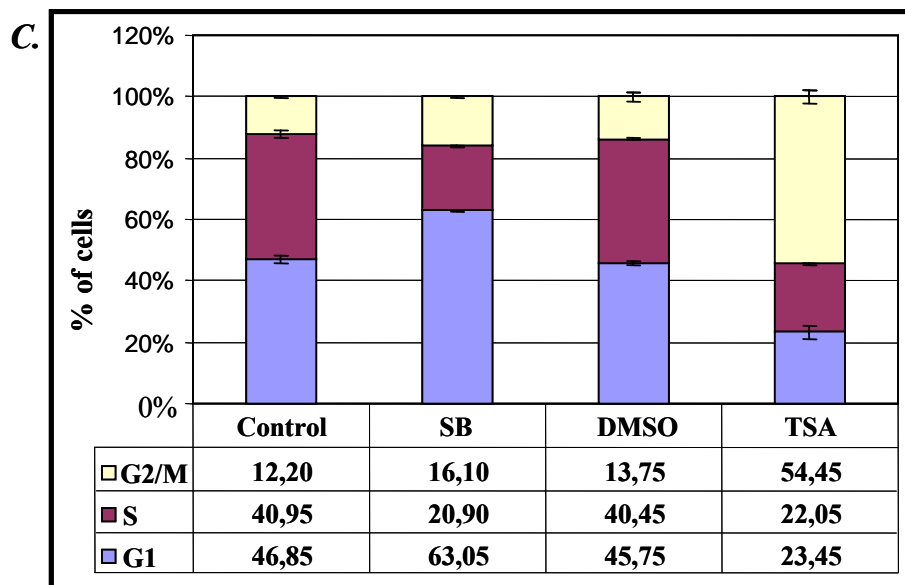
### 3.5.1 Cell cycle analysis after HDAC inhibition

To prove whether the upregulation of p21 upon HDAC inhibition was accompanied by G1 arrest (Richon et al., 2000), cell cycle distribution analyses were performed. HeLa cells were treated with SB and TSA for 24 and 36 hours, harvested, fixed with 70 % ethanol and analysed by flow cytometry. In Figure-3.15 A (24 h) and B (36 h) the proportion of the cells in G1-, S- and G2/M phase are presented. A clear G1 arrest was detected with SB. In particular, exposure with SB for 24 hours increased the percentage of cells accumulating within the G1 fraction from 59,87 % to 68,03% (A) while extended treatment for 36 hours (B) results in an increase from 33,7 % to 54,9 % . After 24 h treatment with TSA there was no significant increase in G1 fraction (A: from 56,9% to 58,8 %), whereupon an increased accumulation in G1 phase was observed after 36 hour incubation (D: 40,53 % to 55,63%). Thus, both HDAC inhibitors induce strong G1 arrest. Nevertheless, the most prominent effect was observed with TSA.



**Figure-3.15: Cell cycle distribution after 24 and 36 hour treatment with SB and TSA**

Flow cytometric analyses was performed in order to quantify the proportion of cells in the cell-cycle phases G1, S, G2/M. Standard deviations are given for three independent experiments. The average mean of three different experiments is given in the table below. Treatment of the HeLa cells was done with 6 mM SB or 330 nM TSA for 24 h (A) and 36 h (B) respectively. C33A cells were treated for 24 h (C). Control: untreated cells; SB: Sodium Butyrate (6 mM); DMSO: Cells treated with 0.1 % DMSO. TSA: Trichostatin A (330 nM).



Previous results indicate too that SB and TSA are potent inhibitors of cell cycle in HPV18 positive cervical cancer cells (Finzer et al., 2003). Therefore it was interesting to analyse, whether HPV negative cervical cancer cells C33-A were also sensitive to the anti cell-proliferative effect of HDAC inhibitors SB and TSA. C33-A cells were treated with SB and TSA for 24 hours and analysed by flow cytometry.

Considering the cell cycle analysis (Figure-3.15 C), C33-A cells were also growth arrested, but at different stages of the cell cycle. SB treated cells accumulated in the G1 fraction from 46,85 % to 63,05 %, whereas TSA treated cells in G2/M fraction (from 13,75 to 54,45%).

This result demonstrates that SB and TSA induce in HeLa cells G1 arrest while in C33-A cells an arrest in G1 as well as in G2/M phase is induced. Furthermore these results indicate that HDAC inhibitor mediated cell cycle arrest occur independent of the presence of HPV.

### 3.5.2 TLCK interferes with sodium butyrate induced G1 arrest

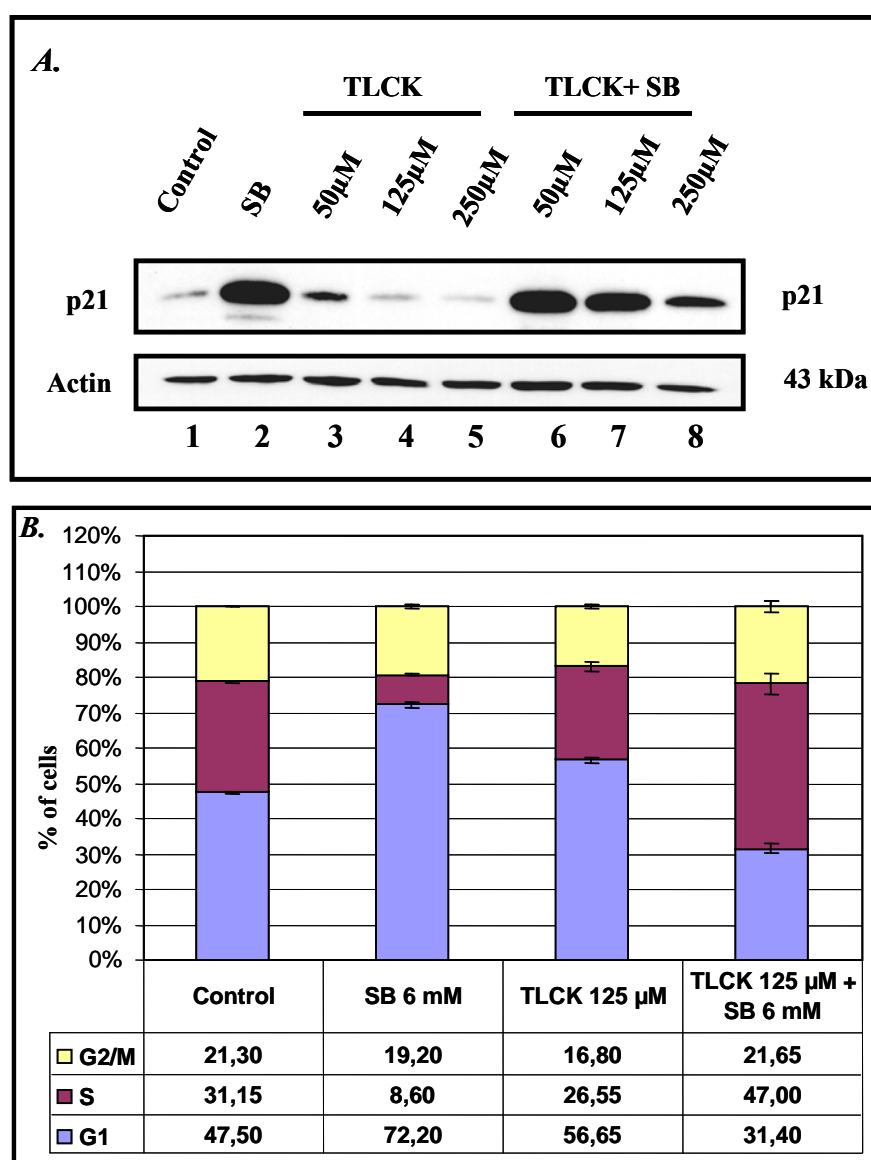
Previous experiments indicate that an SB mediated degradation of pRb necessitates a direct interaction between E7 and pRb to enhance the degradation process. Inhibition of the interaction with TLCK increases the stability (phosphorylation status) of pRb. Since pRb is one of the major regulators of the cell cycle progression, the effects on cell cycle during inhibition of a direct interaction between E7 and pRb were analysed.

As shown in Figure-3.12 A, co-treatment of HeLa cells with TLCK and SB restored pRb which reappeared as a hypo- and hyperphosphorylated forms, while the E7 expression was not affected. To prove whether restored pRb is able to inhibit the G1 arrest in human HPV positive carcinoma cells (Figure-3.15 A and B), expression levels of p21 in HeLa cells were analysed by Western

blotting. Cells were co-treated with TLCK (50  $\mu$ M, 125  $\mu$ M and 250  $\mu$ M) and SB (6 mM) for 16 h. As seen in Figure-3.16 A, concentration dependent decrease of p21 was observed upon co-treatment with TLCK and SB (Lanes 6, 7, 8).

This might lead to reduced G1-arrest induced by HDAC inhibitor. In order to prove this effect in specific cell cycle phases, cell cycle analyses were performed (Figure-3.16 B).

As already shown, SB leads to accumulation of cells in G1 phase (from 47,5 % to 72,2 %) of the cell-cycle. TLCK alone has no prominent effect on the distribution of cells in the different phases of the cell cycle compared to control conditions (from 47,5% to 56,65%). Whereas TLCK and SB co-treatment led to a significant reduction of the percentage of cells in G1 (from 72,20 % to 31,40%), a dramatic increase of cells in S phase (from 8,60% to 47%) compared to SB treatment alone.



**Figure-3.16: TLCK reduces the SB mediated G1 arrest in HeLa cells.**

(A) Western blot analysis of HeLa cells after treatment with TLCK (50  $\mu$ M, 125  $\mu$ M, 250  $\mu$ M), SB (6mM) without or in combination with TLCK for (16 h). Total cellular extracts (75  $\mu$ g) were separated to analyse the posttranslational levels of p21 by Western blot. Protein loading was controlled using anti-actin antibody. (B) Cell cycle distribution in HeLa cells. Flow-cytometric analysis: The bars indicate the percentage of cells within different cell cycle phases (G1, S, G2/M). Standard deviations are given for three different experiments. The indicated cells were incubated with SB (6 mM), TLCK (125  $\mu$ M for) alone and co-incubated with SB and TLCK

(corresponding concentration) for 24 hours. As control, cells with medium-change (DMEM) were used.

This result suggests that TLCK also affects the activity of HDAC inhibitors independently of E7 oncoprotein. Hence, the inhibitory effect of TLCK on HDAC inhibitor induced pRb degradation is apparently not only due to E7 modification but also due to reduced HDACi activity which occurs only at higher TLCK concentrations.

### **3.6 HDAC inhibitors SB and TSA induce apoptosis only in the presence of HPV**

Beside the cell cycle regulatory function, HDAC inhibitors are also able to induce apoptosis in tumor cells (Bolden et al., 2006).

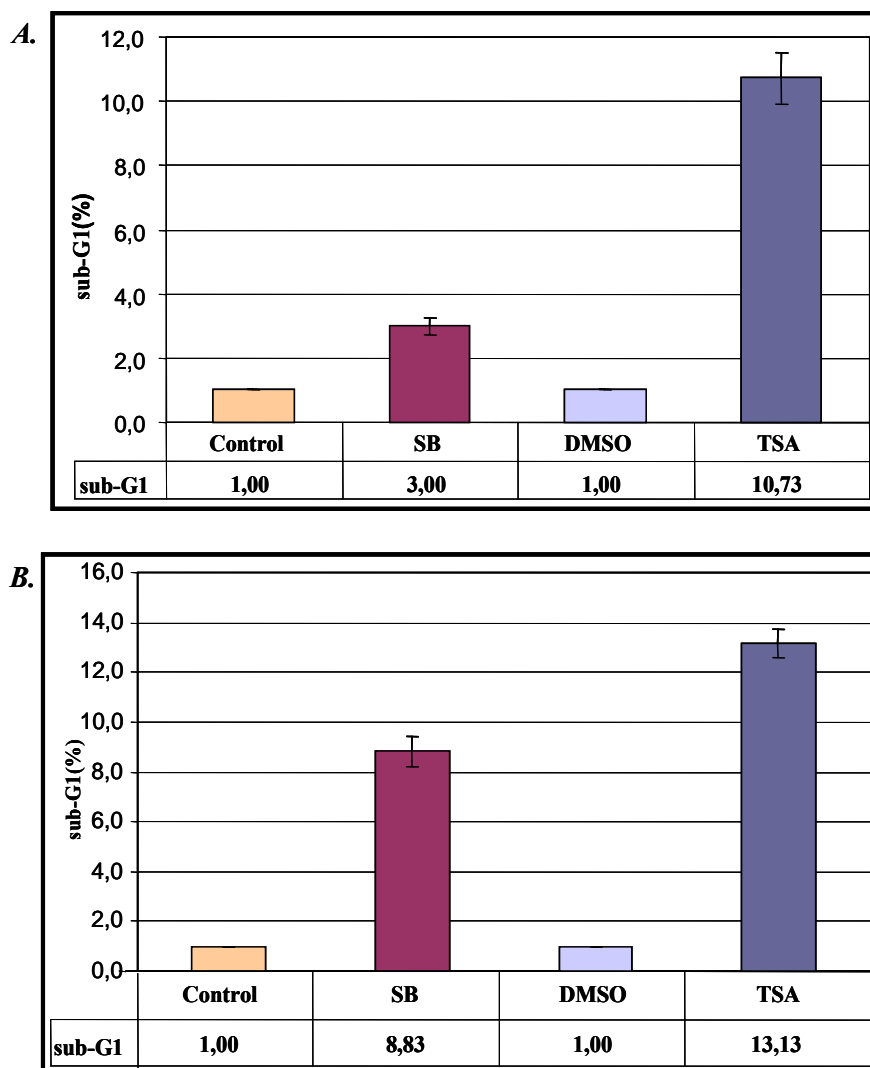
Inactivation of pRb can stimulate proliferation and apoptosis. The “promoter-specific” regulation of pRb/E2F transcription complex involves the expression of S phase genes through phosphorylation of pRb and the expression of the apoptotic genes through the pRb degradation (Chau et al., 2002; Chau and Wang 2003).

Inductions of the genes which are essential for the G1 to S phase transition are dependent on the transcription factor E2F-1. In addition to the proliferative effects, E2F-1 is also involved in apoptosis (Nevins, 1998). E2F can function as a proapoptotic transcription factor if it is freely available in the cell. It induces apoptosis by targeting genes such as the p53 homologue p73 (Phillips and Vousden, 2001).

A pronounced degradation of the anti-apoptotic protein pRb (Haas-Kogan et al., 1995 and Figure-3.1) without any decrease in the transcription factor E2F-1 (see also Figure-3.5) presumably accounts for the final appearance of the distinct apoptotic features.

Therefore the fate of the HeLa cells was analysed by flow cytometry after prolonged HDAC inhibitor treatment. HeLa cells were treated as mentioned in Figure-3.15.

Extending SB incubation time increased the amount of the apoptotic cells. The observed effects were more pronounced in the presence of TSA. As seen in Figure-3.17 A, about 3% of the cell population were accumulated after 24 h with SB in the sub-G1 fraction. This portion of the cell population represents apoptotic cells. This value rose to 9% when the cells were incubated for 36 h (Figure-3.17 B). In the case of the TSA, nearly 11% of the cells accumulate in the sub-G1 fraction after 24 h. This value rose to 13% after 36 hours.

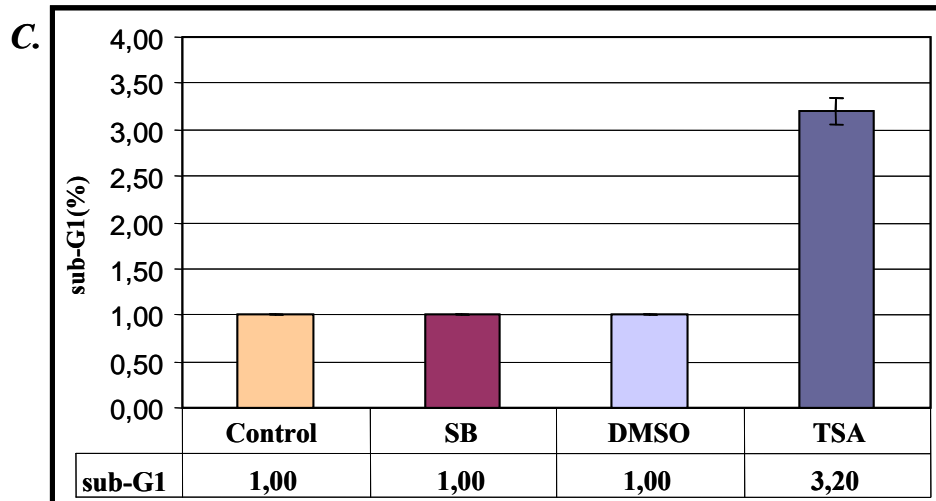


**Figure-3.17 A and B: Induction of apoptosis in HeLa cells after 24 and 36 hour treatment with SB and TSA.**

HeLa cells were treated with 6 mM SB or 330 nM TSA for 24 h (A) and 36 h (B) respectively. The bars indicate the percentage of cells in sub-G1 (= apoptotic cells). Standard deviations are given for three independent experiments. The average mean of three different experiments is given in the table below. Control: untreated cells; SB: Sodium Butyrate (6 mM); DMSO: Cells treated with 0.1 % DMSO. TSA: Trichostatin A (330 nM).

This result demonstrates that treatment of HeLa cells with both HDAC inhibitors induces apoptosis which is increased when cells are incubated for longer than 24 hours.

Since it was observed that interaction between E7 and pRb was essential for the HDAC inhibitor mediated degradation of pRb and also an unaltered level of pRb in HPV negative cervical carcinoma cells by HDAC inhibitor treatment (Figure-3.2), it was mandatory to analyse whether HDAC inhibitors also show apoptotic features in the absence of E7. To study this in greater detail the cervical carcinoma cell line C33-A was used, which is free of HPV infection (Heilman et al., 1980). Flow cytometric analysis revealed that these cells did not accumulate at the sub-G1 fraction with SB, while TSA induced apoptosis to a certain extent (cells in sub-G1= 3%) as seen in Figure-3.17 C.



**Figure-3.17 C: HDAC inhibitors SB and TSA don't induce apoptosis in C33-A cells**

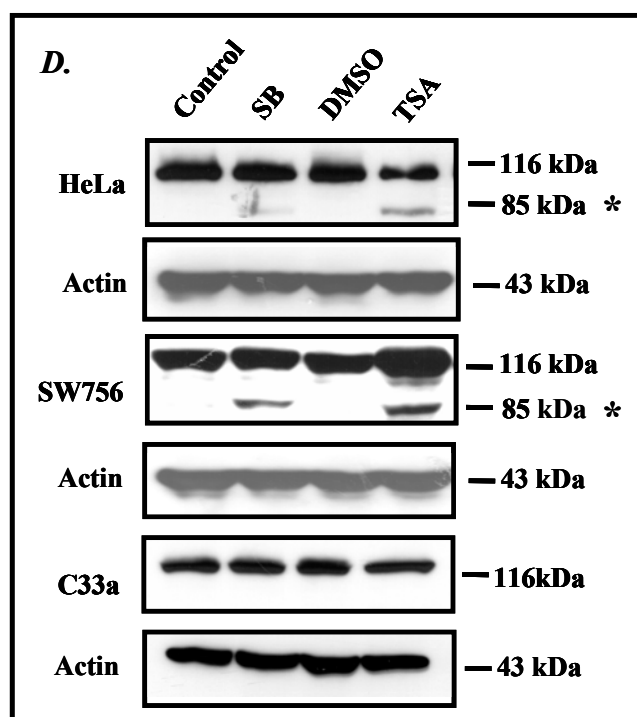
C33a cells were treated with 6 mM SB or with 330 nM TSA for 24 h. (A) The bars indicate the percentage of cells in sub-G1 (= apoptotic cells). Standard deviations are given for three independent experiments. The average

mean of three different experiments is given in the table below. Control: untreated; SB: Sodium Butyrate; TSA: Trichostatin

In order to confirm apoptosis induced by SB and TSA treatment in HPV positive cells, PARP cleavage was used as an internal marker (Finzer et al., 2004). This process can be monitored by caspase-3 activation. Proteolytic cleavage of poly ADP-dependent-ribose polymerase (PARP), a natural substrate for caspase-3, produces two fragments of 116 kDa and 85 kDa (Lazebnik et al., 1994). Western blot analysis demonstrate that the expected fragment of 85 kDa fragment appeared only in HPV positive cells HeLa and SW756, while it is absent in HPV negative cervical cancer cells C33-A (Figure-3.17 D).

**Figure-3.17 D: Cleavage of PARP in HPV 18 positive and HPV negative cells.**

HeLa, SW756 and C33A cells were treated with SB (6 mM) and TSA (330 n) Western blot analysis: 75µg of cellular extracts were separated in 8% SDS-PAGE gels. After incubation with a monoclonal antibody against PARP, equal protein loading was confirmed by incubating the filters with an anti-actin antibody for individual cell lines. Control: untreated cells, DMSO: Cells treated with 0.1 % DMSO. Treatments for SB and TSA were done for 24 h. The characteristic cleavage product (85 kDa fragment) of PARP is marked by asterisks.





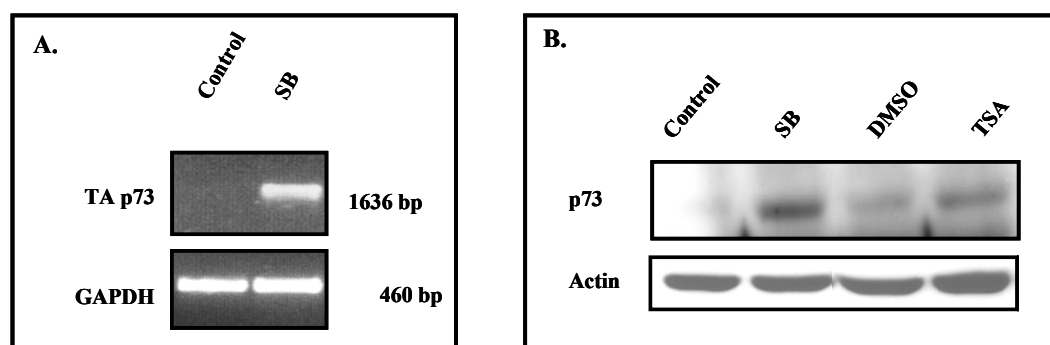
### 3.6.1 SB and TSA induce apoptosis in HPV positive cells via the proapoptotic gene p73

As a chemotherapeutic drug HDACi can induce cell death by activating either an intrinsic or extrinsic apoptotic pathway (Johnstone et al., 2002; Minucci et al., 2006).

Notably it has been described that p73 is one of the E2F-1 responsive genes which exhibits pro-apoptotic properties and is up regulated at the transcriptional level after SB treatment (Finzer et al., 2004)

The p73 belongs to the p53 family that transactivates several genes such as, p21, p53R2, PUMA, BAX involved in cell cycle control and apoptosis (Melino et al., 2002). The tumor suppressor gene TP73 encodes two different proteins which exhibit opposite effects: The p73 isoform TAp73 contains a transactivation (TA) domain which functions as a pro-apoptotic protein, while the  $\Delta$ Np73 has a shorter amino terminus, without the TA domain, functioning as anti-apoptotic protein. The apoptosis induced by TAp73 involves the mitochondrial pathway (Ramadan et al., 2005).

In order to verify whether SB induced apoptosis involves the induction of the p73 upregulation in HeLa cells RT-PCR upon SB treatment for 24 h was carry out. As depicted in Figure-3.18 A, p73 is upregulated at the transcriptional level upon SB treatment. Moreover this transcriptional effect is also monitored by Western blotting for SB and TSA (Figure-3.18 B).



**Figure-3.18 A:** HeLa cells were treated with SB (6 mM) for 16 h. **(A)** RT-PCR of p73 gene. The respective band for p73 (1636 bp) is indicated. As an internal control, GAPDH- PCR was performed. **(B)** Western blot analysis of p73 after treatment with SB (6 mM) and TSA (330 nM) for 24 h. Total cellular extracts (75  $\mu$ g) were separated in a 10 % SDS-PAGE. The membrane was incubated with a rabbit polyclonal antibody specific for p73. Protein loading was controlled using anti-actin antibody.

This result indicates that SB strongly induced the transcription of the p73 in HeLa cells which is involved in the intrinsic pathway of apoptosis.

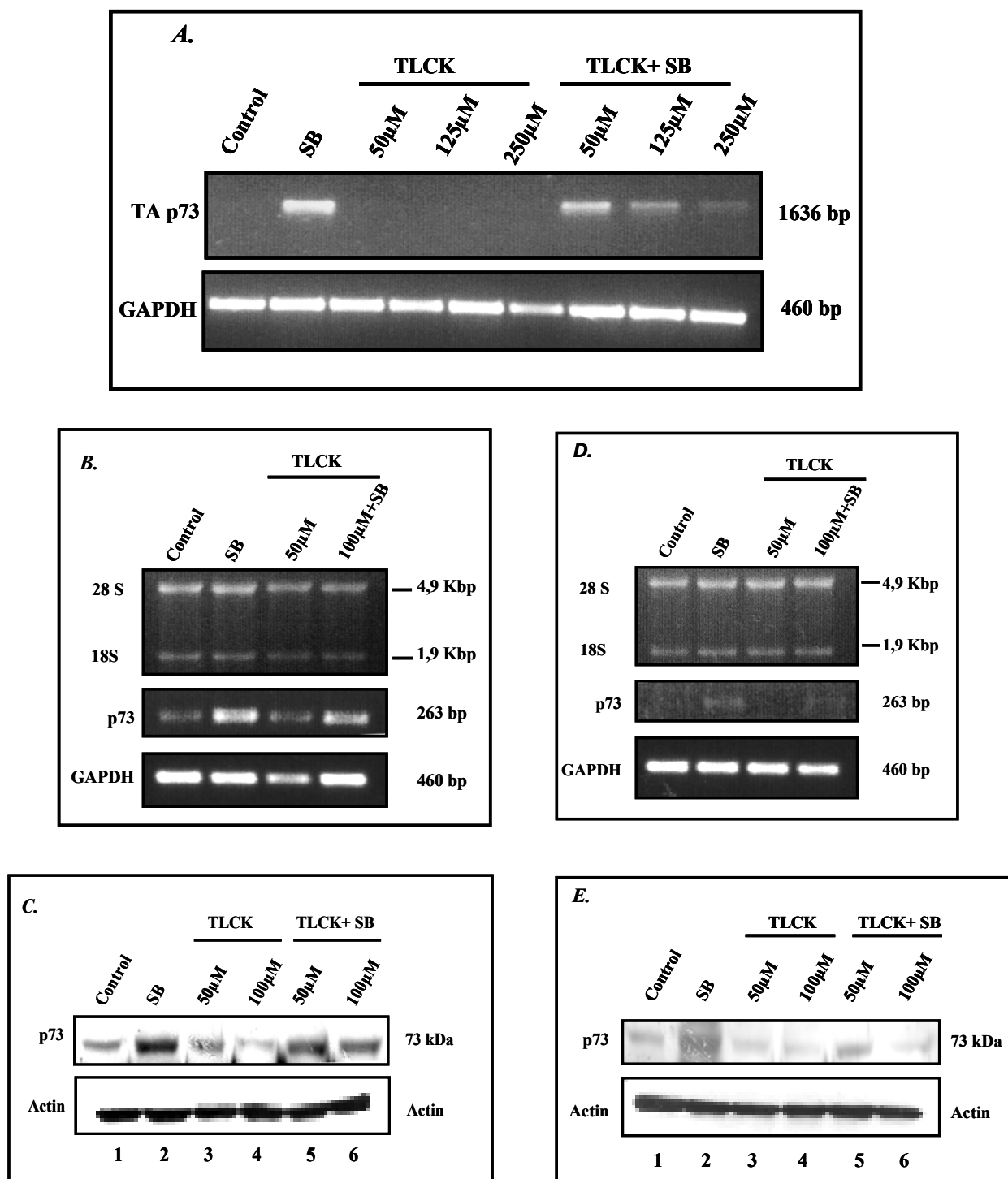
### **3.6.2 TLCK treatment leads to a suppression of the HDAC inhibitor induced proapoptotic p73 transcription**

The previous results showed that the interaction between E7 and pRb is essential for the HDAC inhibitor induced apoptosis in cervical cancer cells (Figure-3.12; 3.13 A and 3.15).

TLCK can disrupt the interaction between pRb and E7 and restores pRb (Figure-3.12; 3.13 A). It was therefore expected that this should also lead to a suppression of E2F-1 induced p73 transcription. Moreover E6 keratinocytes were analysed as a negative control since pRb is not a direct interaction partner of E6.

Therefore the transcriptional level of p73 was investigated by RT-PCR upon co-treatment with TLCK. As shown for HeLa cells (Figure-3.19 A) as well as for E7 immortalised keratinocytes (Figure-3.19 B), TLCK inhibits SB induced p73 mRNA (TAp73) in a strictly concentration dependent manner. In the case of E7 immortalised keratinocytes a higher concentration of 100  $\mu$ M TLCK is needed to completely block p73 induction. This reduction of p73 was also observed at the protein level (Figure-3.19 C). Surprisingly, E6 immortalised keratinocytes also present a moderate up regulation of p73 transcription by HDAC inhibition (Figure-3.19 D) which is accompanied with a slight up regulation on the protein level (Figure-3.19 E).

These results indicate that TLCK mediated E7 modification is also able to impair the induction of p73 which is strongly induced by SB. Consequently, E2F is not able to activate the proapoptotic genes p73. Moreover this result also highlights the role of the presence of E7 for the sensitization of HDAC inhibitor induced apoptosis.



**Figure-3.19: TLCK can inhibit the SB mediated p73 transcription**

HeLa cells were treated with TLCK (50  $\mu$ M, 125  $\mu$ M, 250  $\mu$ M), SB (6 mM) without or in combination with TLCK for 16 h. **(A)** RT-PCR of p73 genes. The respective band for p73 (1636 bp) is indicated. E7- and E6- immortalised keratinocytes were treated with TLCK (50  $\mu$ M and 100  $\mu$ M), SB (6 mM) without or in combination with TLCK. RT-PCR was performed for p73 mRNA expression for E7 and E6 keratinocytes respectively **(B and D)**. The respective band for p73 (263 bp) is indicated. As an internal control GAPDH-PCR was performed. Total cellular extracts (75  $\mu$ g) of E7 and E6 keratinocytes were separated in a 10 % SDS-PAGE. The membrane was incubated with a rabbit polyclonal antibody specific for p73. Protein loading was controlled using anti-actin antibody **(C and E)**.

## IV. DISCUSSION

### 4.1 HDACi mediated pRb degradation requires the presence of the HPVE7

The presented work was specifically focused on the role of E7 in tumor suppressor protein pRb degradation following HDAC inhibition.

Initial observations in the HPV 18 positive cervical carcinoma cells HeLa demonstrated that the degradation of pRb upon SB, occurs only in the presence of E7 oncoprotein. pRb degradation following HDACi administration has previously been reported by Finzer *et al.*, (2001). Analysis of SW756 cells demonstrated that this effect is common to other HPV18 positive cervical cancer cell lines and that it is independent of the viral integration sites, since HeLa cells are known to be positive for multiple truncated copies of HPV18 integrated at four distinct chromosomal loci (Schwarz *et al.*, 1985; Popescu *et al.*, 1987a), while SW756 cells harbour multiple truncated copies of HPV18 located at a single integration site (Popescu *et al.*, 1987b). In addition, the origin of the cancer cells appears to have no effect as HeLa derived from adenocarcinoma (Boshart *et al.*, 1984; Schwarz *et al.*, 1985) and SW756 from squamous cell carcinoma (Freedman *et al.*, 1982). To rule out the possibility that the tumorigenic potential phenotype of the cell rather than the presence of HPV was the cause of this observed phenotype, the Stanbridge cell system and HPV negative cervical cancer cell were used. The presented results showed a HPV dependency, while the tumorigenic phenotype was not influenced by HDACi treatment (Figure-3.2 and 3.3).

Interestingly, in HPV negative cell lines (C33-A and HT-3 cells), no pRb degradation was observed after treatment with HDACi, rather they had different pRb phosphorylation states. Furthermore, these cell lines harbour mutations in the *RB* gene. These mutations (exons 20 in C33-A and exon 13 in HT-3) are located in the pRb growth inhibitory domain, the so called “pocket domain” which is necessary for its binding to viral and many cellular proteins. This domain can be differently hyperphosphorylated (Scheffner *et al.*, 1991). Therefore it is likely that the phosphorylation state of pRb upon HDACi application could be a confounding factor.

In HT-3 cells additional hyperphosphorylated bands were observed (Figure-3.2). This different phosphorylation status of pRb in C33A and HT-3 cells could also be caused by the different mutations. Since differences between the phosphorylation states of both C33-A and HT-3 cells were observed, it is possible that this is also due to the presence of different mutation. Moreover Rb degradation in the presence of E7 in individually E7-immortalised keratinocytes confirms this crucial functional link (Figure-3.4 A), which is also shown by Finzer *et al.*, (2001). Given that the presence of E7 is a prerequisite for HDAC inhibitor induced enhanced degradation of

pRb, it would be interesting to prove whether introduction of HPV E7 in C33-A and HT-3 cells would sensitize the cells for HDACi mediated pRb degradation.

The observed pRb degradation in these cells could be explained by the presence of HPV E7 oncoprotein. E7 preferentially bind to hypophosphorylated form of pRb (Munger et al., 1992; Imai et al., 1991) and can at least abrogate the binding of pRb to E2F. Subsequently, degradation of pRb by E7 promotes entry to S phase (Boyer et al., 1996; Jones et al., 1997a). Though, both mechanisms are dependent on stoichiometry between pRb and E7 and the presence of hypophosphorylated pRb in cervical cancer cells. Here a synergistic effect of HDAC inhibitors and E7 on the destabilisation of pRb was observed.

In contrast to E7 immortalised keratinocytes, hypophosphorylated pRb in E6 keratinocytes following HDAC inhibition was observed. pRb hypophosphorylation following HDACi application has also been described in other cell systems (Wang et al., 2000; Wharton et al., 2000; Emanuele et al., 2004; Sambucetti et al., 1999).

pRb protein inactivation by HPV E6 can be due to hyperphosphorylation and involves increased activity of cyclin A/CDK2. Hyperphosphorylation of pRb allows the transcription of cyclin E and cyclin A which are important for the maintenance of cell cycle progression. Furthermore, strong down-regulation of p21, which inhibits the cyclin A/CDK2 activity, could also contribute to the E6 mediated deregulation of the G1/S transition (Malanchi et al., 2002; Malanchi et al., 2004). E6 is not able to bind and degrade pRb like E7, therefore the presence of hypophosphorylated pRb was due to the upregulation of cyclin-dependent kinase inhibitors such as p21 and p27 after HDACi (for p21 see Fig.-3.14). In turn, this suppresses activation of the cyclin-dependent kinase complexes (Richon et al., 2000; Finzer et al., 2001).

The presence of E7 upon HDACi treatment is likely due to the increased stability of E7, mediated by posttranslational modification such as acetylation (Caron et al., 2005). Studies have shown that E7 can independently interact with pCAF acetyltransferases, (Huang and McCane, 2002; Avvakumov et al., 2003) *in vitro* and *in vivo* which results in disruption or direct targeting of the pCAF complex to new substrates, including other E7-binding proteins (Bernat et al., 2003). The acetyltransferase pCAF, has been reported to function as a coactivator for variety of transcription factors such as p53 (Sakaguchi et al., 1998; Liu et al., 1999).

HDACi do not exert their pleiotropic effect solely by direct regulation promoter activity by histone hyperacetylation (Bolden et al., 2006). It is also known that HDAC molecules can bind, deacetylate and regulate the activity of a number of other proteins, including transcription factors. For example, the gene regulatory activity of transcription factors E2F1, p53, STAT1, STAT3 and NF- $\kappa$ B can be modulated through direct acetylation and deacetylation of the factors themselves (Gu et al., 1997; Martinez-Balbas et al., 2000; Chen et al., 2001; Yuan et al., 2005).

Similarly acetylation by HDACi may contribute to an extended half-life of E7 and increased activity.

However, considering the experiments in which the cell lines HeLa, 444 and CGL-3 were used, a reduction of E7 protein upon treatment with TSA (Figure-3.3) was observed. As previously reported, HPV transcription can be transiently suppressed by HDAC inhibition, but it returns to basal expression levels after 12 to 16 hours treatment (Finzer et al., 2003). Therefore the observed reduction of E7 protein by TSA is a posttranslational effect. TSA has been shown to be a more potent inhibitor of HDAC molecules than SB (Finnin et al., 1999; Matsuyama et al., 2002) which is even active in nanomolar concentrations. For this reason different results could be observed after the same incubation time. Indeed pRb degradation was observed in E7 immortalised keratinocytes upon TSA treatment for 16 hours while E7 viral protein was not degraded (Figure-3.4 A).

Furthermore it has been shown that TSA treatment down-regulates cellular proteins via ubiquitin-dependent proteasomal degradation. For example, Alao et al., (2004) reported about a proteasomal degradation of cyclin D1 after TSA treatment, where the regulatory component of SCF complex (Jackson and Eldridge, 2002), SKP2/p45 is upregulated. From the literature it is known, that SKP2/p45 is also involved in the ubiquitin-dependent proteasomal degradation of E7 (Wang et al., 2001; Oh et al., 2004). It is possible that longer treatment with TSA can also induce a proteasomal degradation of E7 in a SKP2/p45 dependent manner. Nevertheless, the upregulation of the SKP2/p45 was not observed upon treatment with SB, since previous data by our group demonstrate its suppression which in turn upregulates the cyclin-dependent kinase inhibitor p27 (Finzer et al., 2001). Therefore the role of the SKP2/p45 in down-regulation of the E7 protein needs to be further clarified considering the appropriate incubation times.

Interestingly, a reduced E7 protein level was observed in SW756 cells. Compared to the other HPV18 positive cells, this reduction was correlating with a reduced effect on pRb degradation after SB treatment. However, the mechanism behind the slight reduction of E7 (whether it be a posttranslational or transcriptional event), needs to be clarified.

To clarify the functional link between E7 and pRb degradation upon HDACi treatment two different experimental approaches were used:

At first, the transcription of the HPV E7 was knocked down by E7 siRNA in HeLa cells. Since the posttranslational level of pRb is inherently controlled in HPVE7 expressing cells (Boyer et al., 1996), one would expect an upregulation of the pRb protein in response to HPV mRNA suppression. However, only the hypophosphorylated pRb was produced which appears to be completely maintained upon SB treatment (Figure-3.8 A and B). The hypophosphorylation of pRb after transcriptional suppression of HPV18 was also reported by the group of Goodwin

(2000). In contrast, they reported a dramatic up-regulation of the hypophosphorylated pRb and repression of the pRb/E2F regulated genes (Goodwin et al., 2000) upon suppression of the HPV transcription.

However, the presence of hypophosphorylated pRb could also be explained by abolishment of other E7 mediated cellular activities. For example, disruption of the interaction between cyclin A and cyclin E could inhibit the activation of the cyclin/CDK complexes (Zerfass et al., 1995). Additionally it is possible that suppression of HPV E7 leads to the disruption of the activity of the CDK inhibitors p21<sup>WAF/CIP1</sup> and p27<sup>KIP1</sup> which in turn inhibits the activity of cyclin/CDK complexes (Jones et al., 1997). Therefore, to assess the role of the cyclins (A, E, and D) in addition to the role of the CDKi (p21 and p27) under these circumstances needs to be clarified.

As the E6 and E7 proteins of HPV18 are both translated from common polycistronic transcripts (Schneider-Gaedicke et al., 1986), it is technically feasible to concomitantly block the expression of both genes by one single siRNA species (Hall et al., 2003). Indeed recent report from the group of Kuner et al., 2007, showed a silencing of HPV18 E6/E7 by using pSUPER-siRNA directed against sequence motives common to all HPV18 E6/E7-encoding mRNAs. In agreement with the presented result in Figure-3.8 A they were also able to demonstrate the silenced E7 transcription. As there are currently no antibodies available to reliably detect the presumably very low endogenous E6 protein levels in HeLa cells, inhibition of E6 following siRNA expression was assessed functionally. They observed increased p53 levels, which is proteolytically degraded by E6 (Scheffner et al., 1990) and an accompanied increase of p21, which is a transcriptional target for p53 (El-Deiry et al., 1993; Kuner et al., 2007). Interestingly, hyperphosphorylated pRb was observed in response to the siRNA transfection in our results (Figure-3.8). It is conceivable that upregulation of p21 in response to E6 silencing can inhibit the cyclin-CDK complexes which are responsible for the phosphorylation of the pRb (Weinberg, 1995a; Sherr and Roberts 1999). Nevertheless experiments need to determine the relationships between downregulation of the pRb hyperphosphorylation and E6 silencing.

Additionally, to support the hypothesis, the E7 gene was introduced into the HPV negative cell line TE-671, which possesses a full functional pRb. Obviously enhanced degradation of pRb in the presence of HDACi confirms the particular importance for HPV16-E7 in those cells (Figure-3.9). Down-regulation of the hypophosphorylated pRb upon SB treatment indicated the stabilisation of the E7 protein. However, further experiments should be conducted in order to clarify the stabilisation of E7 upon HDAC inhibition. Accordingly, it was confirmed that the delivery of E7 induces an enhanced SB mediated degradation of pRb.

The observed overexpression of the E7 protein by SB *per se* might be explained by the non-specific promoter (in our case CMV- promoter) induction by SB which is reported by Nan et al.,

(2004). Since we observed a concomitant increase of E7 upon HDAC inhibition in E7 positive TE-671 cells, the pRb degradation cannot be attributed to only a SB-mediated effect. Nevertheless, comparison of the TE-671 cells (Iolascon et al., 1996; Xia et al., 2002) with the HPV negative cervical cancer cells C33-A and HT-3 cells, which are harbouring mutations in *RBI* gene, one can predict that SB mediated enhanced degradation of pRb is strongly dependent on the presence of E7 protein (Figure-3.2 and 3.9). It would be interesting to monitor whether these cells could be sensitized to HDACi mediated degradation of pRb after introduction of a truncated E7-form which is unable to bind pRb.

In a further approach, both recombinant pRb and E7 plasmid were transfected in a HPV and pRb negative cell line Saos-2 (Osteosarcoma cell line). Nevertheless, the initial attempts remained without success, due to the unspecific induction of plasmid promoters by HDACi.

#### **4.2 HDACi mediated pRb degradation increases the expression of the negatively regulated cyclin E**

pRb is sequentially phosphorylated during entry into S phase by Cyclin D/CDK4/6 complexes and Cyclin E/CDK2 complexes. As a consequence, E2F can transactivate its responsive genes and finally permits cells to enter into S phase. Under normal conditions, hypophosphorylated pRb binds to E2F and inhibits the transcription of negatively regulated genes (Weinberg et al., 1996). This partially occurs due to the recruitment of the HDAC1 on E2F responsive promoters (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; Brehm and Kouzarides et al., 1999a and 1999b).

As expected, cyclin E gene, which is negatively regulated pRb was upregulated in HeLa, SW756, 444, CGL-3 and in E7 and E6-immortalised keratinocytes upon SB and TSA treatment (Figure-3.4 A). The induction of cyclin E is primarily explained by derepression of the E2F binding sites on the cyclin E promoter (Geng et al., 1996). Therefore the degradation of pRb in HPV18 positive and in E7 immortalised cells upon HDAC inhibition is a prerequisite for induction of cyclin E.

Also an induction of cyclin E expression in control conditions was observed. This is explained mainly due to the presence of E7 which can induce the expression of the cyclin E (Zerfass et al., 1995) as E2F mediated repression is lost.

Since HDAC inhibition was shown to downregulate cyclins D and A, and to suppress CDK2 activity (Finzer et al., 2001), one would expect that this finally leads to hypophosphorylated pRb and turns off the E2F-responsive genes. Thus, the increase of cyclin E expression upon HDAC inhibition is contradictory to its effect on cell cycle control, where it promoted accelerated entry from G1 to S phase (Resnitzky et al., 1994; Ohtsubo et al., 1995). Nevertheless



this shows that HDACi potentiate the effect of E7 on the cyclin E expression, whereas antagonizing the effect of E7 on other cyclins by inducing their degradation (Finzer et al., 2001), such as cyclin D (Alao et al., 2004) and cyclin A (Schulze et al., 1995).

The presence of hypophosphorylated pRb (which was still able to repress the transactivation of E2F), was also associated with upregulation of cyclin E in E6-immortalised keratinocytes. This suggests that another transcriptional control mechanism was involved. Furthermore, this is supported by the fact that E2F was also decreased, which suggests the two mechanisms are not directly linked (Figure-3.5 B). The transcription of cyclin E has been shown to be E2F-dependent, but recently, several investigators have demonstrated that Sp1 transcription factor has a synergistic effect with E2F on the transcription of cyclin E and other E2F responsive genes such as DHFR (Karlseder et al., 1996; Lin et al., 1996; Vogt et al., 1999). Among these studies Kim et al., (2006) provide evidence that HDAC inhibitor apicidin increases hyperacetylation of histones at cyclin E promoter region containing specific Sp1 sites, resulting in an induction of cyclin E expression through Sp-1 dependent and E2F-independent mechanisms. Furthermore, another transcription factor Myc was shown to be involved in upstream regulation of the cyclin E and plays a critical role in sustaining an E2F-independent, G1/S-promoting mechanism by regulating the cyclinE/CDK2 activity (Santoni-Rugiu et al., 2000). According to this, Santoni-Rugiu et al., were able to demonstrate that Myc activates cyclin E transcriptionally and is capable of promoting G1/S transition in parallel to Rb/E2F pathway (2000).

Taking these studies into consideration it is conceivable that transcriptional regulation of cyclin E upon HDAC inhibition in E6 keratinocytes involves additionally other transcription factors.

As shown by Figure-3.5 B, E2F levels remain unchanged in HPV18 positive cells HeLa, 444, CGL-3 and in E7-immortalised keratinocytes. The presence of the E2F upon HDAC inhibition can be explained by the increased protein stability due to the acetylation as it is previously reported by several studies (Bhalla, 2005; Caron et al., 2005; Minucci et al., 2006). Thus, the HDACi mediated increase of E2F stability is due to activated pCAF which has an HAT activity. Consequently, E2F acquires increased DNA binding affinity, enhanced transactivation capacity and prolonged half-life (Martinez-Balabas et al., 2000; Ianari et al., 2004).

The abundance of E2F can be regulated at the level of transcription (Johnson et al., 1994) and on its DNA binding (Dynlacht et al., 1994) capability. Moreover the hypophosphorylated pRb appears to be in fact a specific protector of E2F from proteasomal degradation (Hateboer et al., 1996; Hofmann et al., 1996). Therefore the half-life regulation of E2F is not a monocausal event. The activity of pRb can be increased also by HDACi (Lindemann et al., 2004) via acetylation as a non-histone target. Interestingly, Chan et al., (2001) demonstrated that acetylation of pRb can inhibit its phosphorylation by cyclin E/CDK2. Moreover, studies of the group of Brown (2002)

reported that the role of the six-lysine basic patch of pRb on the rim of the LXCXE binding site is essential to relieve from E2F. Amongst others, the effect on E2F transactivation, the mechanism and biological relevance of pRb acetylation remain unclear.

Previous work reported that the ubiquitin ligase p45 (SKP2) was reduced upon SB treatment (Finzer et al., 2001) which plays a role in ubiquitin-mediated proteasomal degradation of E2F (Marti et al., 1999). It is still questionable why under these condition E2F level is not upregulated, since it was shown that ectopic p45<sup>SKP2</sup> expression can induce premature degradation of E2F (Marti et al., 1999). Whether this discrepancy reflects an equilibrium between the accumulation of E2F and its preferential degradation when pRb is absent remains to be answered. Furthermore, the observed unchanged level of E2F in E7 immortalised keratinocytes needs to be clarified prior to and after delivery of E7-expression vectors.

Interestingly E2F is down-regulated in the presence of the hypophosphorylated pRb in E6 keratinocytes. This seems to be contradicting (Figure-3.5 B) because of the upregulation of the cyclin E in these cells (see above).

One explanation for that could be an autoregulatory loop by which E2F regulates its own expression. It is known that the transcription of the SKP2/p45 is directly regulated by E2F itself. The increased expression would then efficiently induce the degradation of E2F.

Nevertheless the expression levels of E3 ligase p45<sup>SKP2</sup> in E6 and E7 immortalised keratinocytes needs to be investigated to explain the down regulation of E2F upon SB.

Further assumption of an inefficient binding of hypophosphorylated pRb with E2F needs to be confirmed by co-immunoprecipitation analyses. So far evidence still failed to clarify the role of both acetylated pRb and E2F and their interaction between the pRb-pocket C (Whitaker et al., 1998) and E2F transactivation domain upon SB will be the objective for further studies.

#### **4.3 Physical association of pRb and E7 is important for the HDACi mediated degradation of pRb**

To further investigate the crucial role of the direct interaction between E7 and pRb upon HDACi treatment, a strategy employing the postranslational modification of E7 by the serine protease inhibitor TLCK was used. Consequently, co-treatment with TLCK stabilised the hypophosphorylated and hyperphosphorylated pRb (Figure-3.12 A; 3.13 A1). The recovery of the hypophosphorylated pRb was mostly due to the modified E7 which was unable to directly interact. Under normal conditions, the E7 binds pRb via its LXCXE motif (Watanabe et al., 1990; Stirdivant et al., 1992) and is responsible for pRb degradation by proteasome mediated pathway (Wang et al., 2001). Modification of E7 by TLCK is caused by alkylation of the cysteine residue at the LXCXE binding motif (Stöppler et al., 1996) which is directly involved in

the association with Rb pocket domain (Lee et al., 1998). Studies in immortalised E7 keratinocytes clearly demonstrate the individual role of the E7 in this context. Nevertheless the levels of the accumulated pRb are higher in HeLa than in E7-Keratinocytes. Remarkably, the repression activity of the recovered hypophosphorylated pRb correlates with the decreased level of cyclin E expression (Figure-3.12 A).

In addition, the appearance of the hyperphosphorylated pRb in HeLa, E7 and E6 immortalised keratinocytes can be explained by the decreased levels of p21, which was shown to be downregulated in a dose dependent manner with TLCK (Figure-3.16 A). Therefore the activity of Cyclin/CDK2, 4 and 6, which are responsible for the cell cycle dependent phosphorylation of pRb (Weinberg et al., 1995a; Sherr et al., 1995a) needs to be further investigated.

Furthermore, it is conceivable that E7 modification could prevent E7 to perform other cellular activities which are important for the cell cycle progression, such as direct activation of cyclin A, D and inhibition of the CDK inhibitor p21 (Zerfass et al., 1995; Zwerschke et al., 2000; zur Hausen 2002). Moreover previous results by our group demonstrated that HDACi treatment downregulates the expression of cyclins such as D and A (Finzer et al., 2001). Therefore the involvement of the cyclin D and A in the hyperphosphorylation of pRb upon TLCK and SB co-treatment needs to be clarified. Interestingly, p21 is downregulated even in the presence of the modified E7. Thus, that might occur in an E7 independent manner. However, whether this downregulation occurs at transcriptional level or not needs to be demonstrated.

It is obvious that TLCK synergize the activity of E7 in the HPV transformed cells by counteracting the pleiotropic effects of HDAC inhibitors in order to support the cell progression. Nevertheless, a clear demonstration of the reduced association between E7 and pRb upon TLCK co-treatment with HDACi is the objective of further co-immunoprecipitation experiments.

Previous studies have shown that E7 is phosphorylated by casein kinase II (CKII) and, suggest that phosphorylation by CKII is a significant factor in regulating the function of E7 (Barbosa et al., 1990; Storey et al., 1990; Firzlaff et al., 1991). Inhibitors of the CKII class such as TBB (4, 5, 6, 7-tetrabromobenzotriazole) (Sarno et al., 2001), were used to inhibit the E7 function on HDACi mediated pRb degradation. The experiments showed that TBB was not able to counteract the effect of HDACi on pRb degradation. This suggests that phosphorylation of E7 does not contribute to HDACi induced pRb degradation (data not shown).

Other possible strategies can help to understand and confirm the direct involvement of E7 in pRb degradation such as disrupting the interaction between E7 and pRb by using the peptide aptamer technique. Thus, Nauenburg et al., (2001) demonstrated that a specific artificial peptide which can bind with a high affinity to E7 can knock out the function of E7 such as reducing the proliferation of NIH3T3 (Mouse embryonic fibroblast cells) cells expressing E7 protein.

Conversely, posttranslational modification of pRb can also be considered as a strategy in order to study the interaction between these two proteins. For example SUMOylation of pRb disrupts the interaction with E7 (Ledl et al., 2005) and therefore can be used for our purpose.

#### **4.4 Proteasomal degradation of pRb in HPV positive cells**

The degradation of the tumor suppressor protein pRb as a short lived protein (Wang et al., 1994; Knudson and Wang, 1997) is controlled by the proteasome pathway. In normal as well as in HPV transformed cells, pRb degradation requires the presence of the 26S proteasome (Boyer et al., 1996). Thus, treatment of E7 keratinocytes with the proteasome inhibitor MG132 revealed and confirmed the role of the proteasome in HDACi mediated pRb degradation (Figure-3.10). Moreover, the recovered hypophosphorylated pRb further supports the crucial role of E7 protein in this specific enhanced degradation process of pRb. This result is in agreement with the initial observation in HeLa cells which was shown by Finzer et al. 2004. Notably, blocking the proteasome should lead to an increase of hypophosphorylated pRb, also demonstrating the requirement of the proteasome for HDACi mediated degradation of pRb. Since pRb can be posttranslationally acetylated by binding to p300/CBP, which is a transcription factor with intrinsic histone acetyltransferase (HAT) activity, the stability is increased by treatment with HDACi. According to this, SB treatment switches the HAT/HDAC equilibrium within the Rb complex in favour of HAT. Thus Chan et al., (2001) reported that certain lysine amino acids at positions 830 and 884 are enhanced acetylated, which in turn increase the level of hypophosphorylated pRb after MG132 incubation.

However, experiments need to be performed in order to clarify the functionality of the recovered pRb such as binding to E2F and the re-establishment of the inhibitory effects on cyclin E expression. Conversely, the observed decrease of hypophosphorylated pRb in E6 keratinocytes after simultaneous MG132/SB treatment seems to have a synergistic effect with HDAC inhibitor since both of them lead to a decrease of hypophosphorylated pRb (Figure-3.10). It has been previously reported that the cellular level of Rb is regulated by ubiquitin-dependent proteolysis in HPV-positive cervical cancer (Wang et al., 2001) and in other tumor cells, such as human lung cancer (Uchida et al., 2005).

The detailed mechanism of how pRb is degraded by the proteasome system is not completely understood. It has been suggested that E7 may function as an adaptor between pRb and the proteasome without prior ubiquitination. Initial studies revealed an interaction between E7 and the S4 ATPase subunit of the 19S regulatory complex of the 26S proteasome. Furthermore it is known that E7 binds pRb and S4 through independent domains, it is possible that an E7/Rb/S4 quaternary complex forms an intermediate step leading to the proteolytic degradation of pRb

(Berezustkaya et al., 1997). Recent reports indicate that the HPV16E7 associates with the enzymatically active Cullin-2 ubiquitin ligase complex and that the HPV16E7/pRb complex contains Cullin-2 (Huh et al., 2007). Therefore analyses of the association between Cullin-2 and HPV18E7/pRb complex and its role in enhanced degradation of pRb upon HDAC inhibition will be the objective of further studies.

Nevertheless, the initial ubiquitination analyses of pRb upon HDAC inhibition remains unsuccessful (data not shown). However, recent data show that pRb can be degraded via the proteasome in an ubiquitin independent manner (Kalejta et al., 2003; Sdek et al., 2005). Thus several studies demonstrate the involvement of the 20S proteasome in pRb degradation (Orlowski et al., 2003). Interestingly, Tanahashi et al., (2000) demonstrated that the estimated concentration of the 20S proteasome in HeLa cells was approximately twice as high as the amount of the 26S or 20S-11S-REG complexes. Thus it would be interesting to analyse whether the 20S proteasome plays a role in the trimeric complex consisting of Rb-26S / 20S-E7 by HDAC inhibition. The fate of the trimeric complexes of pRb/E7/26S or 20S upon HDAC inhibition is urgently necessary be analysed.

In addition, the contribution of other pRb destabilization proteins to the degradation process following HDAC inhibition needs to be clarified. For example, the cellular oncoprotein gankyrin contains also the LXCXE Rb-binding motif and target pRb for its degradation in either ubiquitin-dependent or ubiquitin-independent proteasome pathways (Higashitsuji et al., 2000 and 2005; Dawson et al., 2006; Nakamura et al., 2007). The exact molecular mechanisms, however, remain elusive.

Since pRb binds many nuclear proteins including MDM2 (Uchida et al., 2005) and BRCA1 (Hashizume et al, 2001), which have ubiquitin ligase activities, these interaction partners could also be investigated in the future. Interestingly, a recent report of Sdek et al., showed that MDM2 binds the C8 subunit of 20S proteasome and promotes Rb-C8 interaction, leading to a proteasome-dependent ubiquitin-independent degradation of Rb both *in vivo* and *in vitro*. This finding further indicates that ubiquitination is not always a prerequisite for the proteasomal degradation of pRb (2005).

Recently, it was found that E7 promotes C-terminal cleavage of pRb, (a 95 kDa fragment (pRb<sup>(1-810)</sup>), by calcium-activated cysteine protease calpain and that this cleavage is required before E7 can promote proteasomal degradation of pRb (Darnell et al., 2007). Considering this recent data and the knowledge that MG132 can also block the calpains, experiments to clarify the role of the calpains in E7 mediated pRb degradation by HDAC inhibition need to be performed.

## **4.5 HDACi mediated cell cycle arrest and induction of apoptosis in HPV positive cells**

### **4.5.1 HDACi mediated cell cycle arrest**

It has previously been shown that HDAC inhibitors can cause cell-cycle arrest in G1 and /or G2 phase and apoptosis in cultured cells (Marks et al., 2001). From a large number of studies it is known that this is often associated with the p53-independent induction of CDKN1A (a p21 promoter) (El-Deiry et al., 1995; Guittal et al., 2004; Varshochi et al., 2005). HDACi mediated cell cycle arrest was concomitant with p21 upregulation in HeLa cells (Figure-3.14 and 3.15) as previously shown by Finzer et al., (2001).

The induction of the CDKN1A might not be solely responsible for the G1 arrest observed, as HDACi-mediated repression of cyclin D and cyclin A genes are likely to contribute to the loss of CDK2 and CDK4 kinase activities and hypophosphorylation of pRb which is sequentially phosphorylated during entry into S phase by cyclin D/CDK4 and CDK6 complexes and Cyclin E/CDK2 complexes (Kim et al., 1999; Qiu et al., 2000; Sandor et al., 2000; Finzer et al., 2001). Surprisingly, transactivation of cyclin E is not associated with CDK2 activity, but likely due to the concomitant increase of p21 levels and subsequent the loss of the pRb repression on cyclin E promoter (Sambucetti et al., 1999; Sandor et al., 2000; Kim et al., 1999).

The cell cycle arrest in the presence of E7 seems to be quite contradictory, as it is known that viral proteins E6 and E7 promote cell cycle progression. From our previous data we know that HPVE7 is not responsible for the observed cell cycle arrest, as it was shown that the HPV transcription was deregulated but the posttranslational level of HPV was not affected by HDAC inhibition (Finzer et al., 2002). Moreover studies with C33-A clearly demonstrate that the presence of HPV is not a prerequisite for HDACi induced cell cycle arrest in cervical cancer cells (Figure-3.15 C). Here we observed a cell cycle arrest in G2/M- phase. Several studies reported that G2/M arrest, which is activated by G2-phase checkpoint, is a much rarer event than HDACi induced G1 arrest (Qiu et al., 2000; Burgess et al., 2001). Nevertheless this demonstrates that HDACi induced cell cycle arrest (G1 and/ or G2 phase) is not dependent on the HPV content. Moreover HDACi induced cell cycle arrest has been shown to be irreversible and resembles replicative senescence (Richon et al., 2000; Archer et al., 1998). Indeed, a study demonstrates that the function of E6 and E7 can be circumvented during the senescence (Chen et al., 1993). Interestingly modification of E7 by TLCK can abolish the HDAC inhibitor induced cell cycle arrest independent of modifying the E7 protein. The observed dose dependent downregulation of p21 confirms the accumulation of cells in S-phase (Figure-3.16 A and B). TLCK mediated p21 downregulation implies that TLCK can reverse the SB mediated effect independent of the E7 modification. Indeed as a general serine protease inhibitor the effects of

the TLCK can not be limited only to the E7 protein modifications, since several serine/threonine kinases are involved in regulating cell proliferation and cell death (see discussion 4.5.2). Experiments addressing the direct role of the E7 in HDACi mediated cell cycle arrest, needs to be analysed with TLCK co-treatment in E6 and E7-immortalised keratinocytes.

#### 4.5.2 HDACi mediated apoptosis

The therapeutic potential of HDACi stems from their capacity to selectively induce apoptosis in tumor cells compared with normal cells. In agreement with previous studies (Drummond et al., 2005; Kelly et al., 2005), treatment with HDAC inhibitor causes an increased apoptotic cell population after longer treatment of HeLa cells (Figure-3.17 A and B).

One explanation for this observation could be the direct induction of the p21 gene at chromatin level. It is well known, that at the restriction point of G1-phase, a decision is made either G1 arrest and/or apoptosis (King and Cidlowski, 1998). Furthermore many regulators of the cell cycle play key roles in apoptosis regulation (Craig et al., 1997; Katayose et al., 1997). Nevertheless, the observed apoptosis cannot be explained by the upregulation of the p21 since several studies reported that p21 upregulation induced by HDACi, reduces the HDACi mediated cytotoxicity (Burgess et al., 2001). The observation of strong degradation of pRb, while E7 protein did not considerably change upon HDACi, strongly indicates that the loss of pRb is the cause of apoptosis. Depending on the cell system and the external stimulus, pRb can be also cleaved by activated caspase-3 during apoptosis, meaning that pRb degradation is a consequence of the apoptotic stimulus (Boutillier et al., 2000; Chau et al., 2002). But previous data by our group demonstrated that the degradation of pRb upon HDACi in HeLa cells was independent of caspase cleavage (Finzer et al., 2004).

Conversely, several studies showed that loss of pRb can sensitize the cells to apoptosis (Du and Pogoriler, 2006) as demonstrated in various tissues of Rb<sup>-/-</sup> mice. In fact, pRb acts not only as a transcriptional corepressor that controls cell cycle progression (Zhang and Dean, 2001), its absence in pRb-deficient cells is associated with increased rates of apoptosis (reviewed by Chau and Wang 2003). One of the several models which try to explain the apoptotic phenotype of *RB*-null cells propose that suppression of apoptosis is a primary function of pRb, independent of its antiproliferative activity (Chau and Wang 2003). Since the inactivation of pRb can stimulate proliferation and apoptosis, and the pRb/E2F complex regulates the expression of S phase and apoptotic genes (Nevins et al., 2001; Muller et al., 2001), it is possible that Rb/E2F promoter complexes could be differentially regulated in response to mitogenic and apoptotic signals. The promoter-specific regulation of Rb/E2F transcription complex at the promoters of S-phase genes is regulated through pRb phosphorylation, whereas at the promoters of apoptotic genes, the main

regulatory step is the degradation of pRb. This model would predict that a fraction of the pRb/E2F repression complexes are stable and functional in proliferating cells (Chau and Wang, 2003).

Loss of pRb leads to accumulation of free E2F and transactivating genes and induction of the intrinsic apoptotic pathway (Qin et al., 1994; Almasan et al., 1995; Lieman 2005).

The degradation of the pRb during HDACi mediated apoptosis obviously requires the presence of the E7 oncoprotein (Finzer et al., 2001 and 2004). It is known that E7 inherently controls the half-life of pRb and enforces its intracellular degradation upon HDACi which in turn diminishes the anti-apoptotic activity of pRb. Notably, reports from a previous study have shown that E7 can also interact with E2F-1 in a pRb-independent manner and thereby increasing the E2F-1 proapoptotic activity (Hwang et al., 2002). This observation was confirmed by immortalised keratinocytes in which only the E7 immortalised keratinocytes were strongly apoptotic while the E6 keratinocytes were not (Stöppler et al., 1998; Finzer et al., 2001). Also a report from Iglesias et al., (1998) confirms the presence of E7 for E2F-1 mediated apoptosis upon sensitisation of the keratinocytes.

Interestingly, HPV negative cervical carcinoma cells C33-A were not sensitive to HDACi induced cell death (Figure-3.17 C). Therefore apoptosis analyses of the HPV-negative cells C33-A confirmed that the cells were not sensitive to HDAC inhibitor treatment. Moreover, PARP cleavage of the HPV positive cells clearly contributes to the role of HPV in apoptosis upon HDACi treatment (Figure-3.5.6).

These data indicate an essential role of HPV presence in apoptosis in cervical carcinoma cells induced by HDAC inhibitor SB and TSA. Moreover loss of pRb upon HDACi provides some evidence of their anticancer activity which is accounted for transcription-independent effect (Cohen et al., 2004; Subramanian 2005).

A large number of independent studies strongly support a role for the mitochondrial apoptotic pathway in HDACi-mediated tumor cell death. These data show that overexpression of BCL2 or BCL-XL, both blocked the intrinsic apoptotic pathway, inhibit apoptosis mediated by diverse HDAC inhibitors irrespective of cell type (Ruefli et al., 2001; Peart et al., 2003; Mitsiades et al., 2003; Zhao et al., 2005).

Since HDAC inhibitors stabilize the E2F protein by acetylation (Caron et al., 2005) it is conceivable that the anti-proliferative effect could occur by inducing the E2F/p14<sup>ARF</sup>/p53 pathway. Thus E2F-1 triggers the activation of p14<sup>ARF</sup>, which in turn leads to stabilization of p53 by inhibiting its E3 ligase MDM-2 (Zhang et al., 1998; Pomerantz et al., 1998). However this possibility can be excluded, because the protein level of p53 remained unaffected up to 32 h in the presence of HDAC inhibitors and elevated p21<sup>CIP1/WAF-1</sup> (Finzer et al., 2004). Nevertheless



p53 can be also acetylated by HDAC inhibitors (Bolden 2006), which would contribute for its stabilisation and strengthen its potential as an inducer of apoptosis. Even though the levels of p53 can be reduced by E6 oncoprotein inherently (Münger and Howley 2002; zur Hausen, 2002), the residual p53 levels could still participate in apoptosis. Hence, experiments where cells transfected with p53 specific siRNAs showed that the apoptotic rate was not influenced upon SB treatment (Finzer et al., 2004).

Since the p53-dependent mechanism can be ruled out in HDAC inhibitor induced apoptosis, other candidate molecules were investigated which can be also induced by E2F transcription factor. Previous reports considering the microarray analyses on HeLa cells where E2F activity was induced by HDAC inhibitor SB, did not show any induction of the potential target genes like p14<sup>ARF</sup>, Apaf-1 (Moroni et al., 2001) and Bcl-2 (Gomez-Manzano et al., 2001) (Finzer et al., unpublished data). Nevertheless this supports the notion that the range of induced E2F genes is not activated in all cell systems (Stanelle et al., 2002).

Phillips and Vousden (2001) reported an E2F1 induced apoptosis via the p53 homolog p73 (Irwin et al., 2000). While p53 is posttranslationally stabilized by MDM2 and p14<sup>ARF</sup>, the p73 activation by E2F-1 occurs via binding to E2F-1 responsive site within the p73 TA promoter, which finally acts exclusively on transcription. We observed an upregulation of p73 at transcriptional level as demonstrated in Figure-3.18 A (HeLa) and in Figure-19 B (E7 keratinocytes respectively). This correlates with the previous observation of apoptotic analyses (Figure-3.17 A, B and Finzer et al., 2001).

The slight upregulation of p73 in E6 keratinocytes (Figure-3.19 D, E) can be explained by the fact that p73 is not a target for E6 oncoprotein, unlike p53 (Melino et al., 2002), which indicates that p53 and p73 are differentially recognized by viral proteins. Also the reduced level of E2F in E6 keratinocytes upon HDACi confirms that this induction of p73 involves other transcriptional regulation than via E2F. Actually it is shown that p73 can be induced by other transcription factors such as c-Myc (Zaika et al., 2001). However the interplay between c-Myc and p73 is still controversial (Watanabe et al., 2002).

Interestingly, co-treatment with TLCK did not only influence HDAC inhibitor mediated enhanced pRb degradation in the presence of E7 oncoprotein but also reduced transcription of the apoptotic p73 in HPV positive HeLa cells and in E7 immortalised keratinocytes (see Figure-3.19 A, B). This would explain the recovery of the pRb protein and thereby reduced sensitivity to apoptosis. The dose-dependent decrease of the p73 transcription following TLCK and SB co-treatment, significantly confirms the reduction of the G1 arrest in HeLa cells (see Figure-3.16). The strong accumulation in S phase supports that SB mediated G1 arrest can be reversed (see 3.16 B).

As a general serine protease inhibitor, TLCK mediated effects cannot be restricted to modifications of E7 protein. It has been shown that TLCK and TPCK (also a serine protease inhibitor) can inhibit a wide range of general cellular functions including chemotaxis (Barna et al., 1995), gene expression (Griscavage et al., 1995), aortic relaxation (Rodriguez et al., 1996), and apoptosis (Yoshida et al., 1996; Mitsui et al., 2001; Okada et al., 2004).

As previously mentioned several serine/threonine kinases are involved in regulating the cell proliferation and cell death. Kuninaka et al., (2007) demonstrated that the serine protease Omi/HtrA2 targets WARTS kinases to control the cell proliferation. WARTS are serine/threonin proteases which interact with the mitochondrial serine protease Omi/HtrA2 (Srinivasula et al., 2003; Salvesen and Duckett, 2002) and induce their protease activity. Omi/HtrA2 was previously identified to be released into the cytosol by apoptotic stimuli and induced cell death through its own serine protease activity, attenuating the activity of IAPs (inhibitor of apoptosis proteins) (Suzuki et al., 2001; Hegde et al., 2002; Martins et al., 2002; Verhagen et al., 2002).

Moreover, Yang et al., (2007) reported that serine/threonine kinases Akt1 and Akt2 (Franke et al., 1995; Burgering et al., 1995; Cross et al., 1995) phosphorylate the mitochondria-released HtrA2/Omi on serine 212 *in vivo* and *in vitro*, which results in attenuation of its serine protease activity and pro-apoptotic function. Interestingly, mitochondrial release of the Omi/HtrA2 was previously demonstrated by the group of Garcia-Morales (2005) following apoptotic stimulus with HDAC inhibitors. Further studies should address the apoptotic fate of the cells upon TLCK and SB co-treatment in E6 and E7- immortalised keratinocytes. Furthermore it would be interesting to evaluate the role of p73 in HPV negative cervical carcinoma cells and how these cells would be affected upon co-treatment with TLCK. Also measurement of the transmembrane potential by cytochrome C release upon SB (Finzer et al., 2004) and TLCK co-treatment would help to understand the role of the mitochondrial pathway in the used cell system.

The splice variant,  $\Delta$ Np73 transcripts of p73 gene which lacks the transactivation domain (exon1-3) function as an antiapoptotic protein (Yang et al., 2002).  $\Delta$ Np73 is a dominant negative inhibitor of the TAp73 which was shown to be upregulated after longer treatment with SB (24h) and therefore accounting for reduced expression of TAp73 (Finzer et al., 2004). Determining the expression level of this antiapoptotic splice variant in E6 immortalised keratinocytes would be interesting, since it is known that these cells did not undergo apoptosis by HDAC inhibition (Finzer et al., 2001).

p73 induced apoptosis involves the induction of PUMA (a BH3 only protein), which in turn promotes Bax mitochondrial translocation and cytochrome C release in a time dependent manner (Melino et al., 2004). However, Bax protein levels did not correlate with the kinetics of apoptosis (Ramadan et al., 2005). Whereas p73-induced and PUMA mediated mitochondrial translocation

of Bax is kinetically compatible with the induction of cell death (Ramadan et al., 2005). This might explain why Bax expression was not observed in response to SB treatment (Finzer et al unpublished data). p73 is localized in the nucleus, and remains nuclear during the induction of apoptosis. This indicates that the effect of p73 on Bax is indirect. Thus a direct interaction of p73 with PUMA would be interesting to demonstrate by immunoprecipitation upon SB treatment in our cellular systems. Nevertheless the localisation of the p73 in nucleus might be also examined upon SB.

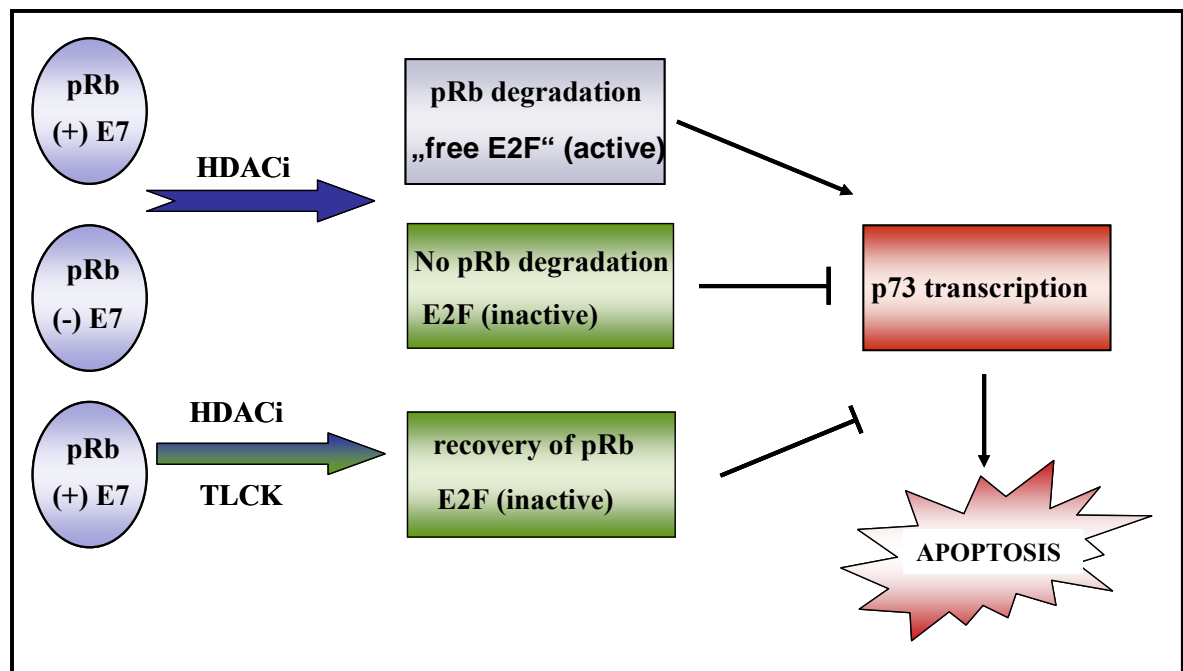
### **Conclusion and Future Perspective:**

In this present study it was shown that the presence of HPV E7 is a prerequisite for HDAC inhibitor SB and TSA mediated pRb degradation. This degradation mainly occurs by a proteasome dependent pathway. Nevertheless, possible co-factors such as E3 ligases, which could be involved in enhanced degradation of pRb or other mechanism contributing to its degradation, should be determined. Moreover these data confirm that the presence of HPV E7 sensitizes the cells for SB mediated apoptosis which can be reversed by posttranslational modification of the HPV E7.

Furthermore co-treatment of SB with TLCK demonstrates that the HDAC inhibition mediated degradation of pRb can be rescued. This results in an increase of the repressive function of pRb on E2F transcription factor which subsequently reduces the transcription of the apoptotic gene p73. Down-regulation of p21 by TLCK further interferes with SB mediated cell cycle arrest. Finally the HDAC inhibitor mediated apoptosis can be inhibited by TLCK. Subsequently this study bears new future aspects in understanding the role of serine proteases during HDAC inhibition.

HDACi show a promise as single agent anticancer drugs in HPV-induced carcinogenesis: they first force HPV-positive cells to arrest the cell cycle, and subsequently trigger the intrinsic apoptosis by targeting the pRb/E2F/p73 pathway without impairing oncogene suppression. Given the range of molecular and biological responses that these agents can elicit and minimal toxicity to normal cells, the combination with other agents such as with proteasome inhibitor MG132, could prove to be their most useful application (Bolden et al., 2006).

Figure-4.1 illustrates the main themes of this work.



**Figure-4.1: HDAC inhibitor induced cell cycle and apoptosis is influenced by TLCK.**

HDAC inhibition of HPV positive HeLa cells induces the expression of the cyclin–dependent kinase inhibitor p21 resulting in G1 arrest. Prolonged treatment with SB triggers the induction of apoptosis only in E7 positive cells (HeLa and E7 immortalised keratinocytes). Enhanced degradation of pRb occurs only in E7 containing cells upon HDAC inhibition. Loss of pRb leads to a “free E2F” which induces the transcription of the proapoptotic genes p73. TLCK treatment influencing the SB mediated effect: first restoring the pRb level and reducing the expression of the p21. Cell cycle arrest is inhibited by entry in the S phases. Secondly the re-establishment of the hypophosphorylated pRb acts again as a repressor protein and reduced the expression of the p73.

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