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Identification of epigenetic mechanisms involved in transcriptional activation of silent HPV-16 genomes

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Dedicated to

my family, especially my grandparents.

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1 Summary

1.1 English

An important initial step towards cervical cancer is the integration of HPV DNA copies into the host genome. Subsequently, the viral DNA gets targeted by methylation leading to its transcriptional silencing (Jeon et al., 1995; Yu et al., 2005). Later on, demethylation of these inserted HPV genomes correlates with further progression of cervical cancer (Badal et al., 2003). Therefore, characterisation of epigenetic mechanisms involved in oncogene re-expression is required to potentially counteract cancer development.

In this study, the contribution of both epigenetic mechanisms, DNA demethylation and incorporation of histone variant H3.3 for the re-activation of the viral DNA transcription was analyzed. The cervical cancer cell line CaSki harbors approximately 600 mostly methylated HPV-16 copies and provides an excellent *in vitro* model to study epigenetic mechanisms in the context of HPV induced carcinogenesis. DNA demethylation was induced by treatment of these cells with 5-Azacytidine (DAC), which led to demethylation of approximately 50% of the silent HPV-16 copies. In addition, clones stably expressing a Myc-tagged H3.3 were generated and characterized in combination with DAC treatment.

On protein level, DAC treatment of CaSki cells was accompanied by an unexpected down-regulation of the oncoprotein E7, where intracellular half life was rescued after exposure to proteosomal and calpain inhibitors. Additionally, for the first time, an interaction of E7 with, the transcription activating histone arginine methyltransferase, CARM1 was detected after E7-IP by Mass Spectrometry and Western blot. On transcriptional level, a slightly down-regulation of the E7 oncogene was detectable after DAC treatment of CaSki cells. Importantly, the same treatment induced E7 transcription in CaSki clones expressing Myc-H3.3, which was verified by both qPCR and Northern blot.

In summary, this study demonstrates that DAC treatment induces down-regulation of oncoprotein E7 in CaSki cells. In addition, the data imply that DNA demethylation alone is not sufficient for re-activation of silent HPV genomes, since induction of E7 transcription strictly requires both, DNA demethylation and incorporation of the histone variant H3.3 into the URR of HPV-16.

1.2 German: Zusammenfassung

Das Cervixcarzinom ist die zweithäufigste malignombedingte Todesursache bei Frauen weltweit. Pathogenetisch kommt dabei der Integration humaner Papillomviren (HPV) in das Genom ihrer Wirtszellen eine entscheidende Rolle zu (Jeon et al., 1995; Yu et al., 2005). Anschließend erfolgt die Methylierung der meisten integrierten viralen Genome, wobei die Transkription viraler Onkogene durch einige nicht-methylierte Kopien noch gewährleistet bleibt. Im Rahmen der weiteren Krankheitsprogression werden Anteile der zunächst methylierten viralen DNA dann erneut demethyliert (Badal et al., 2001). Die genaue Entschlüsselung des Zusammenwirkens dieser epigenetischen Mechanismen, deren Interaktionen zur Reaktivierung ausgeschalteter Genome führt, ist daher zum Verständnis der Entstehung und Progression des Cervixcarzinoms von entscheidender Bedeutung.

In der vorliegenden Arbeit wird die Rolle der epigenetischen Mechanismen, DNA-Methylierung und Einbau des Histon Varianten H3.3 bei der Reaktivierung von HPV-16 DNA untersucht. Dabei wurde als optimales in vitro Model die Zervixkarzinomzelllinie CaSki ausgewählt. CaSki Zellen beinhalten ca. 600 integrierte und überwiegend methylierte HPV-16 Genome (Mincheva et al, 1987). Die Demethylierung von ca 50% der viralen DNA-Kopien wurde durch Behandlung mit 5-Desoxy-Azacytidin (DAC) erreicht. Zusätzlich wurden CaSki Klone hergestellt, die den Histone 3.3 Varianten stabil exprimieren.

Anschließende Untersuchungen ergaben, dass auf Proteinebene die Behandlung mit DAC zur Herunterregulation des Onkoproteins E7 führt. Inkubation mit Calpain- und Proteasom-Inhibitoren führte zur Hemmung der E7 Degradation in den DAC behandelten Proben. Zusätzlich wurde die Interaktion von E7 mit der Histone-Methyltransferase CARM1 mittels Immunpräzipitation und anschließender Massenspektrometrie und Western Blot nachgewiesen. Auf transkriptioneller Ebene wurde eine leichte Herunterregulation der E7 mRNA in CaSki Zellen nach DAC Behandlung detektiert. Hingegen erfolgte bei gleicher Behandlung, in den H3.3 stabilen CaSki Klonen, der Einbau des H3.3 Varianten in der regulatorischen Region (URR) des HPV-16 und damit die Induktion der E7 mRNA Transkription. Dies konnte mittels Chromatin-Immunpräzipitation und Northern Blot detektiert werden.

Zusammenfassend konnte gezeigt werden, dass die Behandlung mit DAC zur Herunterregulation von E7 in CaSki Zellen führt. Zusätzlich wurde nachgewiesen, dass zur erneuten Induktion der E7 Transkription aus ausgeschalteten HPV Genomen, neben DNA Demethylierung der Einbau der Histon Varianten H3.3 zwingend notwendig ist.

2 Introduction

2.1 Human papilloma viruses (HPV) induced cervical cancer

Cervical cancer is the second most common cancer among women worldwide (Parkin and Bray, 2006) and the fifth leading cause of cancer mortality. Worldwide, approximately 470,000 new cases of cervical cancer are diagnosed annually, appearing usually among woman with a mean age of 52 years (Pagliusi, World Health Organisation; Fehrmann and Laimins, 2003).

Persistent and long-lasting infection with one of the 15 high risk human papilloma viruses (HPV) is the necessary cause for development of cervical cancer (Pisani et al., 2002). In 95% of cervical tumors high risk HPV DNA is detectable. The most common oncogenic HPVs are HPV-16 and HPV-18, known to cause approximately 70% of cervical cancers (Munoz et al., 2003). HPV is a common sexually transmitted virus. Young and middle aged healthy women usually undergo only transient infections, which are cleared by the immune system (Wallin et al., 1999; Snijders et al., 2006). Nevertheless, a small proportion of women unable to overcome the infection are committed to finally developing cancer. Additional risk factors are immuno-suppression, cigarette smoking, changing sexual partners and co-infection with the human immunodeficiency virus (HIV) (Waggoner, 2003; Castle et al., 2004; Hellberg et al., 2005).

Cervical cancer development is divided into well defined pre-malignant stages, which are recognized by cytological examinations of exfoliated cervical cells (zur Hausen, 2002). The “Pap test” was first described by Papanicolaou and is still the key diagnostic tool for cervical cancer screening. Abnormalities detected by Pap smears are further analysed by histological examination of cervical material and detection of viral DNA by PCR (Saslow et al., 2007). Pre-malignant stages are characterized as different cervical intraepithelial neoplasias ranging from CIN1 (mild dysplasia) to CIN2 (moderate dysplasia) and to CIN3 (severe dysplasia/ carcinoma in situ) (Waggoner et al., 2003; Saslow et al., 2007).

Prevention of cervical cancer in developed countries is based on well established and extended cancer screening programs, leading to a notable decrease (zur Hausen, 2002). Another promising approach is the population based prophylactic immunization program with bivalent HPV (types 16 and 18) and quadrivalent HPV (types 6, 11, 16 and 18) vaccines which started in 2006 (Schiller and Davies, 2004, Lowy and Schiller, 2006, Garcia and

Saslow; 2007). Nevertheless, despite these ambitious approaches for prevention, cervical cancer is still not curable. Therefore, further research for better understanding the oncogenic properties of high risk HPV and subsequently curing cervical cancer needs to be done.

2.2 The PV genome structure and transcriptional control

Papilloma viruses (PV) are small non-enveloped double stranded DNA viruses that belong to the family of Papillomaviridae (de Villiers et al., 2004). The circular DNA genome of PV has a size of approximately 8 kb containing three major regions: early, late, and an upstream regulatory region (URR, also called long control region: LCR). These regions are divided by two polyadenylation (pA) sites: early pA (AE) and late pA (AL). The URR covers a 850 bp (10% of the HPV genome) long non-coding region, harbouring the origin of replication and multiple regulatory transcription factor binding sites for transcriptional initiation of the viral early (Bernard et al., 2002) and late promoters (Grassmann et al., 1996). About 50% of the viral genome consists of the early region, which encodes six open reading frames for the transcription of the early viral proteins E1, E2, E4, E5, E6 and E7. Downstream of the early region is the late region, which covers almost 40% of the viral genome and encodes the major (L1) and the minor (L2) capsid proteins (Zheng et al., 2006).

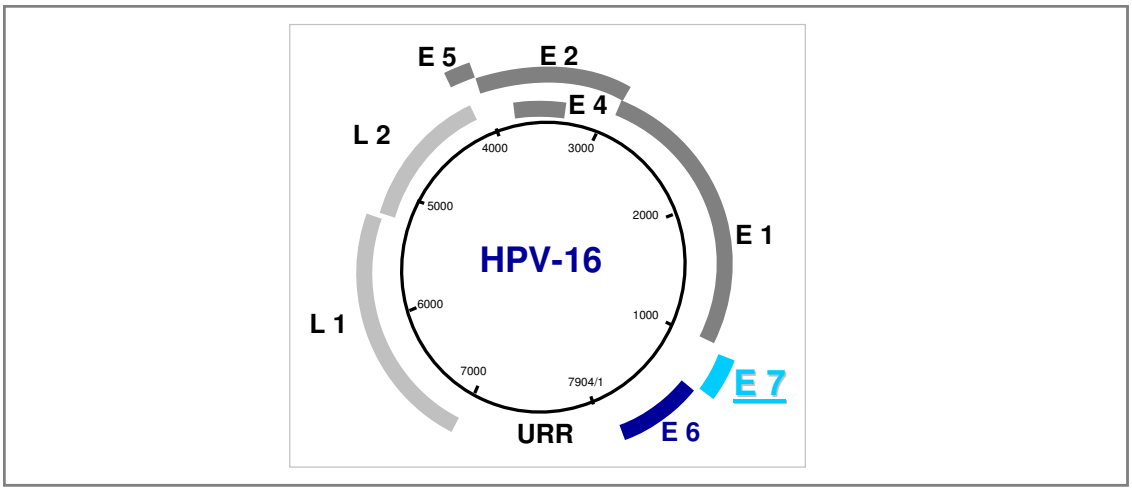

***Figure 2.1* The genome of the human papilloma virus type 16 (HPV-16).**
The different open reading frames for the viral proteins and the upstream regulatory region (URR) are shown.

2.2.1 Normal PV infection and productive replication

The PV replication is tightly controlled and regulated by the keratinocyte differentiation (Bedell et al., 1991). The virus usually enters through microlesions of skin and mucosa and reaches the basal layers of a stratified squamous epithelium. Infection occurs in keratinocytes of the basal layers, which are still able to proliferate. After infection the viral genome persists as a stable episome (Stubenrauch and Laimins, 1999; zur Hausen, 2002). Upon limited expression of six non-structural viral regulatory proteins (E1, E2, E4, E5, E6 and E7) from the early region of the viral genome, enhanced proliferation of the infected cells followed by their lateral expansion is induced (Stubenrauch and Laimins, 1999; zur Hausen, 2002; Doorbar, 2006). Hereby E5 plays an important role, as it can form complexes with various proliferation stimulating factors like the epidermal growth factor receptor (EGFR), the platelet-derived growth factor (PDGF) and the colony stimulating factor-1 receptor (CSFR) (Hwang et al., 1995). After reaching the suprabasal layers, the late viral gene expression is initiated. Thereby, the proteins E1 and E2 are involved in viral DNA replication and the regulation of early transcription. The viral protein E2 plays different roles in viral genome replication and maintenance. The most important ones are the recognition and binding to DNA within the palindromic motif: AACCg(N4)cGGTT in the URR of the viral genome (Dell et al., 2003), and the anchoring of viral episomes to mitotic chromosomes for correct segregation (You et al., 2004). E2 binding facilitates the recruitment of E1, a helicase, which in turn supports the binding of cellular proteins necessary for DNA replication (Masterson et al., 1998; Conger et al., 1999; Loo et al., 2004). This results in amplification of the viral genome. In addition, the formation of the structural capsid proteins (L1, L2), which takes place exclusively in the nucleus, is induced (Day et al., 1998; Zhao et al., 2000; Buck et al., 2004). Finally, in the upper layers of the epidermis or mucosa the viral particles are assembled and released, whereby the viral protein E4 associates with cytokeratin filament collapse in a productive infection (Doorbar et al., 1991; Wang et al., 2004).

2.2.2 High risk PV and the multi-step progression to malignancy

The frequency of infection with a high-risk HPV among young women is usually 20-40 %, reaching up to 60%, depending on geographical location (Woodman, et al., 2001;

Richarsson et al., 2003). Although infections with high risk HPV are very common, only women who cannot clear the virus and develop a persistent active infection, lasting years or decades, will finally develop cervical cancer (Richardson et al., 2003; Cuschieri et al., 2003; Parkin et al., 2002). During this time, progression is accompanied by changes in the expression pattern of viral proteins. In CIN1, the viral DNA is still episomal and the typical sequential expression of the viral proteins (Figure 2.1), resulting in viral release, is observed (Klaes et al., 1999; Fuji et al., 2005; zur Hausen, 2002). A crucial step toward malignancy is

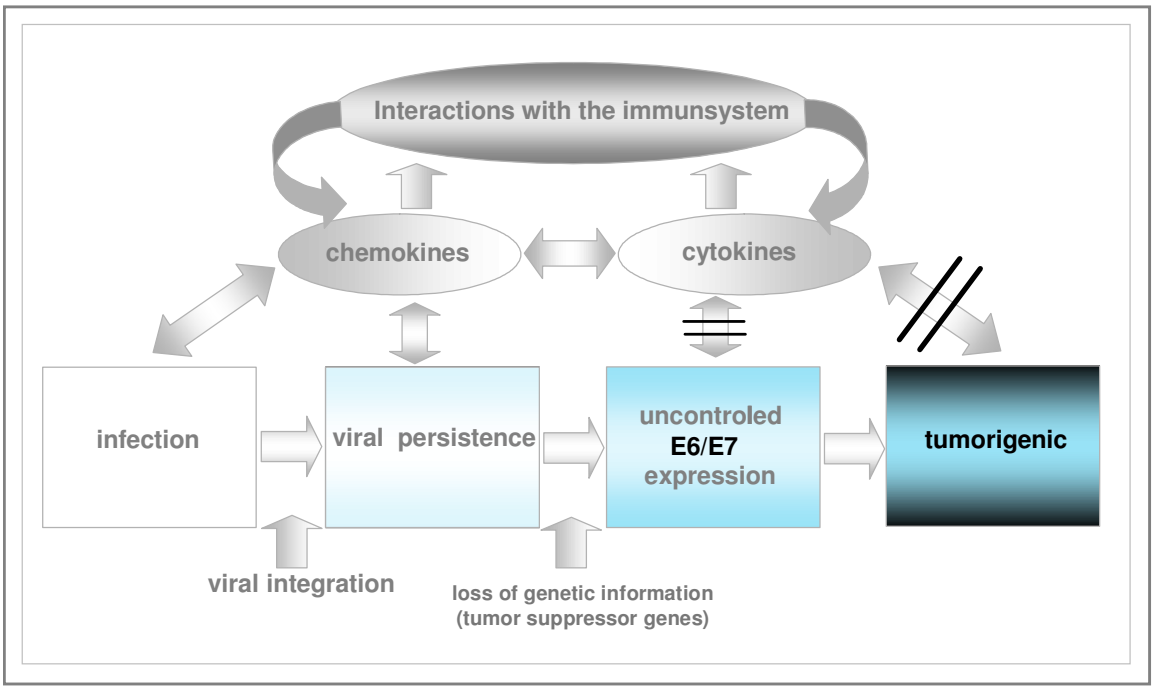

***Figure 2.2* The multi-step process leading to cervical cancer.**
The different steps towards malignancy are illustrated with regard to the crucial steps like viral integration, loss of both genetic information in form of tumor suppressor genes and control by the immunesystem.

the integration of the viral genome into the genome of the host cells (Jeon et al., 1995; Yu et al., 2005). Upon further progression, the productive stages of the virus life cycle are shifted towards thinner layers of the epithelial surface until the viral reproduction is no longer supported, and the oncoproteins E6 and E7 are expressed throughout the malignant tissue (zur Hausen, 2002; Doorbar, 2006). Furthermore, integration of the viral genomes into the host genome is preferentially accompanied by the disruption of the region containing the open reading frame for the protein E2 (Choo et al., 1987). This leads to the loss of expression of the proteins E1, E2 and E5 (Corden et al., 1999). E2 can act as a negative regulator of the E6/ E7 transcription, by occupying sequences in the HPV promoter region and therefore limiting the binding of necessary host transcription factors. (Lewis et al., 1987; Tan et al., 1992; Tan et al.,

1994). Deregulation of viral protein expression and additional pivotal changes in the intracellular control mechanisms of the host cells result in an uncontrolled proliferation (Woodman et al., 2007).

A prerequisite for progression to cancer is the escape of the malignant cells from detection by the immune system, a phenomenon which is called immune surveillance (Stagg et al., 2007). One key event is loss of expression of the chemokine MCP-1, which is induced in healthy cells by TNF-α (Figure 2.2) (Rösl et al., 1994). Thereafter, the recruitment of effector cells of the immune system to the sites of viral infection ceases, allowing the uncontrolled growth of malignant cells (Kleine et al., 1995; Kleine-Lowinsky et al., 1999; Kleine-Lowinsky et al. 2003).

2.2.3 The AP-1 transcription factors and their involvement in the transcription of HPV

2.2.3.1 The AP-1 transcription factors

The host cell activator protein 1 (AP-1) complex plays a significant role in the transcriptional induction of the HPV URR. The complex is built up by different dimer compositions from the members of the JUN, FOS, activating transcription factor (ATF) (Bakiri et al., 2002) and musculoaponeurotic fibrosarcoma (MAF) protein families (Matsushima-Hibiya et al., 1998; Li et al., 1999). The c-Jun and c-Fos family members belong to the immediate early genes (IE) (Herschman et al., 1991). These genes are induced within minutes after treatment with the appropriate stimuli, like epidermis growth factor EGF (Kajanne et al., 2007), tumor necrosis factor (TNF-α) (Brenner et al., 1989) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Espino et al., 2006). Hence, AP-1 activity is controlled by a variety of physiological, pathological and oncogenic stimuli, creating a huge diversity of biological functions. Therefore, AP-1 is involved in the induction and regulation of cellular responses reaching from proliferation to apoptosis (Angel and Karin, 1991; van Dam and Castellazzi, 2001). Whereas Jun proteins are able to homo- (Grondin et al., 2007) and heterodimerize (Turner et al., 1989), the Fos family members are only able to heterodimerize (Smeal et al., 1989). Depending on the stimulus and the duration of induction, different proteins dimerize to build the AP-1 transcription factor (Hess et al., 2004). The composition of the dimerisation defines which genes are further induced (Chalmers et al., 2007). FOS and JUN proteins have been first described as the viral oncoproteins v-Fos and v-

Jun in the Finkel-Biskis-Jinkins osteosarcoma virus (Curran et al., 1982) and avian sarcoma virus 17 (Maki et al., 1987), respectively. AP-1 proteins are characterized by a basic leucin zipper domain (bZIP). The leucin zipper is needed for the dimerisation, whereas the basic domain allows the interaction with the DNA backbone (Turner et al., 1989; Smeal et al., 1989). There are different DNA sequences which are recognized by AP-1. The strongest sequence is the TPA response element (TRE), but different dimers also bind to the cAMP-respose element (CRE), the MAF recognition elements (MAREs) and the antioxidant-response elements (AREs) (Sng et al., 2004; Hess et al., 2004).

2.2.3.2 The AP-1 transcription factors and their involvement in the transcription of HPV

The AP-1 transcription factor is required for the transcription of HPV (Offord and Beard, 1990). HPV-16 harbours 3 AP-1 binding sites and HPV-18 2 sites in their URR (Figure 2.3). Inactivation by mutagenesis of one site in HPV-18 leads to suppression of the HPV transcription (Butz and Hoppe-Seyler, 1993). In addition, similar to the regulation of other cellular events, the composition of the AP-1 transcription factor complex is essential for the HPV transcription (Soto et al., 1999). Previous experiments with the antioxidant pyrrolidine-dithiocarbamate (PDTC) have already shown that induced changing of the AP-1 composition influences the HPV transcription (Rösl et al., 1997). Besides AP-1 several other cellular transcription factors regulate the HPV transcription from the HPV enhancer region (Chong et al., 1991, Figure 2.3).

Taking advantage of a hybrid cellular system, which allows the comparison between premalignant and malignant cervical cancer cells, it has been shown that the AP-1 composition is pivotal for HPV transcription (Soto et al., 1999). HPV-18 positive HeLa cells were fused to normal fibroblasts resulting in the cell line 444 which is not tumorigenic in immunodeficient nude mice (Soto et al., 1999). In contrast, HeLa cells grow and form tumors under the same experimental settings (Soto et al., 1999). Treatment with TNF-α induces down-regulation of HPV expression only in 444 cells (Rösl et al., 1994), due to enhanced expression of Fra-1 and the accompanied changes in the AP-1 composition from predominantly Jun/Jun or Jun/Fos to Jun/Fra-1 dimers. Neither this alteration in the AP-1 composition, nor the suppression of viral transcription is detectable in HeLa cells or in tumorigenic segregants from the 444 cells, namely CGL3, after incubation with TNF-α (Soto et al., 1999).

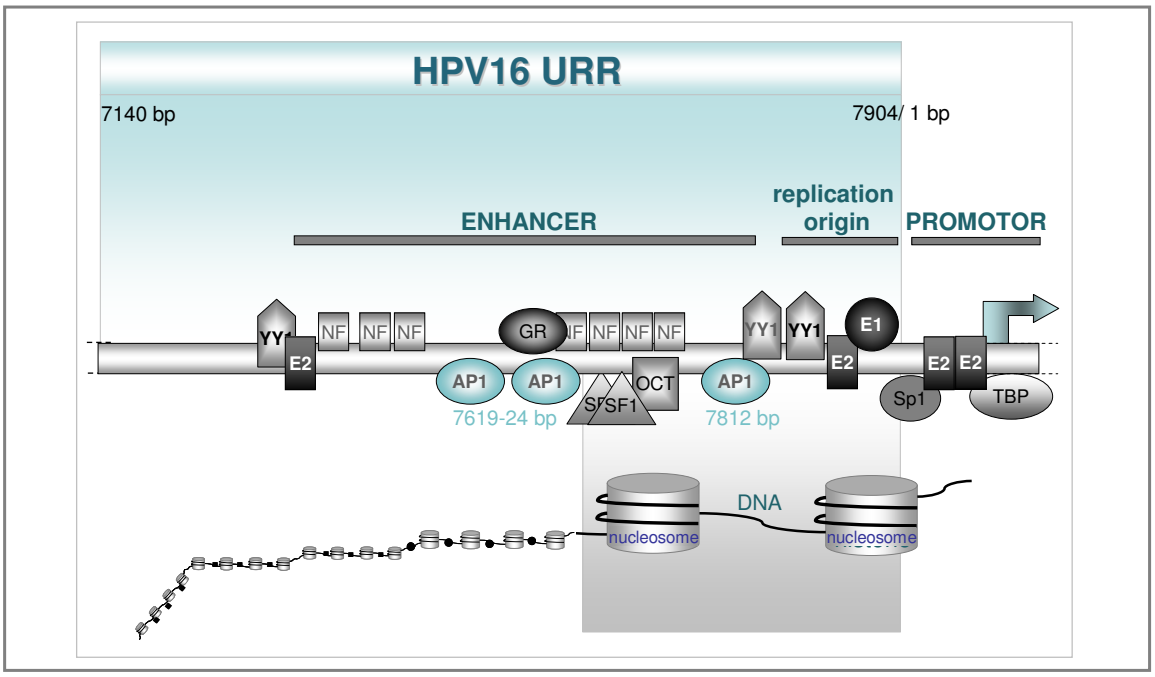

***Figure 2.3* Schematic overview for the HPV-16 URR.**
Binding sites for transcription factors and repressors in the enhancer, replication origin and promoter region are illustrated. The arrow marks the transcriptional start site. Two important nucleosomes which are directly located close to the promoter and enhancer regions are also shown. Viral proteins are written in white letters, whereas host cellular proteins are black. GR: glucocorticoid receptor, AP1: activator protein 1, NFI: nuclear factor 1, TF1: transcription enhancer factor 1, OCT: octamer binding factor 1, TBP: TATA-box binding protein, YY1: Yin Yang 1, SF1: steroidogenic factor 1

2.2.3.3 MAPK- regulated AP-1 activation

Mitogen activated protein kinases (MAPK) are proline-directed serine/threonine kinases, which have been shown to induce AP-1 activation (Whitmarsh and Davis, 1996). The MAPK cascades are induced by various extra-cellular stimuli like growth factors, cytokines and cellular stress and, in addition to other factors, regulate the AP-1 transcription factor complex (Hazzalin and Mahadevan, 2002). After signal transduction MAP-Kinase-Kinase-Kinases (MAPKKK) are activated by phosphorylation and they in turn activate MAP-Kinase-Kinases (MAPKK) resulting in phosphorylation of the MAPK (Sng et al., 2004; Young et al., 2006). Several signal transduction pathways have been related to the final activation of three distinct MAPK: extracellular-regulated kinase (ERK) (Chalmers et al., 2007), c-Jun N-terminal kinase (JNK) and p38 (Hazzalin and Mahadevan, 2002). The activation of MAPK

leads to their translocation to the nucleus and subsequent activation of their specific substrates (Chen et al., 1992; Brunet et al., 1999; Mizukami, 1997). JNK, for example, phosphorylates c-Jun on Ser-63 and Ser-73 within its N-terminal activation domain (Hibi et al., 1993). This stabilizes and activates c-Jun, which results in the enhanced transcriptional activation of c-Jun dependent genes (Musti et al., 1997). Additionally, c-Jun phosphorylation allows the recruitment of the coactivator CBP to transcriptional active sequences (Arias et al., 1994). For signal termination the kinases are deactivated through dephosphorylation by antagonistic phosphatases (Sng et al., 2004).

2.3 The high risk HPV oncoproteins E6 and E7

The transforming potential of the oncoproteins E6 and E7 *in vitro* has been evidently proven by the immortalisation of primary human keratinocytes (Münger et al., 1989; Hawley-Nelson et al., 1989). The high-risk HPV E6 and E7 oncoproteins are sufficient for induction of immortalisation (Münger et al., 1989; Hawley-Nelson et al., 1989).

Further activation of oncogenes (DiPaolo et al., 1989) or sustained maintenance in tissue culture (Dürst et al., 1989) are necessary for the cells to become entirely transformed. These experimental approaches again underline the requirement of a multi-step progress to carcinogenesis (DiPaolo et al., 1989; Dürst et al., 1989; Snijders et al., 2006). Transgenic mouse models expressing the oncoproteins under tissue specific promoters have shown that E6 usually induces mostly malignant tumors, whereas E7 was predominantly found in benign tumors (Herber et al., 1996; Song et al., 1999). Nevertheless, when expressed together E6 and E7 where able to induce malignant tumors (Arbeit et al., 1993; Griep et al., 1993; Comerford et al., 1995).

The HPV E6 protein is about 150 amino acids long and contains two zinc-finger motifs (Cole et al., 1987; Barbosa et al., 1989). E6 can induce transformation by targeting p53 to proteasomal degradation (Scheffner et al., 1990). During this process E6 first binds to the ubiquitin ligase E6AP, particularly to its N-terminal recognition domain (Huibregtse et al., 1993). This interaction alters the specificity of E6AP toward p53, which leads to p53 degradation (Huibregtse et al., 1993). Additionally, E6 can directly bind to the p53 C-terminus (Werness et al., 1990; Li and Coffino, 1996), thereby inhibiting the stabilisation or

E7 domain	interaction	function	author
zinc-finger motif	c-Jun (AP-1)	transcription	Antinore et al., 1996
pRb-binding domain (AA 31-32)	pRb	cell cycle progression	Berezutskaya et al., 1997
pRb-binding domain (AA 31-32) zinc-finger motif	TBP	transcription	Phillips et al., 1997
C-terminus	S4 subunit of the 26S proteasome	protein degradation	Berezutskaya and Bagchi, 1997
zinc-finger motif (Cys-X-X-Cys motif)	HDAC, Mi2b	transcription Histone deacethylation	Brehm et al., 1999
N-terminus	MPP2	transcription	Lüscher-Firzlaff et al., 1999
---	IRF-1	transcription	Park et al., 2000
C-terminus	Skip	transcription	Prathapam et al., 2001
---	BRCA1	---	Avvakumov, 2003
---	SOCS-1	E7 ubiquitination degradation	Kamio et al., 2004
N-terminal	Cullin1, Skp2	E7 ubiquitination degradation	Oh et al., 2005
Zinc-finger motif	HPV16 E2	E7 stability and localisation	Gammoh et al., 2006
N-terminal	p600	transformation, anchorage inde-pendent growth	Huh et al., 2005
Zinc-finger motif	BRCA1	---	Zhang et al., 2006
PEST domain (AA 25-36)	IRF-9	Circumvention of IFN-a signaling	Antonsson, 2006
---	Nm23-H1	Invasiveness apoptosis resistance	Mileo et al., 2006
C-terminus	Siva-1	apoptosis resistance	Severino et al., 2007
HPV 18 :C-terminal: Cys-X-X-Cys motif	c-Myc	transcription	Wang et al., 2007
zinc-finger domain	Dnmt1	DNA methylation	Burgers et al., 2007
Overlall structure	FHL2	transcription cell transformation	Campo-Fernandez et al., 2007

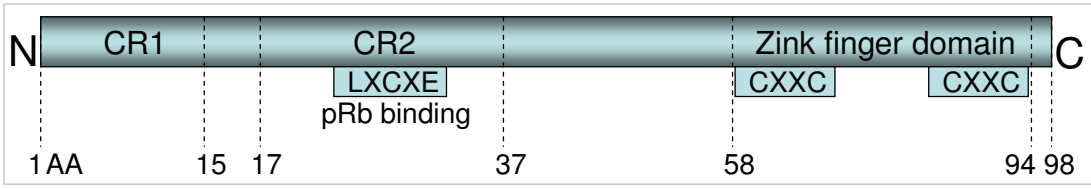

Figure 2.4. E7 interaction partners and a schematic overview of the E7 structure.
The already discovered E7 interaction partners are shown in chronological order with the respective interaction domain and its function.

functional activities of p53 like DNA binding (Lechner et al., 1994; Thomas et al., 1995). p53 plays a pivotal role as a sensor of cellular stress (Derry et al., 2001) and DNA damage (Gatz et al., 2006; Bunz et al., 1998) and is mutated in 40 % of different cancer types (Lengauer, 1998; Hainaut et al., 1998). Its degradation leads to an increase of mutations and the affected

cells lack the ability to undergo apoptosis through the intrinsic pathway. Both effects are prerequisites to malignant progression (Fridman and Lowe, 2003; Mihara et al., 2003). An additional interaction partner of E6 is the transcriptional activator p300/CBP (Patel et al., 1999; Zimmermann et al., 1999), which may alter transcription in infected cells.

The HPV-16E7 protein is 94 amino acids long and can be divided in three functional regions: the N-terminal conserved region (CR) 1 and CR2 regions and the C-terminal zinc-finger domain. CR1 and CR2 have structural and functional homologies to regions of the adenovirus E1A and Polyomavirus large T antigen, whereas the zinc-finger domain does not show any similarities to E1A or the large T antigen (Phelps et al., 1988; Barbosa et al., 1990). E7 has been described to interact with various cellular proteins including retinoblastoma protein 1 (pRb) (Dyson et al., 1989; Münger et al, 1989; Figure 2.4). Under normal conditions the pRb family members, p110, p107 and p130 interact with E2F thereby inhibiting its function (Dyson, 1993). Upon pRb phosporylation in uninfected normal cycling cells, conformational changes of pRb leads to the release of transcriptionally active E2F, which in turn induces the transcription of proteins for cell cycle progression from G0 to G1 and into the S phase (Dyson, 1998). E7 is able to disrupt the pRb-E2F complex by binding to pRb (Dyson et al., 1989). The free E2F is now able to induce cell cycle progression. This transition to the S-phase is important for ensuring viral DNA replication in normal viral life cycle (Banerjee et al., 2006; Flores et al., 2000). In addition, interaction of high risk HPV with pRb induces its proteosomal degradation (Münger and Howley, 2002; Gonzalez et al., 2001). Another E7 interaction has been shown between the S4 ATPase subunit of the 19S regulatory complex of the 26 S proteasome and E7 (Berezutskaya and Bagchi, 1997). This is of particular interest as it supposes that E7 functions as an adaptor between pRb and the proteasome (Berezutskaya and Bagchi, 1997). Until today several E7 interaction partners were discovered and analysed, which are summarized in Figure 2.4.

2.4 Epigenetic and cancer

2.4.1 Epigenetic changes through cancer progression

The term “epigenetic” defines inherited patterns of altered gene expression which are independent of the primary DNA sequence itself. There are three main interrelated

mechanisms of epigenetic inheritance: DNA methylation, genomic imprinting and histone modifications.

In mammals, DNA methylation occurs only at cytosines located 5´ to guanosine (CpGs) and is a key element of epigenetic regulation inducing transcriptional silencing (Bird et al., 2002). CpG islands are accumulated in promoter and enhancer regions of nearly 50% of the genes in the mammalian genome (Gardiner-Garden et al., 1987). Furthermore, regarding the whole genome, only 20% of the statistically expected CpGs are present (Schorderet and Gartler, 1992). This specific CpG distribution underlines the particular importance of gene regulation by DNA methylation.

Several reports have shown the important role of the DNA methylation status in cancer progression (Costello et al., 2000; Esteller et al., 2000; Weber et al., 2005). Particularly, cancer cell genomes show simultaneously both global hypomethylation (Gaudet et al., 2003; Eden et al., 2003, Rodriguez et al., 2006) and tumor suppressor gene-promoter-specific hyper-methylation (Jones and Baylin, 2002; Gokul et al., 2007; Nomoto et al., 2007). Feinberg et al. provided the first evidence of genome wide DNA hypomethylation in cancer samples compared to normal cells (Feinberg et al., 1983). Hypomethylation seems to be linked to genomic instability, structural changes in chromosomes and increased gene expression (Ehrlich et al., 2002; Rodriguez et al., 2006; Eden et al., 2003). Gene specific activation by hypomethylation was defined for the oncogenes R-ras in gastric cancer and for cyclin D2 and maspin in pancreatic cancer (Nishigaki et al., 2005; Oshimo et al., 2003; Akiyama et al., 2003). In HPV induced malignancies, hypomethylation includes HPV genomes resulting in carcinogenic progression. For example, in invasive carcinomas only 6 % of HPV-16 DNA is methylated whereas 50 % of asymptomatic smears show hypermethylated HPV-16 DNA (Badal et al., 2003). These results were further confirmed by De Capoa et al. (2003). Furthermore, the oncoproteins E6 and E7 induce numerical and structural chromosome instability (Duensing and Münger, 2002), which might be enhanced by hypomethylation of the host genomes. Another study demonstrated that the URR and the E6 coding region remained unmethylated in almost all analysed samples with patient material from various stages of cervical cancer progression (Turan et al., 2006). In addition, direct comparison of HPV-18 L1 methylation revealed that L1 was methylated only in caricinomas and can therefore be considered as a biomarker for neoplastic progression (Turan et al., 2006).

In contrast to the genome wide hypomethylation, promoters of important tumor suppressors are hypermethylated in many cancer types, whereas in normal cells the same CpG islands are not methylated (Jones and Baylin, 2002; Gokul et al., 2006; Nomoto et al., 2007).

Of particular interest is that about 50% of the genes which are mutated in the germline, and thus cause familiar forms of cancers, are also silenced by methylation in a variety of sporadic cancers. Among these genes is the well known breast cancer 1 (*BRCA1*) (Birgisdottir et al., 2006), which is mutated in breast cancer. Other affected genes, which are also mutated in several cancers, are the retinoblastoma 1 (*RB1*) (Simpson et al., 2000) and *p16* (Blanco et al., 2007).

In cervical cancer a growing number of methylated genes have been detected. For example suppressor of cytokine signaling (SOCS-1), which is involved in E7 proteasomal degradation after IFN-gamma treatment of cervical carcinoma cells, is methylated in 50% of cervical carcinomas (Widschwendter et al., 2004). Another interesting link between epigenetic and cervical cancer development has been suggested by the recently discovered interaction between E7 and the DNA methyltransferase1 (DNMT1). The E7 binding enhances the activity of DNMT1 (Burgers et al., 2007). This might be a hint for the involvement of the viral protein in methylation of tumor-suppressor genes. Furthermore, E7 might induce the transcription of DNMT1 by inducing E2F activity. This has been already shown for other viral proteins, like the adenovirus E1A and the polyomavirus BKV large T antigen (TAg) (McCabe et al., 2006). E7 is known to induce E2F activity by the degradation of pRb. Interestingly, DNMT1 transcription can be induced by E2F1 (McCabe et al., 2005). Therefore, E7 might not only induce the transcription of proteins important for cell cycle progression through E2F but, as an additional benefit, the transcription of DNMT1 resulting in methylation of tumor-suppressor genes.

Future efforts promise the identification, by microarray based technologies, of novel tumor-suppressor genes and their subsequent characterization in the context of tumor progression. Epigenetic changes are likely to be an essential first step towards malignancy, since additional mutations, needed for further progression, are more easily acquired after global hypomethylation and hypermethylation of tumor-suppressor genes. Moreover, analysis of the genome methylation patterns will provide biomarkers for defined tumors, for risk considerations, early detection of neoplasias and accessibility or response to cancer therapies.

2.4.2Induction of DNA demethylation by DNA-methytransferase inhibitors

Since promoter specific DNA hypermethylation of tumor-suppressor genes has been detected in many cancer types, the usage of DNA methyltransferase inhibitors for cancer treatment has become a promising new field of investigation (Mund et al., 2006).

DNA methyltransferases (Dnmts) are a family of enzymes that methylate DNA by adding a methyl-group to the carbon-5 position of cytosine residues (Bestor, 2000). In mammals, four Dnmt are currently known, namely Dnmt1, Dnmt2, Dnmt3a, Dnmt3b (Bestor et al., 2000; Tang et al., 2003). Nevertheless, only three of them are fully active. Dnmt1, which is the major Dnmt, has been predominantly shown to maintain the methylation patterns after DNA replication and associates with the replication machinery during the S-phase (Leonardt et al., 1992). Dnmt1 interacts with several proteins involved in epigenetic regulation like HDACs (Rountree et al., 2000), HP1, SUV39H1 (Fuks et al., 2003), MeCP2 (Kimura et al., 2002) and transcription factors like E2F1, pRb (Robertson et al., 2000) and p53 (Esteve et al., 2005). Dnmt3a and Dnmt3b are *de novo* methyltransferases (Okano et al., 1999).

The first inhibitors of Dnmt, the nucleotide analogs 5-aza-2'-deoxycytidine and its analog 5-azycytidine (AC), were synthesized by Sorm and collegues (Sorm et al., 1964). DAC is only incorporated into DNA, in contrast to AC which also incorporates into RNA and therefore induces cellular activities independent of demethylation. However, DAC is up to 10 times more toxic that AC in cultured cells and animals (Bouffard et al., 1994). Despite higher toxicity, DAC is usually preferred in both research and clinic, for induction of demethylation, due to its ability to strictly bind only to DNA (Jüttermann et al., 1994). Both DAC and AC develop their activity following incorporation into the DNA thereby trapping the Dmnt to DNA by covalent irreversible binding to the catalytic core, which subsequently decreases the amount of free enzyme molecules in the cells resulting in demethylation (Santi et al., 1984).

DAC is also known as Decitabine. Several reports have proven that Decitabine treatment induces demethylation in hypermethylated promoters of tumor-suppressor genes leading to their reactivation, and hence phenotypic reversion of cancer cells (Magdinier et al., 2000; van der Velden et al., 2001). In clinical studies, Dnmt inhibitors are commonly used for treatment of chronic myelogenous leukaemia, sickle cell anemia and myelodyplastic syndrome (MDS). Clinical studies for the treatment of other cancer types are proceeding, though DAC is not stable in aqueous solution and needs to be administrated frequently rendering the treatment inconvenient. Therefore, additional demethylating drugs are currently

under study for improving cancer treatment concerning the mode of application and toxicity (Lyko and Brown, 2005).

2.4.3 Involvement of DNA methylation in PV gene expression

The HPV-16 genome contains 112 CpG sites, mainly located on the URR, although taking into consideration the genome size (7,900 bp) and the C+G content (40%), about 400 CpG sites would be expected. This observation is also true for all common HPV types (Myers et al., 1994). The enhancer and promoter region of HPV-16 harbours 11 CpG dinucleotides. Detailed analysis of the CpG patterns showed that CpG methylation occurs predominantly in the URR and in part of the L1 ORF (Rajeevan et al., 2006). The distribution pattern underlines that methylation of HPV genomes is important for regulating viral protein expression. Indeed, experiments with *in vitro* methylated HPV-18 URR-directed reporter gene expression showed a down-regulation of HPV transcription, which was detected by down-regulation of CAT activity (Rösl et al., 1993). Additional analysis further verified that CpG methylation plays an important role for controlling the transcription of the viral genomes (List et al., 1994). Methylation of PV DNA was first detected for the PV1a (Danos et al., 1980, Burnett, 1984).

2.4.4 The CaSki cells line as an experimental in vitro model for HPV-16 genome demethylation

The HPV-16 positive CaSki cells are of epidermoid origin and were established in 1977 from a cervical cancer metastasis in the small intestines (Pattillo et al., 1977). CaSki cells harbor approximately 600 copies of the viral genome, which are incorporated, mostly in tandem, at about 11 sites into the host genome (Mincheva et al., 1987). These incorporated HPV genomes are mostly methylated and therefore transcriptionally silent (Rajeevan et al., 2006). Only one minor HPV DNA-positive locus on chromosome 14 is transcriptionally active as nascent HPV RNA was detected by tyramide-FISH (Van Tine et al., 2001). In its URR the HPV-16 genome altogether carries 11 CpG sites. Methylation analysis by bisulfite treatment revealed, that these CpG sites in CaSki cells are heavily methylated in the promoter

and the enhancer region (Kalantari et al., 2004). Later, analysis by pyrosequencing provided the tool for detecting the exact methylation status of each CpG site within the URR from each individual HPV genome among the total 600 copies in CaSki cells (Rajeevan et al., 2006).

2.4.5 Epigenetic modulation of histones

Besides DNA methylation the recruitment of proteins involved in chromatin modification results in the appropriate chromatin configuration for silencing (Burgers et al., 2003). It is currently known that DNA methylation affects histone modifications and vice versa (Johnson et al., 2002; Zhang et al., 2005). However, future experiments will clarify whether methylation leads to the initial silencing or if it is a consequence of earlier chromatin remodelling events.

The basic units of chromatin are the nucleosomes. They are built up by an octamer consisting of 2 out of 4 highly conserved core histones, namely H3, H4, H2A and H2B. Z DNA of 146 bp length is wrapped in 1.6 turns around each nucleosome. The four canonical histone proteins contain a central, globular domain, which is connected with the DNA and an amino-terminal tail (Kornberg et al., 1974; Finch et al., 1977). The linker histone H1 is located between nucleosomes and binds nucleosomes and DNA for further chromatin condensation (Thoma et al., 1979). Besides their function as scaffold proteins, histones are integrated in a complex network of major cellular regulatory mechanisms for setting of epigenetic landmarks. Thereby, the N-terminal tails of the histone proteins, which extend beyond the nucleosomal core particles, serve as modules for post-translational covalent modifications (Januwein and Allis, 2001; Kouzarides, 2007). These include acetylation of lysines, methylation of lysines and arginines, phosphorylation of serine and threonines, ubiquitination and sumoylation of lysines and ADP-ribosylation of glutamic acids (Januwein and Allis, 2001; Kouzarides, 2007). Since the modified N-termini significantly increase and supplement the information potential of the genetic code, this level of epigenetic marking has been termed the histone code (Strahl and Allis, 2000). The histone code is enriched by the incorporation of core histone variants. In addition, the extension of the modifications plays a pivotal role, as lysines can be mono, -di, or trimethylated at the ε-amino group and mono,-or di-methylated at arginine residues (Januwein and Allis, 2001).

For simplification, histone modifications can be divided in two different parts, one dealing with transcriptional activation, the other with gene silencing. Acetylation of lysines

(Eberharter et al., 2002) and arginine methylation (Bauer et al., 2002) on histones H3 and H4 residues are accompanied with transcriptional activation. One of the most well studied mark is H3 lysine 9 acetylation (H3K9ac). Methylation of lysines plays a dual role. While H3K4me (Santos-Rosa et al., 2002), H3K36me (Banister et al., 2005) and H3K79me are marks for gene activation, H3K9me (Nakajama et al., 2001) and H3K27me (Cao et al., 2004) are linked to gene silencing (Figure 2.5, Figure 2.6). An interesting aspect of histone imprints is that they can impinge on each other. For example, in gene activation phosphorylation of H3 at serine 10 (H3S10phos) facilitates the acetylation of H3K14 (Lo et al., 2000) and H3K9 (Rea et al., 2000) resulting in an open chromatin conformation (Cheung et al., 2000). Additionally, acetylation of H3K9 excludes the methylation at H3K9. H3K9me serves as a mark for gene silencing (Rea at al, 2000). Therefore it has been postulated that histone modifications act as molecular switches, enabling or blocking the setting of other covalent marks (Fischle et al., 2003).

A prerequisite for gene silencing and chromatin condensation is the deacetylation of histones (Chen et al., 2000; Landry, 2000). Subsequently, the region is marked for gene silencing for example by the well studied methylation of H3 at lysine 9 (H3K9me) (Nakayama *et al.*, 2001, Rea et al., 2000). Several protein families are involved in setting

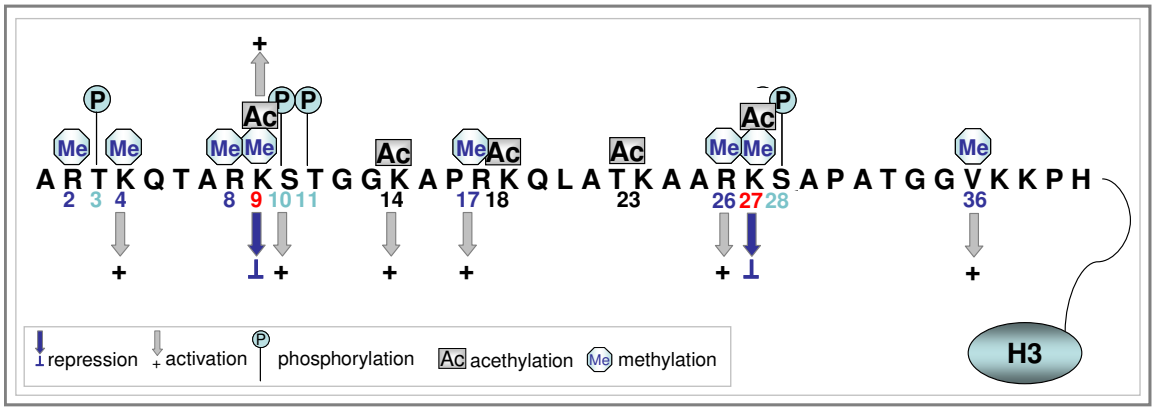

***Figure 2.5** N-terminal residues of histone H3 and reported modifications.*
The different epigenetic marks are shown. The residues which are known to undergo modifications are numbered. Red numbers represent important marks. Arrows represent the outcome of the mark, where (+) means activation and (-) repression.

the N-terminal modifications on histones. Intensive research in this field has also shown that most of the modifications are dynamic and enzymes and mechanisms which remove the histone marks have been described. Only the enzymes responsible for demethylation of arginines have not yet been identified.

2.4.6 Histone variants

Histones are the basic units of the nucleosomes. The expression of the canonical histones, H3, H4, H2A and H2B is tightly regulated and occurs only during the S-phase of the cell cycle from the intronless histone genes (Ahmad et al., 2002, Osley, 1991). These canonical histones are deposited into the DNA during a process only linked to DNA replication (Osley et al., 1991). In addition to the canonical histones other histones, so called histone variants or `replacement histones` have been discovered. They are transcribed from orphan genes throughout the cell cycle (Sarma and Reinberg, 2005). Therefore they can be incorporated into nucleosomes independently from DNA replication. Histone variants are known for H1, H3 and H2A. The difference between the canonical histones and their variants can range from some amino acids to full additional domains (Sarma and Reinberg, 2005). Given their expression throughout the cell cycle the variants are incorporated into chromatin at particular sites and they play an important role in epigenetic marking. In the last years interesting new insights in the biology and functionality of these histone variants have been achieved. Five histone variants for H2A have been described, H2A, H2AX, H2AZ (Redon et al., 2002) macroH2A (Chadwick et al., 2001) and H2ABBD (Gautier et al., 2004). Five different variants for histone H3 have also been discovered so far, namely H3.1 (the canonical histone 3), H3.2, H3.3, CENP-A (Castillo et al., 2007) and H3.1t (Trostle-Weige et al., 1984). All of them exhibit different functions. CENP-A is the histone H3 variant which exclusively localizes to centromeres. While it has high similarity to the C-terminal part of H3.1, its N-terminal region strongly differs compared to H3.1. H3.1t is only expressed in testis (Trostle-Weige et al., 1984).

Although the histone H3 variants H3.1 and H3.3 differ only in 4 amino acids, their incorporation into the chromatin is highly regulated and influences the differentiation between transcriptionally active and silenced loci (Ahmand, 2002; McKittrick et al., 2004; Johnson et al., 2004; Chow et al. 2005).

Four residues in the globular domain, which are different between H3.1 and H3.3, are responsible for the differential incorporation during the cell cycle and the S-phase restricted expression of the H3.1. H3.1 has been described to be incorporated into the nucleosomes in a strictly replication dependent (RD) (Tagami et al., 2004) manner, since these four amino acids hinder the incorporation outside the S-phase. As H3.3 varies in four amino acids, it can be incorporated throughout the whole cell cycle (Tagami et al., 2004). This restriction in the H3.1 incorporation profile ensures that, if nucleosomes that are disrupted or lost during

transcription, the new nucleosomes will also contain H3.3. Hence, H3.3 is incorporated during transcription as a common component of active chromatin and serves in marking transcriptionally active regions (Ahmad and Henikoff, 2002). Histone 3 can be modified on its N-terminal region at several residues (Figure 2.5). Further experiments have confirmed, that H3.3 is enriched for covalent histone modifications associated with active chromatin, such as acetylation at Lys9 (K9), Lys18 (K18), Lys23 (K23), di- and tri-methylation at Lys-4 (K4) (Santos-Rosa, 2002) and methylation at K79 (Strahl and Allis, 2000). In contrast, repressive modifications are enriched on the H3.1 histone (McKittrick et al. 2004). H3.1 and H3.3 associate with different chromatin-remodelling complexes *in vivo*. The mechanism of H3.3 deposition involves a specialized chaperone complex containing the protein histone cell cycle regulation defective homolog A (HIRA). In contrast, the replication-dependent incorporation of H3.1 is effected by a replication specific complex that includes chromatin assembly factor (CAF-1) (Verreault, 1996; Tagami et al., 2004; Loppin et al., 2005). It has been postulated that when H3.3-containing chromatin is more easily transcribed, increased transcription may lead to additional replacement of H3.1 by H3.3 (Tagami et al., 2004). Therefore, although during replication, the H3.3 nucleosomes will be diluted by newly deposited H3.1 nucleosomes, the genomic domains with higher H3.3 content will preserve their active status also after the replication, and the H3.1 nucleosomes will once again gradually be replaced during ongoing transcription (Nakayama et al., 2007). Regarding this model, H3.3 behaves as an epigenetic mark for transcriptionally active chromatin. Several cell lines have been tested for their incorporation patterns of H3.3. For a mouse pre-B-cell line it has been demonstrated that promoters, but not coding regions, are the principal sites of H3.3 incorporation (Chow et al., 2005). Additionally, the same distribution was also detected for the folate receptor (FR) and Vascular Endothelial Growth Factor-D (VEGF-D) promoter. Moreover, the incorporated H3.3 enhanced the expression of FR and VEGF-D (Jin et al., 2005). However, other genes included in the studies of Jin et al., like beta-globin, did not show such promoter restricted incorporation. Another set of experiments showed an enrichment of H3.3 in dihydropholate reductase (dhfr) and β-actin promoters after transient H3.3 expression throughout the cell cycle (Daury et al., 2006). Taken together, all data underline the pivotal role of histone modifications and histone variants in setting and maintaining epigenetic inheritance.

The epigenetic research field has become highly important and for the study of cancer development. As epigenetic serves as a mechanism for acquiring new, advantageous inherited changes, independently of DNA mutations, which commits normal cells to turn into tumor

cells. Progression to cancer has been always described as a multi-step process where different abnormal cellular processes finally lead to the uncontrolled proliferation of the cells. Therefore, it has been suggested that modifications of the epigenetic state might be the one initial step, leading to cellular instability and finally decisive DNA mutations during cancer development. Besides this, epigenetic changes are also able to mimic genetic mutations by silencing tumor suppressor genes or reactivating oncogenes (Esteller et al., 2000; Ehrlich, 2002; Ballerstar and Esteller, 2005).

2.4.7 Interaction between different epigenetic mechanisms

Several proteins contain domains for recognition of histone N-terminal modifications. They are mainly embedded in multi-protein-complexes, which are responsible for chromatin and transcriptional regulation (Rosenfeld et al., 2006). These complexes include transcriptional coactivators or corepressors which, upon recruitment by transcription factors, induce the adequate chromatin remodelling response. Thereby the recognition specificity, activity and histone modifications that are carried out by these complexes are determined by the proteins within the complex and their recruitment partners (Rosenfeld et al., 2006). In addition, the modulatory roles of covalent modifications on histones or protein-complexes, dictate inclusion or exclusion of specific interactive subunits from these complexes (Wang et al., 2004). Signal-induced interactions of coactivators and corepressors with sequence specific transcription factors can be controlled on different levels, including cofactor expression and post-translational modifications of both cofactors and their targets (Rosenfeld et al., 2006). For example CBP/p300 are known transcriptional coactivators with histone acethylation activity and they are part of transcriptional activator complexes. CBP/p300 interact with several transcription factors like NFkB (Covic et al. , 1999) and are recruited to promoters with specific sequences for these transcription factors, where they are also responsible for histone acethylation leading to transcription activation (Strahl and Allis, 2000). On the other hand, the well investigated nuclear receptor corepressor (NCoR) complex is involved in transcriptional repression (Perissi et al., 2004). Large protein complexes including NCoR and HDACs induce transcriptional repression (Wang et al., 2000). Well studied chromatin binding domains are the bromodomain (Haynes et al., 1992), chromodomain (Delmas et al., 1993), Trp-Asp (WD) WD40 repeats (Couture et al., 2006), Tudor domain (Callebaut and Marnon, 1997) and the plant homeo domain (PHD) finger domain (Tripoulas et al., 1996).

The bromodomain binds acetylated lysines on histone tails (Owen et al., 2000) and is therefore usually connected to transcriptional active regions (Syntichaki et al., 2000). The chromodomain recognizes methylated lysines (Jacobs and Khorasanizadeh, 2002), a mark which is mainly associated with transcriptional repression, specifically in context of H3K9me (Cheutin et al., 2003).

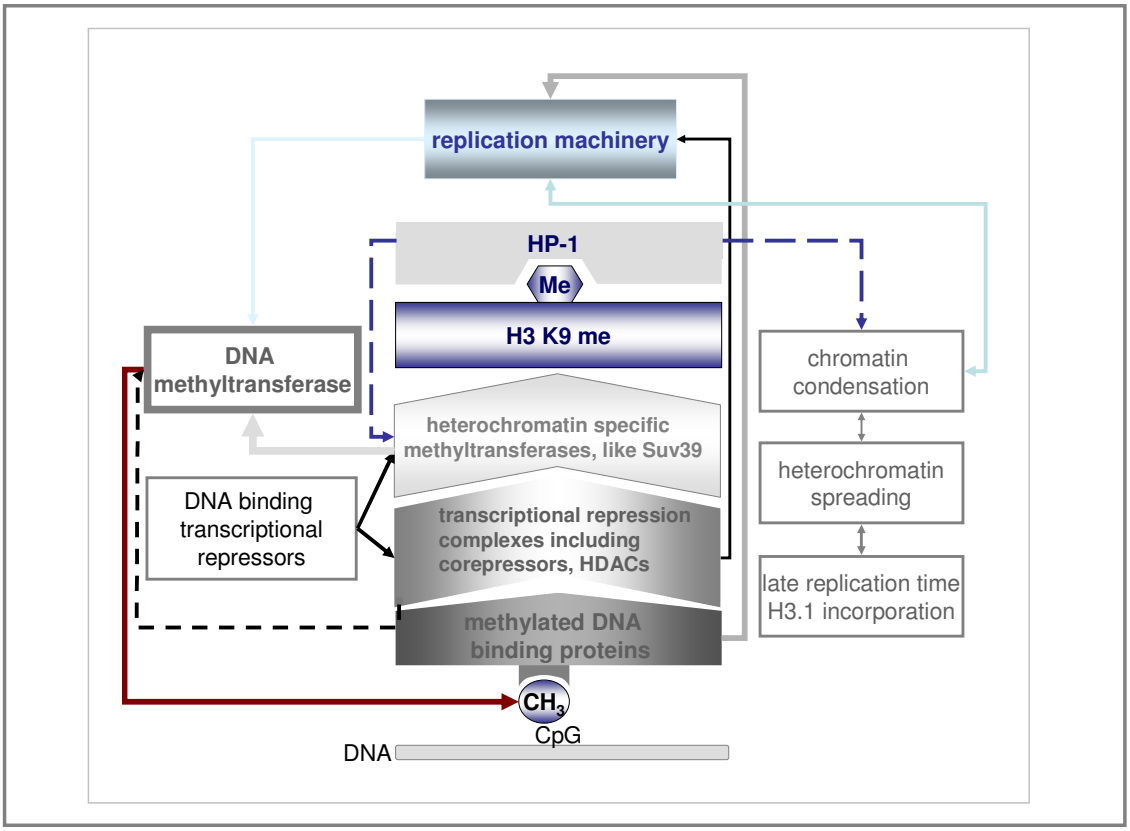

***Figure 2.6* Interaction between different mechanism and factors involved in establishing and maintaining heterochromatin.**
Upon CpG methylation **by** DNA methyltransferases methylated DNA is in turn recognized by specific binding proteins (Nendrich and Bird, 1997), which are able to recruit transcriptional repression complexes. The activity of these complexes results in deacetylation of histones (Feng and Zhang, 2001) and setting of the new repressive mark H3K9me by histone methyltransferases. This is further recognized by HP-1, which binds to H3K9me leading to chromatin condensation (Lachner and Januwein, 2002, Steward et al., 2005). The newly established heterochromatin can spread (Ebert et al., 2006) and is replicated late during the S-phase (Wu et al., 2005), where predominantly H3.1 is incorporated (Nakayama et al., 2007). This is influenced by the replication machinery, which can also interact with other factors. These mechanisms all influence each other (Fuks et al., 2003, Fujita et al., 2003) and therefore build up a tightly regulated network, illustrated by the arrows.

The WD40 repeats and PHD fingers also bind to methylated lysines (Ruthenberg et al., 2006, Li et al., 2006), whereas Tudor domains are capable of recognizing both methylated lysines (Kim et al., 2006, Huang et al., 2006) and arginines (Cote and Richards, 2005). Importantly, protein domains that bind to methylated amino acids have been described to differently associate with the various states of methylation (mono,-di-tri-methylation) (Forneris et al.,

2005). Since methylation is known to be involved in both transcriptional repression and activation, depending on which amino acid residue is methylated, domains recognizing methylation are also involved in both mechanism (Kouzarides, 2007). A brief overview for interacting molecules and their function and influences on each other in establishment and maintenance of heterochromatin are summarized in Figure 2.6.

2.4.8 The Lysine Methyltransferases and Demethylases

Lysine methyltransferases are enzymes able to specifically recognize and methylate lysines on histones and other proteins. They harbor a **S**uppressor of variegation 3-9 (Su(var)3-9), **E**nhancer-of-zeste E(z), **T**rithorax (Trx) (**SET)** domain which functions in transferring a methyl group from S-adenosyl-L-methionine (AdoMet) to the amino group of a lysine residue, leaving a methylated lysine residue and the cofactor by-product S-adenosyl-L-homocysteine (AdoHcy). The SET domain was first detected as a conserved sequence in three Drosophila melanogaster proteins: a modifier of position-effect variegation Su(var)3-9 (Tschiersch et al., 1994, Rea et al. , 2000), the Polycomb-group chromatin regulator E(z) (Jones et al., 1993), and the trithorax-group chromatin regulator trithorax (Trx) (Stassen et al., 1995). Besides these SET domain protein families, the disruptor of telomeric silencing (DOT1) family, which do not possess a SET domain, are also responsible for histone methylation, specifically H3K79 (Feng et al., 2002, Ng et al., 2002).

The histone methyltransferase (HMT) SUV39H1 is one prominent SET domain containing protein known to stabilize heterochromatic regions by setting the most general histone mark for gene silencing; the H3K9me (Rea et al., 2000). This high affinity binding site is specifically recognized by the chromodomain of heterochromatin associated protein 1 (HP1) (Lachner and Januwein, 2002, Steward et al., 2005). So far, three HP1 proteins have been identified in humans HP1α, HP1β and HP1γ. While HP1α and HP1β are predominantly associated with pericentric heterochromatin, HP1γ interacts with H3K9me in euchromatin (Eisenberg, 2000). Knockout mice for SUV39H1 are prone to develop cancer by impaired heterochromatin and genomic stability (Peters et al., 2001).

The Polycomb group PcG proteins are transcriptional repressors, which can be separated into two complexes: the initiation complex, Polycomb repression complex 2 (PRC2) and the maintenance complex, PRC1. One core protein of the PRC2, namely EZH2 is responsible for gene silencing by methylation of the Lys 9 and Lys 27 on H3 (Cao et al.,

2002; Müller et al., 2002). Notably, EZH2 is overexpressed in many cancers (Varambally et al., 2002), correlating with bad prognoses in prostate and breast cancer (Kleer et al., 2003).

The Trithorax proteins are well described as positive regulators of transcription by setting positive methylation marks on histones. One member of the trithorax group has been upregulated upon 11q23 translocations in acute leukemias (Tkachuk et al., 1992). Mixed-lineage leukemia (MLL1) is a H3 Lys 4- specific methyltransferase (Nakamura et al., 2002), this mark correlates with transcriptional activity. In addition MLL1 is interacting with the the HAT CBP from the SWI/SNF nucleosome remodelling complex (Ernst et al., 2002), thereby proceeding transcriptional preinitiation, nucleosome remodelling, histone acetylation and histone methylation (Nakamura et al., 2002).

Lysine demethylation leads to the removal of lysine methylation marks on histones. LSD1 demethylates both H3K4 and H3K9 and hence induces transcriptional repression (Shi et al., 2004) or activation (Metzger et al., 2005). Other H3K9 demethylases are JHDM2A (Yamane et al., 2006), JMJD2B (Fodor et al., 2006), JMJD2C (Cloos et al., 2006) and JMJD2D (Shin S, Janknecht R, 2007).

2.4.9 Arginine Methyltransferases and demethylases

Arginine methylation plays a role in transcriptional regulation (Fujiwara et al., 2006), translation and DNA repair (Imhof et al., 1999; Bauer at al., 2002). Like lysine methylation, arginine methylation promotes either activation or repression. This post-translational modification is carried out by the protein arginine methyltransferases family (PRMTs) (Kouzarides, 2007). So far, nine members have been discovered. One prominent member is the coactivator-associated arginine methyltransferase 1 (CARM-1, PRMT4). Like other family members CARM1 is able to methylate histones, but also other proteins. Therefore CARM1 has been described as a strong coactivator of transcription and an important protein in other cellular processes. For example in the regulation of alternative splicing by methylation of splicing factors like Spliceosome-associated protein SAP49, Small ribonucleoprotein particle protein B (SmB) and U1C (Cheng et al., 2007). Furthermore the RNA-binding proteins HuR (Hongwei, 2002) and HuD belong to the CARM1 substrates (Fujiwara et al., 2006). As a transcriptional coactivator CARM-1 is responsible for H3R17 and H3R26 methylation on the estrogen receptor-regulated pS2 gene and was also described to act as a positive regulator of the Cycline E gene (Bauer et al., 2003; El Messaoudi et al.,

2006). Furthermore, CARM-1 is known to associate with nuclear-receptor associated factors and NFkB (Covic et al., 2005). Another important observation is that CARM1 knock-out mice die just after birth (Yadav et al., 2003). Arginine methylation is reversed by PAD proteins. The direct CARM1 counteracting protein, PAD4 is responsible for the demethylation of H3R17 (Wang et al., 2004).

2.4.10 Histone acetylation and deacetylation: the HAT and HDAC enzymes

For many genes, a key step in transcriptional regulation is the highly modulated switch from gene repression to gene activation. Signal transduction pathways and other communication networks in the cell are transmitted to the chromatin remodelling complexes, which in turn, depending on several parameters like protein amounts, kinetics, modifications and interactions, subsequently activate or repress gene transcription (Rosenfeld et al., 2006). During this process, histone acetylation is an essential step in positive transcriptional control (Turner et al., 2000). While histone methylation, depending on which amino acid the methyl-group is added, serves as a mark for both active and silent regions, histone acetylation is always associated with actively transcribed genes (Strahl and Allis, 2000). Therefore Histone acetyltransferases (HAT) and deacetylases (HDAC) included in holo-complexes play an important role in transcriptional regulation. Additionally, besides various histones HATs can also modify non histone substrates. HAT complexes have overlapping substrates, but the results of acetylation and their regulatory functions varies (Rosenfeld, 2006). For example, the HAT complex SAGA preferentially modifies H3K9 but to a less extend also H3K14, whereas the NuA3 complex preferentially acetylates H3K14 (Baker et al., 2007, Grant et al., 1999, Lee and Workman, 2007). Histone acetylation is reversed by HDACs. Deacetylation of histone tails by HDACs is inducing transcriptional repression. Chromatin remodelling complexes, which also include HDACs are required for switching to the silent chromatin (Lee and Workman, 2007). In case of CpG methylation, methyl-CpG-binding –protein 2 (MeCP2) specifically binds to the methylated CpG and subsequently recruits the chromatin remodelling complex Sin3, which also includes HDAC activity (Jones et al.,. 1998).

2.5 Aim of this study

Demethylation of intergrated HPV genomes correlates with further progression to cervical cancer (Badal et al., 2003). In addition, investigation of oncogene expression levels reveals a close relationship between their increase and a poor survival prognosis in cervical cancer patients. Interestingly, the viral DNA copy number is not related to the oncogene expression level, implying that a number of copies are not transcriptionally active (de Boer et al., 2007). Therefore, our understanding of epigenetic mechanisms, which are involved in oncogene re-expression, is required for creating new unanticipated approaches for treatment of cervical cancer.

The aim of my study was to investigate the role of epigenetic mechanisms, namely DNA demethylation and histone composition of nuleosomes for re-induction of HPV expression. For addressing this question, two different approaches were chosen taking advantage of the cervical cancer cell line CaSki, which harbours 600 mostly methylated HPV-16 copies.

- First, for demethylation of these silenced HPV in CaSki cells were treated with DAC and the effects were monitored both on protein and transcriptional level.
- Second, stable cell clones expressing the histone variant H3.3 were generated in CaSki cells and characterized.
- In addition mass spectrometry analysis was carried out for identifying new potential E7 interaction partners.

3 Materials and Methods

3.1 Chemicals

Acrylamid/bis-Acrylamid (29:1), 30% solution	Sigma, Deisenhofen
Agarose for gel electrophoresis	Sigma, Deisenhofen
Ammoniumpersulfate (APS)	Sigma, Deisenhofen
Boric acid, p.a.	Sigma, Deisenhofen
Bovine Serum Albumin	Sigma, Deisenhofen
Bradford reagent (Biorad Protein Assay)	Biorad, Munich
Bromphenolblue	Sigma, Deisenhofen
Chloroform,p.a.	Merck, Darmstadt
Diethylpyrocarbonate (DEPC)	Sigma, Deisenhofen
Dimethysulfoxide 100% (DMSO)	Merck, Darmstadt
di-Sodiumhydrogenphosphate-Dihydrate,p.a.	Roth, Karlsruhe
DTT (DL-Dithiothreitol)	Sigma, Deisenhofen
E-64	Roche Diagnostics, Mannheim
ECL- reagent (Renaissance)	NEN Lifescience Products Inc., Boston
EDTA	Roche Diagnostics, Mannheim
EGTA	Sigma, Deisenhofen
Acidic acid, p.a 100%	Merck, Darmstadt
Ethanol, p.a.	Merck, Darmstadt
Ethidium Bromide solution, 1%	Flunka, Deisenhofen
Formaldehyde p.a. 37%	Sigma, Deisenhofen
Foramide, p.a.	Flunka, Deisenhofen
Gycerine, p.a. 86 %	Roth, Karlruhe
Glycine	Sigma, Deisenhofen
Glycogen	Sigma, Deisenhofen
Urea	Merck, Darmstadt
HEPES	Gerbu, Gaiberg
Isoamylalcohol (3-methyl-1-butanol)	Merck, Darmstadt

Isopropanol (2-propanol), p.a.	Merck, Darmstadt
Calciumacetate, pure	Merck, Darmstadt
Kaliumchloride, p.a.	Merck, Darmstadt
Kaliumdihydrogenphosphate,p.a.	Roth, Karlsruhe
Milk powder	Merck, Darmstadt
Magnesiumchloride-hexahydrate,p.a.	Merck, Darmstadt
Methanol, p.a.	Merck, Darmstadt
2- Mercaptoethanol	Sigma, Deisenhofen
Sodiumacetate, p.a.	Merck, Darmstadt
Sodiumazid	Sigma, Deisenhofen
Sodiumchloride,p.a.	Merck, Darmstadt
Sodiumdodecylsulfate (SDS)	Roche Diagnostics, Mannheim
3-(N-Morpholino-) propansulfonacid (MOPS)	Gerbu, Gaiberg
Nodident P40, (NP-40) 10% (w/v)	Roche Diagnostics, Mannheim
Pefabloc SC	Roche Diagnostics, Mannheim
Phenol in TE-buffer (pH 7,5)	Roth, Karlsruhe
Ponceau	Serva, Heidelberg
Proteinase Inhibitor Mix	Roche Diagnostics, Mannheim
Hydrochlorideacid, conc.37%, p.a.	Merck, Darmstadt
TEMED	Sigma, Deisenhofen
Tris (Tri(hydroxymethyl)aminoethane)	Sigma, Deisenhofen
Triton X-100 (t-Octylphenoxypolyethoxyethanol)	Sigma, Deisenhofen
Tween 20	Sigma, Deisenhofen

3.2 Reagents and Media

3.2.1 cell culture: tumour cell lines

5`- Desoxyazacytidine (DAC)	Calbiochem, Bad Soden
Dimethylsulfoxide (DMSO)	Merck, Darmstadt
Dulbecco`s modified Eagle`s Medium (DMEM)	Gibco, Eggenstein
Cyclohexamide (CHX)	Roche Diagnostics, Mannheim

Fetal Calf Serum (FCS)	Gibco, Eggenstein
Geneticin (G-418)	Calbiochem, Bad Soden
MG-132 (Proteasom–Inhibitor, in solution)	Calbiochem, Bad Soden
Penicillin 10.000 U/ml	Gibco, Eggenstein
Phosphate buffered saline (PBS)	Gibco, Eggenstein
Trypanblue	Sigma, Deisenhofen
Trypsin/EDTA solution	Gibco, Eggenstein
Tumor Necrosis Factor- (TNF-α)	Strathmann Biotech, Hannover

Media for freezing:
10% DMSO
30% FCS
60 % DMEM

3.2.2 bacteria culture

Bacto-Agar	Roche Diagnostics, Mannheim
Bacto-Trypton	Difco, Detroit
Yeast extract for molecular biology	Difco, Detroit
Ampicillin, Gibco	
100mg/ml in dH_2O, stored at -20°C	
Kanamycin, Gibco	
100mg/ml in dH_2O, stored at -20°C	
Luria-Bertani –Media (LB)	
0.1 % (w/v) sodiumchloride	
0.1 % (w/v) Bacto-Trypton	
0.5 % (w/v) Yeast extract	pH 7.2 (NaOH)/ autoclaved
LB- Plates	
LB-Media, 2% (w/v) Bacto-Agar	

3.2.3 Nucleotides, Primers and Oligonucleotides

[α- ^{32}P] dCTP (10 mCi/ ml)	Amersham-Pharmacia, Braunschweig

[γ- ^{32}P] dATP (10 mCi/ ml) Amersham-Pharmacia, Braunschweig

Desoxynucleotides (dATP, dTTP, dCTP, dGTP) Roche Diagnostics, Mannheim

Gene	Primer- Sequence 5`-3`	annealing	length
c-fos	F- AACTTCATTCCCACGGTCAC- R - CCTTCTCCTTCAGCAGTTG – De-Castro Arce et al., 2004	55°C	397 bp
c- jun	F-GCATGAGGAACCGCATCGCTGCCTCCAAGT- R-GCGACCAAGTCCTTCCCCACTCGTGCACACACT- De-Castro Arce et al., 2004	55°C	409 bp
fra-1	F-GCGCCTAGGCCTTGTATCTCCCTTTCCCC- R-CCGCTCGAGGCGAGGAGGGTTGGAGAGCC- De-Castro Arce et al., 2004	65°C	211 bp
Jun B	F-GCCCTTCTACCACGACGACTC- R-CTGCACCTCCACCGCTGCCA- De-Castro Arce et al., 2004	63°C	344 bp
Jun D	F-GGTGCCCGACGTGCCGAGCTT- R-GTACGCCGGGACCTGGTGC- De-Castro Arce et al., 2004	61°C	307 bp
GAPDH	F-TGGATATTGTTGCCATCAATGACC- R-GATGGCATGGACTGTGGTCATG- De-Castro Arce et al., 2007	65°C	460 bp
GAPDH promotor	F- CCCAACTTTCCCGCCTCT- R- CAGCCGCCTGGTTCAACTG- De-Castro Arce et al., 2007	62°C	293 bp
HPV-16 URR1.1	F-CCGAATTCTGTTGCATGC- R-CCTAACAGCGGTATGTAAGG-	49°c	250 bp
HPV-16 URR1.2	F-CCTTACATCCCGCTGTTAGG- R-GCTTGTAAATGTGTAACCC-	49°C	202 bp
HPV-16E7	F-CCCAGCTGTAATCATGCATG- R-TGCCCATTAAACAGGTCTTCC-	60°c	269 bp
H3.3	F-GGGATCCATGGCCCGAACCAAGC- R-CTCGAGAGCTCTCTCTCCCCGTATCCG-	57°C	420 bp

The PCR-primer- sequences, if not otherwise mentioned, have been created using the Primer3 program from the Whitehead Institutes for Genome Research, Cambridge, MA, USA

(http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). All primers have been verified for secondary structures by the program Genewalker and the sequences have been blasted by NCBI-blast/human genome for binding similarities. The primer-synthesis has been performed either by the nucleotide synthesis facility of the DKFZ or by Invitrogen.

3.2.4 Plasmids

Name	Backbone	Insert
pCMV-Myc-H3.3	pCMV-Myc-tag3B, Invitrogen	cDNA Histone H3.3
pOZ/h3.3 plasmid	pOZ, kindly provided by Dr. Nakatani	cDNA Histone H3.3

3.2.5 Enzymes

Enzyme	**company**	**product #**	**concentration**	**recognition site**
DNA				
Taq DNA Polymerse	Invitrogen	10342-020	5 U/µl	---
T4 DNA ligase	NEB	M020L	400 U/µl	---
RNA				
Rnase A	Roche	11119915001	500 µg/ml	---
SuperScript™ II reverse-transcriptase	Invitrogen	18064-022	200 U/µl	---
Protein				
proteinase K	Invitrogen	25530-049	20 U/mg	---
restriction- enzymes				
BamHI	NEB	R0136S	20 U/µl	5'...G▼GATCC...3' 3'...CCTAG▲G...5'
HhaI	NEB	R0139L	20 U/µl	5'...GCG▼C...3' 3'...C▲GCG...5'
Xho I	NEB	R0146S	20 U/µl	5'...C▼TCGAG...3' 3'...GAGCT▲C.. 3'

3.2.6 Markers and Kits

DNA- Standard, Gene Ruler 100 bp DNA-Ladder	MBI-Fermentas, Vilnius
DNA 6x Loading Dye Solution	MBI-Fermentas, Vilnius
Protein- Standard Benchmark	Gibco, Eggenstein
Effectene Transfection Reagent	Qiagen, Hilden
HexaLabel Plus DNA Labeling Kit	MBI-Fermentas, Vilnius
Plasmid Purification Maxi-Kit	Qiagen, Hilden
One Shot TOP 10 E.coli	Invitrogen, Karlsruhe
QIAquick Gel Extraction Kit	Qiagen, Hilden

3.2.7 Antibodies

antibody	**company**	**product #, Lot #**	**Isotype**	**use**
α-c-Jun	Santa Cruz	sc-1694, I3004	polyclonal/rabbit	1 : 10000
α-p-c-Jun	Santa Cruz	sc-822x, H199	murine IgG	2µg (EMSA) 1 :10000 (WB)
α-c-Fos	Santa Cruz	sc-52x, E268	polyclonal/rabbit	2 µg (EMSA) 1 : 10000 (WB)
α-Fra-1	Santa Cruz	sc-605x, F229	Polyclonal/rabbit	2 µg (EMSA) 1 : 10000 (WB)
α-p53	Santa Cruz	sc-126, A1204	murine IgG 2°	1:5000 (WB)
α-pRb	BD-Pharma	554136, 37341	Murine IgG1	1: 500 (WB)
α-NM2 E7	kindly provided by Dr.M.Müller		monoclonal/mouse	1 : 2000 (WB) 3 µg (IP)
α-JNK	NEB cell signaling	9252, 1	polyclonal/rabbit	1 :1000 (WB)
α-p-JNK	NEB cell signaling	9251S, 3D	polyclonal/rabbit	1 :1000 (WB)
α-p38	NEB cell signaling	9212, 3	polyclonal/rabbit	1 :1000 (WB)

α-p-p38	NEB cell signaling	9211S, 6	polyclonal/rabbit	1 :1000 (WB)
α-actin,	ICN Biotechnology	69100, 7979E	monoclonal/mouse	1 :100000 (WB)
α-mouse IgG-HRP	Promega	W4021	polyclonal/rabbit	1 : 10000 (WB)
α-rabbit IgG-HRP	Promega	W401B	polyclonal/goat	1 : 10000 (WB)
α- Myc	Santa-cruz	9E10	polyclonal/goat	1 : 10000 (WB)
α- Myc	abcam	9132-100 325228	polyclonal/goat	2 µg ChIP

3.2.8 Cell lines

cell lines	
HeLa	human, cervix/adeno carcinoma, HPV-18 positive, 50 HPV-18 copies per cell Boshart et al., 1984
SiHa	human, cervix/adeno carcinoma, HPV-16 positive, 1-2 HPV-16 copies per cell Baker et al., 1987
CaSki	human, epidemoid carcinoma, HPV-16 positive, ca 600 HPV-16 copies per cell Pattillo et al., 1977
HeLa x SiHa hybrid	human, HPV-18 and HPV-16 positive, Soto et al., 2000
HeLa x CaSki hybrid	human, HPV-18 and HPV-16 positive, Soto et al., 2000
CaSki x SiHa hybrid	human, HPV-16 positive kindly provided by Prof. Dr. Rösl

3.2.9 Solutions and Buffers

DNA/ RNA	
Poly(dI-dC) - 1 mg/ml (10U= 500 µg) in TNE buffer - annealing: 5 min at 85°C, cool at RT - aliquots stored at -20°C	Biogel-Suspension - 1 x TE buffer - 5 mM sodium chloride - 0.2 (w/v) SDS - 50 % (w/v) Biogel
TAE (50 x) - 2 M Tris-HCl - 0.25 M sodium acetate - 0. 05 M EDTA pH 8.0 - pH 7.8	TBE (10x) - 0.9 M Tris base - 0.09 M boric acid - 0.02 M EDTA pH 8.0
TBS (1x) - 10 mM Tris-HCl pH 7.5 - 100 mM sodium chloride	TNE (1x) - 10 mM Tris-HCl pH 8.0 - 1 mM EDTA - 100 mM sodium chloride
RNA Loading Buffer(2x) - 50% (v/v) Formamide - 2.2 M Formaldehyde - 1x MOPS-Buffer pH 7.0 - 1% (w/v) Ficoll (MG 400.000) - 0.02 % Bromephenolblue - dissolved in DEPC-dH2O - aliquots stored at -20°C	DNA lysis buffer (3x) - 1.5 % (v/w) SDS - 150 mM Tris/HCl pH 8.0 - 150 mM EDTA
EMSA binding buffer (5x) - 50 % glycerine - 60 mM HEPES pH 7.9 - 20 mM Tris pH 8.0 - 300 mM potassium chloride - 5 mM EDTA - 100 µg/ml BSA - add protease inhibitors before use	Hybridisation washing buffer - 2 x SSC - 0.1 % (w/v) SDS
Hybridisation buffer - 50 % Formamide - 10 U/ml tRNA - 5 % SSC - 0.1% Denhardt`s solution - 20 mM sodium phosphate buffer pH 6.5 - 1 % (w/v) SDS	SSC (20x) - 3 M sodium chloride - 0.3 M tri-sodium citrate de-hydrate pH 7.0

ChIP Lysis buffer - 5 mM Hepes pH8.0 - 85 mM KCl - 0.5% NP40	ChIP buffer - 0.01 % SDS - 1.1% Triton X-100 - 1.2 mM EDTA pH 8.0 - 16.7 mM Tris pH 8.0 - 167 mM NaCl
ChIP IP buffer - 500 mM NaCl - 50 mM Tris pH 8.0 - 5 mM EDTA pH 8.0 - 0.5 % NP-40	ChIP acetylation buffer - 0.1 % SDS - 1% Triton X-100 - 20 mM Tris pH 8.0 - 150 mM NaCl
ChIP Lithium Chloride buffer - 250 mM LiCl - 10 mM Tris pH 8.0 - 1 mM EDTA - 0.5 % NP-40 - 0.5 % sodium, desoxycholate	TE - 10 mM Tris pH 8.0 - 5 mM EDTA
ChIP cocktail buffer - 320 mM sodium chloride - 80 mM Tris, pH 8.0 - 20 mM EDTA - 100 µg/ ml proteinase K	
Proteine	
Towbin buffer - 25 mM Tris - 192 mM glycine - 0.1% (w/v) SDS - 15 % (v/v) methanol	Western blot loading buffer - 10 % SDS - 5 mg bromphenol blue - 12.5 % (v/v) 2-β-Mercaptoethan. - 5 mM EDTA pH 8.0 - 50 % glycerine - 300 mM Tris pH 6.8 - store at -20°C
TBS-T - 1x TBS pH 7.6 - 0.1% (v/v) Tween 20	PBS - 123 mM sodium chloride - 17.6 mM di-sodium hydrogen-phosphate-dihydrate - 10 mM potassium dihydrogen-phosphate - pH 7.5
Buffer A (also RNA) - 10 mM HEPES pH 7.9 - 10 mM KCl - 0.1 mM EDTA pH 8.0 - 0.1 mM EGTA pH 7.9 - store at -20 °C	Buffer C - 20 mM HEPES pH 7.9 - 25 % glycerine - 400 mM sodium chloride - 1 mM EDTA pH 8.0 - 1 mM EGTA pH 7.9

RIPA buffer - 10 mM Tris pH 8.0 - 150 mM NaCl - 1 mM EDTA - 1 % NP40 - 0.1 % SDS - add PMSF (1 mg/ml) : 100 µl in 10 ml RIPA buffer before usage	IP lysis buffer - 1 % Triton X-100 - 50 mM Tris-HCl pH 7.5 - 500 mM NaCl - 5 mM EDTA
silver stain	
fix solution: - 50% EtOH - 12% HAc - 0.05 % formaldehyde	staining solution: - 200 mg/ 100 ml $AgNO_3$ - 75 µl/ 100 ml formaldehyde
developing solution: - 60g/l Na_2CO_3 - 0.5 ml/l formaldehyde - 4 mg/l $Na_2S_2O_3$	stop solution: - 50 % methanol - 12 % HAc
Rapid mini-preparation of plasmid DNA	
Reagent I - 10 % glucose (50 mM) - 25 mM Tris-HCl pH 8.0 - 10 mM EDTA pH 8.0	Reagent II - 1% SDS - 125 mM NaOH
Reagent III - 29.5 ml acetic acid (for 100 ml final volume) - 3.125 M potassiun acetate	

3.2.10 Materials and Equipment

autoradiography cassettes	Sigma, Deisenhofen
Centrifuges biofuge pico	Heraeus, Hanau
Varifuge RF	Heraeus, Hanau
5417R	Eppendorf, Hamburg
RC-5 Superspeed with GSA, SS34	Sorvall/ Du Pont, Bad Homburg
Cell scraper	BD Biosciences, Heidelberg
Cell culture flasks	Grainer, Nürtigen
Cryotubes	Grainer, Nürtigen

Eppendorf Tubes	Eppendorf, Hamburg
Filterpaper Whatman 3MM	Schleicher & Schüll, Dassel
Glass pipettes	Renner, Darmstadt
Sterile filter	Millipore, Eschborn
Cell culture flasks	Grainer, Nürtigen
Bacteria shaker	Infors, Bottmingen
Beta-Counter QC 2000	Bioscan, Washington D.C.
Balance AE 160	Mettler, Gießen
Balance BL 610	Sartorius, Göttingen
Developing Machine CURIX 60	AGFA Laborgeräte, Gera
EPS 600	Amersham-Pharmacia, Freiburg
Geiger Counter LB 1210B	Berthold, Wildbad
Gel Electrophoresis Chamber	Renner, Dannstadt
Gel Dryer M.483	Bio-Rad Munich
Hybridisation oven Hybrid Mini 38	Saur Laborbedarf, Reutlingen
Incubator B5061 EC/CO2	Heraeus, Hanau
Microscop CK2	Olympus, Hamburg
Microwave oven Micromat 241	AEG, Nürnberg
Neubauer Cell Counting Chamber	Bender & Hobein, Bruchsal
Nylonmembrane GeneScreen Plus	NEN Lifescience, Boston
Pipette tips	Steinbrenner Laborsyst., Wiesebach
PCR-Thermocycler PTC-200	MJ Reserch
Plastic cuvetts , one way	Grainer, Nürtigen
pH-Meter Calimetric 765	Knick, Berlin
Pipettes	Eppendorff, Hamburg
Pipetboy acu	Integra Biosciences
Plastic wrap	Toppits, Minden
Sterile filter	Millipore, Eschborn
Shaker Rotamax 120	Heildolph, Rust
Spectrophotometer Ultrospec III	Amersham-Pharmacia, Freiburg
Sterile Hood BioGard Hood	Baker Company, Sanford
Sonifier 250	Branson/Heinemann, Schwäbisch Gmünd
Sonifier Bioruptor	Diagenode, Liège, Belgium
syringes, one way	BD Bioscience, Heidelberg

Thermomixer compact	Eppendorf, Hamburg
Transilluminator 254 and 366 nm	Vetter, Wiesloch
Water Bath	Julabo, Seelbach
Western-Blot Chamber Hoefer, SemiPhor	Amersham-Pharmacia, Freiburg
Rotomax 120	Heildolph, Rust
Rockomat	Technomara, Zürich
Vortex Reax 1DR	Heidolph, Rust
X-ray films ECL/MP	

3.3 Cell culture: tumor cell lines

All used human tumor cell lines were adherent growing cells. The stocks from nitrogen tank storage were thaw rapidly. Next the cells were dissolved in 25 ml DMEM (*Dulbecco`s modifies eagle medium*) supplemented with 10% FCS (fetal calf serum), 100 µg/ml Penicillin, 100 µg/ml streptomycin, and centrifuged at 1300 rpm for 4 minutes. Then cells were resuspendend in 30 ml medium and plated on cell culture flaks. To avoid cellular overgrow, the cultures were splitted twice per week. The cells were washed once with 10 ml PBS and incubated in 2 ml Trypsin/EDTA solution until the cells were de-attached from the plates. The reaction was stopped adding 10 ml medium. Then cells were diluted 1:10 into new medium.

3.3.1 Cryo-conservation of tumor cells

For conservation 1x 10^7 cells were trypsinized and centrifuged in media at 1500 rpm for 4 minutes. The cell pellets were quickly resuspended in 5 ml freezing-media. Then the cell suspension was aliquoted into cryo-tubes and cooled down to -80°C. Later the cryo-vials were transferred to liquid nitrogen tanks for long term conservation.

3.3.2 Seeding of tumor cells for experiments

The cells were always seeded one day before starting the experiment. For seeding cultured cells were trypsinized, dissolved in 10 ml media and counted using a Neubauer-counting chamber. In order to distinguish between living and dead cells 100 µl cell solution was stained with 100 µl trypanblue prior counting. Then the solution was added into the Neubauer-counting chamber and cells from each chamber quadrate were counted. The cell number was then calculated by the following formula: total counted cells divided by 4 (number of the quadrates) and multiplicated by 2 (dilution in trypanblue). The acquired number X corresponds to Xx10^4cells/ml. For the DAC experiment that lasted 4 days 1x10^6cells were seeded per 60 cm^2 plate or 2.5x10^6cells per 145 cm^2 plate.

3.3.3 Transfection and establishment of stable cell lines

All transfection experiments were carried out with Effectene transfection kit using the protocol provided by the company for six well plates. The cells were seeded one day before transfection and were transfected using 2 µg plasmid DNA per reaction and well. Three days after transfection the cells were trypsinized, seeded on a 60 cm^2 plates and incubated in the medium containing G418 (Geneticin) for selection. CaSki cells were grown in 700 µg/ml G418, whereas the selection medium for SiHa included 1000 µg/ml G418. If necessary the dead cells were washed away with PBS and surviving cells were splitted. After 2 weeks surviving cells were counted, diluted at a concentration of 10 cells/ml and plated for subcloning on 96 well plates containing 100 µl (= 1 cell) or 200 µl (= 2 cells) per well in G418 media. The remaining cells were frozen as transfected stocks. Cell growing was monitored over several weeks. At the end each well was checked for cells and wells containing only one colony were harvested. Thereby, cells were trypsinized and all cells from one well were further grown on 8 cm^2 and then 60 cm^2 plates. After growing the clones were frozen for conservation and 8 clones from each experiment were tested for the Myc-histone-variant expression from the stable inserted plasmid.

3.4 Protein isolation and analysis of cultured cell lines

Protein was isolated using three different methods depending on the further experimental procedure. For EMSA and Western blot proteins were isolated using the nucleus-cytosol separation protocol (5.4.1). Proteins were isolated from the nuclear fraction, whereas the cytosolic extract was used for RNA isolation. For the detection of MAPK activation whole cell lysates were prepared using SDS-lysis buffer (5.4.2). All other Western blots proteins were isolated using the RIPA protocol (5.4.3).

3.4.1 Nucleus-cytosol separation

The advantage of this method is that upon separation of the nuclei from the cytosol proteins from each fraction can be analysed independently. For the EMSA experiments the nuclear fraction was used. Prior to EMSA, Western blots were performed in order to detect any changes in protein content between treated and untreated samples. For the nucleus-cytosol

separation cells were harvested from 60 cm^2 plates. All following steps were carried out at 4°C. The cells were washed twice with PBS. Then they were incubated with 1.2 ml of buffer A containing 75 µl/ml proteinase inhibitor cocktail (stock: 1 tablet/ml) for 15 minutes. Next, the cells were harvested from the plates using a cell scraper and transferred to a 2 ml tube. Then 0,625% of the non ionic detergent NP-40 was added and the solution and votexed for 10 seconds. For further nucleus cytosol separation the solution was centrifuged for 10 seconds at 13000 rpm. For RNA extraction 1 ml from the supernatant containing cytosolic extract was transferred to a 15 ml tube and mixed with 2 ml RLT buffer from QIAGEN RNA extraction kit. RLT buffer was prepared prior use by adding 10 µl/ml β-mercaptoethanol. The supernatant-RLT-buffer solution was further prepared or frozen at -80°C for conservation. The pellet, containing the nuclei, was resuspended in 200 µl of the high-salt buffer C following incubation for 15 minutes. Then solution was centrifuged for five minutes at 14000 rpm. The supernatant with the nuclear proteins was transferred to a new tube and frozen at –80°C.

3.4.2 Protein extraction using SDS-lysis-buffer

For the analysis of MAPKs activation the proteins were isolated using SDS-lysisbuffer (Klotz et al., 1999). The cells were harvested from 60 cm^2 plates after the appropriate treatment. For detection of MAPKs activation a positive control was generated by treating the cells for 30 minutes with 100U/ml TNF-α. All following steps were carried out at 4°C. Cells were washed twice with PBS and subsequently harvested in PBS using a cell scraper. Next, cells were centrifuged for 4 minutes at 2000 rpm. The pelleted cells were resuspended in 500 µl SDS-lysis-buffer. The lysates were sonified 2 times for 10 seconds at 50% power capacity and level 3 (Sonifier 250, Branson). Subsequently, the lysates were heated for 5 minutes at 99°C and centrifuged for 30 seconds at 13000 rpm. An aliquot was used to determine the protein concentration by the Bio-Rad DC Protein Assay. The rest was supplemented with 2-Mercaptoethanol (0.74 M final concentration) and frozen at -80°C.

3.4.3 Protein extraction using the RIPA protocol

Cells were harvested from 60 cm^2 plates after the appropriate treatment. All following steps were carried out at 4°C. Before start 10 µl PMSF/ ml was added freshly to the RIPA buffer. The cells were washed twice with PBS. Cells were harvested in PBS using a cell scraper and transfer to a 15 ml tube. Then, cells were centrifuged for 5 minutes at 1500 rpm. The pellet was resuspended in 400 µl RIPA buffer/PMSF and sonified 1 time for 10 seconds at 50% power capacity and level 3 (Sonifier 250, Branson). The lysate was then incubated on ice for 30 minutes and then transferred to a 1.5 ml tube followed by centrifugation for 10 min at 4°C and 13000 rpm. Supernatants were collected and frozen at –80°C.

3.4.4 Histone isolation

Preparation of a total histone fraction from nuclei was accomplished by extraction with 0.2 M H2SO4. This acidic extraction removes histones from DNA. Different H3.3 clones were grown up to 95% confluent in 60 cm^2 cell culture dishes. Then, cells were washed 2 times with PBS and trypsinized for 5 minutes at 37°C. Next, cells were collected in 2 ml tubes and centrifuged for 5 minutes at 1500 rpm followed by cell lysis in buffer A (5.4.1). For nuclei isolation, the samples were centrifuged for 1 minute at 13.000 rpm and the supernatants were discarded. Subsequently, the nuclei-containing pellet was incubated with 100 µl 0.2 M H2SO4 at 4°C over night. Following this step, the samples were centrifuged for 10 minutes at 13000 rpm, 4°C and the supernatant was transferred to a new tube. Then, histones were precipitated in 900 µl ice-cold acetone at 4°C over night. The samples were centrifuged for 10 minutes at 13.000 rpm and pellets were dissolved in 60 µl dH2O. For electrophoresis and Western blotting 40 µl loading buffer was added to the samples and they were incubated at 99°C for 5 minutes for denaturation prior separation in a 15% polyacrylamide gel.

3.4.5 Detection of protein concentration by Bradford and Lowry

3.4.5.1 Detection of protein concentration by the Bradford method (Bradford et al., 1976)

The method takes advantage of the dye Coomassie Brilliant Blue 250, which has its maximum absorption at 595 nm in complex with proteins. The measurements were carried out in a photometer. Since this method is highly influenced by detergents, like SDS, it was used to quantify proteins in nuclear extracts and RIPA buffers.

To generate a standard curve 1-12 µg BSA (Bovine Serum Albumine) were added to a final volume of 800 µl dH2O and mixed with 200 µl *Bradford Reagent*™. To determine the protein concentrations 2-3 µl sample were added to 797-798 µl dH2O and mixed with 200 µl *Bradford Reagent*™. Then standards and diluted samples were measured in a photometer at 595 nm. Using the acquired data from the standards the protein concentrations of the samples were calculated.

3.4.5.2 Detection of protein concentration by Lowry

The Bio-Rad DC (detergent compatible) Protein Assay is based on the Lowry protein detection principle (Lowry, 1951, Legler et al, 1985), which takes advantage from the interaction of the Folin-Ciocalteu-Phenol-Reagent with proteins. The interaction of the reagent with tyrosine-residues leads to blue colouring which can be detected at 750 nm. This reaction is compatible with higher detergent concentration: maximal 10% SDS and maximal 2% NP-40. Therefore it was used for detection of proteins in SDS-lysisbuffer.

Similar to the Bradford method the standards were made using BSA. Thereby standards and samples were diluted in 200 µl dH2O and mixed with 100 µl reagent A (1 ml reagent A + 20 µl reagent C) followed by 800 µl reagent B. The probes were then incubated for 15 minutes at RT and analysed in a photophotometer at 750 nm. Again the standards were used as references to calculate the protein concentrations of the samples.

3.4.6 Western blot Analysis

3.4.6.1 Protein separation by SDS-PAGE

For analysis the proteins were separated by molecular weight in polyacrylamide matrix. In order to migrate, proteins need to be incubated with SDS (sodium dodecyl sulphate) and denaturated prior loading to the gel. SDS binds to the amino acids residues in an equal amount and charges the proteins negatively. Therefore, they are able to migrate through the gel-matrix to the cathode. Thereby they get separated by their molecular weight, as small proteins move faster than the bigger ones. For the experiments the discontinuous SDS-PAGE (Laemmli, 1970) was used, where the samples are loaded on a matrix composed of two gels: the upper concentrating and the lower separating gel. After entering the lower gel the proteins separate according to their molecular weight. For the loading 50-75 µg protein extract from RIPA and 30 µg protein from nuclear extracts were adjusted. Proteins were diluted in dH2O and SDS-loading buffer (1x final concentration). They were denatured at 99°C for five minutes, cooled on ice, centrifuged at 13.000 rpm for 30 seconds and loaded on the gel or stored at -20°C for up to one week. After loading, the current was first adjusted at 15 mA/gel, allowing migration of the proteins trough the upper gel. Next, for migration trough the separating gel the current was increased to 45-60 mA/gel. For the identification of the molecular weight, size-standard (Benchmark™) was also loaded on the gel.

3.4.6.2 Western blot

Western blot is the subsequent step after the separation of the proteins in the polyacrylaminde gel. The proteins were immobilized on PVDF (Polyvinylidendifluorid) membranes using the `semi-dry` method (Towbin et al., 1979, Gallagher et al., 1997). PVDF membrane was first activated in 100% methanol for 1 minute, washed in dH2O and incubated for at least 15 minutes in Towbin buffer. The blot was built up as follows: Cathode, 9x filter-paper (Whatman 3MM), PVDF membrane, polyacrylamide gel, 9x filter-paper (Whatman 3MM) and anode. The blotting was carried in a Western blot chamber (SemiPhor, Amersham-Pharmacia), where the cathode is located on the bottom plate and the anode is arranged on the top of the blot. The protein transfer proceeded at 4°C for 70 minutes with a current of 1.2 mA / cm2 membrane.

3.4.6.3 Ponceau- staining and Immunodetection

To monitor protein transfer the membrane was stained with the Ponceau-red-dye and then washed with dH_2O. This dye binds reversible to proteins. After the staining the membrane was incubated for 1 hour at RT in blocking-milk (TBS-Tween, 5% low fat milk) in order to block free binding sites on the PVDF membrane. Then the membrane was incubated with the appropriate first antibody in blocking-milk over night at 4°C on an over-head turner (Rotamax 120). After this period the membrane was washed 3 times for 8 minutes in TBS-Tween to remove the unbound antibodies. This was followed by incubation of the membrane with the specific secondary antibody for 1 hour at RT on an over-head turner (Rotamax 120). Subsequently, the membrane was washed and detection of the HRP-labelled secondary antibody was carried out using the ECL-System from the NEN Life Science Products Inc. The chemiluminescence was detected by autoradiography on films during an exposure time of 20 seconds to 5 minutes depending on the signal strength.

3.4.6.4 Membrane stripping and storage

In order to reuse the PVDF membrane for further analysis or for actin control the membrane was first washed in TBS, then in water for 5 minutes, followed by incubation for 5 -10 minutes in 0.2M NaOH. Subsequently, the membrane was washed first in dH2O and then TBS for 5 minutes. After these steps the membrane was again blocked and incubated with a different antibody. For storage the membrane was left in TBS for up to 2 weeks at 4°C and then dried.

3.4.7 Silver stain protocol

Silver staining is a sensitive method for detection of proteins (Blum et al., 1987). The polyacrylamide gel, harbouring the separated proteins, was incubated for 20 minutes in fix solution and washed in 50 % ethanol 3 times for 10 minutes. Pre-treated in freshly prepared 0.02% $Na_2S_2O_3$ solution for 1 minute and rinsed 3 times for 30 seconds. For impregnation the gel was incubated for 10 minutes in staining solution and rinsed. Next, the gel was developed until first bands were visible and washed 2 times for 2 minutes. For stopping the reaction the

stop solution was poured to the gel and incubated for 5 minutes. The gel was washed in H2O for one hour at room temperature and dried.

3.4.8 Mass Spectrometry

For mass spectrometry (MS) analysis (Chait et al., 1992; Nelson et al., 1997) E7 co-immunoprecipitated proteins from DAC and MG132 treated and untreated samples were separated on a 12 % polyacrylamide gel. The one-dimensional gel was cut into 14 slices per sample. Each individual slice was digested by trypsin and further analyzed by MS. This analysis was carried out by the MS core facility of the German Cancer Research Center (DKFZ, Funktionelle Proteomanalyse B100). The identified ions were submitted to a data base search (Mascot Search) for the corresponding peptides/proteins and the matches were qualified by probability Based Mowse Score. Individual ions scores > 37 indicate identity or extensive homology ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. Only proteins representing a score greater than 37 were further included as potential E7 interacting partners. For example, peptides detected for the protein coactivator-associated arginine methyltransferase 1 (CARM-1), in the DAC treated sample, reached a score of 38, as shown in the table below.

gil40288288 Mass: 66382 **Score: 38** Queries matched: 3

coactivator-associated arginine methyltransferase 1 [Homo sapiens]

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
141	605.71	1209.40	1209.62	-0.22	0	25	0.78	1	R.CLFQSPLFAK.A
303	700.27	1398.52	1398.75	-0.22	0	33	0.11	1	R.LLTIGDANGEIQR.H
304	700.72	1399.43	1398.75	0.68	0	(33)	0.15	1	R.LLTIGDANGEIQR.H

3.5 Preparation and analysis of nucleic acids

3.5.1 Detection of nucleic acids concentration

The concentration of nucleic acids was determined by photometry in quartz quvetts. Thereby one takes advantage of the optical density of these molecules, which reaches the maximum at 260 nm. DNA or RNA was diluted 1:100 in dH2O. The extinction of the diluted nucleic acid solution was measured at 260/280 nm. A value of 1.0 corresponds to 50 µg/ml DNA, 40 µg/ml ssDNA, 33 µg/ml DNA-oligonuclotides and 40 µg/ml RNA (Sambrook et al., 1989). To qualify the purity of the analysed samples, the OD260/OD280 quotient was calculated. Samples without protein contaminations have a quotient from 1.8 to 2.0.

3.5.2 Separation of nucleic acids by gel electrophoresis

RNA and DNA molecules are negatively charged, due to their phosphate groups, and able to migrate within an electric field towards the cathode. This characteristic is used for their electrophoretic separation within an agarose gel. Nevertheless, DNA and RNA electrophoresis is performed using different protocols. For DNA, the agarose gels are prepared by dissolving agarose in 1 x TAE (Tris-Acetat-EDTA) buffer. Depending on the size of the DNA fragments, different agarose concentrations are used. DNA molecules between 0.5- 7 kb are separated in 1.0 % (w/v) agarose gels, whereas molecules of 0.2- 3 kb are separated in 1.5 % (w/v) agarose gels. For the detection of both DNA fragments and RNA under UV-light (λ = 254 nm), the DNA or RNA intercalating molecule ethidiumbromide is added to the agarose solution before polymerisation. As standard, 5 µl of 100 bp DNA ladder marker (GeneRuler™ 100 bp ladder) was loaded on the gel. The DNA migration was carried out by 7-10 V/cm and the DNA were visualized on a transilluminator. For documentation a photo was taken for each DNA or RNA gel.

To avoid secondary structures of the RNA molecule, its electrophoresis is carried out under denaturating conditions, in 1x MOPS buffer and 1.0 % agarose. All material used in RNA electrophoresis should be RNase free. 5 µg RNA was added to DEPC-dH2O and RNA loading buffer. The solution was incubated at 65°C for 10 minutes to denaturate the RNA. After preparation of the agarose-MOPS-buffer-gel the samples were loaded on the gel. For

electrophorsis 1x MOPS-DEPC-dH2O buffer was used. The RNA migration was strictly carried out at 60V for 2 hours. The risbosomal 18S and 28S RNA was used as a size marker.

3.5.3 Extraction of DNA from tumor cells

For DNA extractions 90-95% confluent cells in 60 cm^2 cell culture dishes were washed with PBS and lysed in 3 ml DNA-lysis-buffer. The lysate was transferred to a 15 ml tube and incubated at 56°C for 1 hour with 100µg/ml proteinase K. Subsequently 3 ml phenol, pH 8.0 was added to the samples. At this point the samples were either stored for up to 1 week at -80°C or processed adding 3 ml CIA (Chloroform/Isoamylalcohol, 1:24). The samples were transferred to a 15 ml phase lock gel (heavy) tube and mixed for 20 minutes at RT in an over head shaker. Next, samples were centrifuged at 4200 rpm at RT for the separation of the aqueous phase (containing the DNA) from the organic phase (containing phenol/CIA) and the interphase with proteins. Due to the phase lock gel, which is located after this step between the interphase and the aqueous phase, the DNA was easily isolated and transferred to a new 15 ml tube. The DNA was then precipitated by incubation with 0.8 V isopropanol (100%) and 2 M ammonium acetate, pH 5.2 for 1 hour at -20°C. The DNA was pelleted by centrifugation at 4.500 rpm for 1 hour at 16°C. Subsequently, the salt was removed by washing the DNA pellet 2 times with 70% ethanol. Last the DNA was air dried, resuspended in dH2O and stored at -20°C.

3.5.4 Detection of DNA demethylation by digesting with HhaI

For the DNA demethylation assay one took advantage of the methylation sensitive enzyme HhaI (DeCastro-Arce et al., 2007). This enzyme is only able to specifically digest DNA within its recognition sequence, when this DNA is unmethylated. Therefore, it was possible to analyse if the DNA was demethylated in DAC treated cells. After DNA isolation from both DAC treated and control cells, 10 µg DNA was digested with 100U HhaI (NEB) and 10% BSA in 1x NEB buffer 4 over night at 37°C in a total reaction volume of 100 µl. In order to detect the presence of contaminants that may inhibit the restriction activity independent of methylation 5 µl from the 100 µl reaction volume were transferred to a new tube which contained 1 µg pBluescript plasmid, dissolved in 5 µl reaction buffer. These

control samples were also incubated over night at 37°C and directly analysed on an agarose gel. The HhaI activity was determined by the complete digestion of the pBluescript plasmid.After the over night digestion, DNA was recovered by phenol/CIA precipitation. Thereby the samples were supplemented with 100µl H_2O. This solution was transferred into a phase lock gel tube (Eppendorf). 200 µl phenol (pH 7.5) and 200 µl CIA were added to the samples and mixed for 20 minutes. The samples were centrifuged at 4300 rpm for five minutes. The upper phase was transferred to a new tube containing the precipitation mix, 400 µl ethanol absolute, 20 µl Sodium acetate (3M pH 4.8) and 1 µl glycogen. The solutions were gently mixed for 5 minutes and incubated at -80°C for 2 hours. Next, the samples were centrifuged at 13.000 rpm for 1 hour. The supernatants were discarded. Pellets were washed one time with 70% ethanol, centrifuged at 13000 rpm for 10 minutes and dried at room teperature. Then the pellets were resuspended in 50 µl dH_2O. To ensure equal DNA amounts 5 µl of the digested DNA was checked on 1% agarose gel.

3.5.5 Amplification of DNA

3.5.5.1 Polymerase chain reaction (PCR)

The polymerase chain reaction enables the specific exponential amplification of a given DNA sequence (Mullis et al., 1986). The specifity is ensured by the oligonucleotide primers. The amplification is carried out by the DNA dependent DNA polymerase Taq from Thermus aquaticus, which catalyse the reaction at 72°C. The PCR reaction is divided in three different crucial steps, denaturation of the double stranded DNA, annealing of the primers to their specific sequence and elongation from the primers to amplify the particular sequence by the polymerase Taq. These steps are repeated for several cycles (25-35) to reach the exponential amplification of the specific DNA sequence. For the PCR reaction the following final concentrations were used: primers: 1.25 mM, $MgCl_2$ 0.8 µM, dNTPs :200 µM and 0.625 U Taq-polymerase in a 25 µl final volume. After the PCR 5 µl of the 6x DNA loading buffer was added to the samples and the DNA was visualized on an agarose gel. The ChIP PCR was carried out in the Promega master mix following the manufacturer instructions.

3.5.5.2 Quantitative PCR

For the quantitative PCR the LightCycler® 1.5 Instrument by Roche was used. During the PCR the SYBR green fluorescent dye is incorporated into the newly synthesised DNA. After the PCR a melting curve is generated, by slowly heating of the amplicon. This leads to denaturation (melting) of the amplified DNA, which changes fluorescence, and is measured by the machine. Thereby one takes advantage of the fact, that each double-stranded DNA has its own specific melting temperature (T_m), in order to characterize the specifity of the PCR amplification/quantification. This is defined as the temperature at which 50% of the DNA becomes single stranded. The PCR occurs in specially designed glass capillaries. The amplification cycles were as follows: denaturation: 95°C 10 minutes, amplification: 95°C for 10 seconds, 60°C for 15 seconds, 72°C for 10 seconds. The amplification set up was repeated for 50 cycles. The melting curve program was: 95°C, 50°C for 15 seconds and slow heating (0.1°C/s) until 95°C for one cycle. As standard a GAPDH-plasmid was used in concentration from 15 ng-150 pg.

3.5.6 RNA Isolation from tumour cells

The RNA was extracted from cytosolic fractions during nucleus cytosol separation (5.4.1). After centrifugation of the nuclei- buffer A-solution, the supernatant was transferred to a 15 ml tube and mixed with 2ml buffer RLT from the Quiagen RNAeasy kit. The following steps were carried out following the manufactures recommendations. Briefly, the sample was transferred in 700 µl portions to one Qiagen RNAeasy kit spin column and centrifuged at 13000 rpm for 30 seconds. The column was washed one time with 700 µl RW1 buffer and two times with 500 µl RPE buffer. For removal of the ethanol, the column was centrifuged at 13000 rpm, for 1 additional minute. Subsequently, the RNA was eluted from the column by adding in 30 µl DEPC-H_2O (or Qiagen RNase free water). The RNA solution was frozen at –80°C. The RNA concentration was determinated either by using a photometer or the nano drop technique. Then the RNA was visualized on an agarose gel.

3.5.6.1 Reverse transcription of RNA

In order to analyse the expression patterns of a gene on transcriptional level by PCR or quantitative PCR the RNA templates need to be reverse transcribed into cDNAs. This reaction is carried out by the viral enzyme Reverse Transcriptase, which is a RNA dependent polymerase. For initiating the polymerase reaction random primers were used at a concentration of 200 ng pro reaction. First the random primers and 1µg RNA were incubated in a total volume of 12.5 µl for 10 minutes at 70°C for denaturation of the RNA. After 7 µl Master Mix containg 1x RT-buffer, 10 mM DTT, 1 mM dNTP Mix and 1 µl RNase Inhibitor was added to the RNA samples and incubated for 10 minutes at 25°C for the primer annealing. 100U Reverse Transcriptase Superscript II was added to the samples. Next the polymerase reaction was carried out for 50 minutes at 42°C followed by 15 minutes at 70°C. For the PCR reactions 1 µl from the 20 µl RT-reaction volume were used.

3.5.6.2 Northern blot

For directly detection of a specific mRNA the isolated RNA was separated on an agarose gel and transferred to a Gene Screen + Nylonfilter. The mRNA of interest was detected by hydridisation with a radioactive labelled probe. Therefore 50-100 ng of the specic probe was labelled using the HexaLabel DNA labelling Kit (MBI Fermentas), following the manufacturer instruction.

3.5.6.3 Radioactive labelling for Northern blot

For radioactive labelling, the probe was first denaturated by incubation at 99°C for 5 minutes. Afterwards the reaction buffer containing 25µCi (α-32P) dCTP, unlabeled nucleotides (dATP, dGTP, dTTP), 1U DNA-polymerase (Klenow-fragment) and random primers was pipetted to the sample and incubated for 30 minutes at 37°C. To stop the reaction 1x volume of 2x TNE-buffer was added to the sample. Next, the probe was purified from free dNTPs, polymerase and salt by separation over a chromatography column. Prior to hybridisation the membrane was incubated in 10 ml hybridisation buffer which contains tRNAs, for blocking free unspecific binding sites on the membrane, for 2-4 hours at 42°C. The radioactive labelled probe was denaturated at 99°C for 5 minutes and added to the

membrane within the hybridisation buffer. After over night incubation the membrane was washed with hybridisation washing buffer at 68°C and exposed. Specific hybridisation was detected after expose X-ray-films to the membrane for 1-2 days at -70°C.

3.5.7 Analysis of DNA-Protein-Complexes

3.5.7.1 Electrophoretic Mobility Shift Assay (EMSA)

The electrophoretic mobility shift assay (EMSA) is a protein-DNA-binding assay in which the transcription factors bind to their specific DNA recognition sequence (Lewis and Conradi, 1996; Soto et al., 1999). EMSA takes advantage of the observation that an unbound DNA fragment migrates faster during electrophoresis through a polyacrylamide gel. Proteins bound to the fragment significantly slower its migration. This phenomenon is called gel retardation. Additionally, using antibodies specific for the transcription factors this method allows the identification of the bound proteins. The antibodies bind to the protein-DNA-complexes generating a much slower migration described as supershift. Radioactive labelling of the DNA fragment ensures the visualization of the specific band patterns by autoradiography.

3.5.7.2 Annealing and labelling of oligonucleotides

The oligonucleotides were synthesised and delivered as sense and anti-sense ssDNA-oligonucleotides. In a PCR-Thermocycler 2 µg of each oligonucleotide strand were annealed in TNE-buffer for 10 minutes at 85°C followed by cooling down 0.5 °C every 30 seconds. This slow temperature decrease enables the complementation of the strands generating dsDNA-oligo-nucleotides. 200 ng oligonucleotides were radioactive labelled with ^{32}P-γ-ATP. The enzyme T4-Polynucleotide-Kinase transfers the radioactive phosphate to the dephosphorylated 5`-end of the oligonucleotides. This reaction was carried out for 30 minutes at 37°C and stopped by addition of 4 µl 6x loading buffer.

In order to purify the labelled oligonuclotides from unincorporated ^{32}P-γ-ATP -molecules the reaction was separated on a 15% polyacrylamide gel for 1.5 hours in 1x TBE-buffer at RT and 200V (ca:35mA). Afterwards the gel was exposed for 30 seconds- 1 minute to an X-ray film.

The developed film showed the position of the fragment. The fragment was cut out of the gel, sliced in small pieces and incubated at 4°C in 500µl TNE-buffer on a shaker over night.

Labelling of oligonucleotides for EMSA:

dH_2O	2 µl
200 ng oligonucleotides/ µl stock (annealed)	2 µl
T4 buffer 10x	1 µl
^{32}P-γ-ATP 10µCi/µl	4 µl
T4 Kinase (400U/µl)	1 µl
Total amount:	10 µl

3.5.7.3 DNA-protein-complexes and gel electrophoresis

Radioactive labelled and purified oligonucleotides were incubated with nuclear extracts. During this DNA binding reaction the transcription factors from the nuclear extracts bind specifically to the oligonucleotides. In order to diminish unspecific DNA-protein complexes the binding buffer contains BSA and Poly(dI-dC). The reaction occurs at RT for 30 minutes. Afterwards, for the super shift detection, the antibodies were added to the reaction and incubated for 1h at 4°C. Then the samples were loaded on a 5.5% polyacrylamide gel and the electrophoresis was performed at 4°C for 30 minutes at 280 V followed by 1 hour and 350 V. The gel was dried on 3MM Whatman paper for 1 hour at 80°C and subsequently exposed to an x-ray film for detection of different complexes by autoradiography.

3.5.8 Chromatin Immunoprecipiation

Chromatin immunoprecipitation (ChIP) is a powerful experimental tool to identify proteins associated in vivo with specific regions of the genome (DeCastro-Arce et al., 2007). Thereby specific antibodies recognizing a specific protein or a specific modification of a protein are used. The initial step of ChIP was the cross-linking of protein-protein and protein-DNA in live cells with 1% formaldehyde. After 20 minutes incubation at RT the fixation was stopped by 0.125M glycine. After cross-linking, the cells were washed one time with 10 ml ice cold PBS, following by incubation in trypsin for 20 minutes at 37°C. Then the cells were harvested

using a cell scraper and transferred to a 2 ml tube and centrifuged at 1500 rpm for 5 minutes. The cell pellet was lysed in 500 µl ChIP lysis buffer containing proteinase inhibitor cocktail (Roche). The crude extracts were sonicated to shear the DNA for three times 10 minutes at 4°C. Next, 25 µl of each sample were de-cross-linked by incubation in 300 mM NaCl over night at 65°C. After purification DNA concentration and sonification efficiency was determinated. For pre-clearing 25 µg of chromatin were incubated with 40 µg salmon sperm DNA and 40 µl Protein A/G-agarose beads (1:1) for two hours at 4°C. Next, the complexes were immunoprecipitated over night using 2 µg of the anti-Myc-antibody and the appropriate isotype control. Then DNA-protein-antibody complexes were bound to protein-A/G-agarose-beads during incubation for 2-3 hours at RT. Afterwards, the beads were washed with the following buffers: one time with IP buffer, two times with acetylation buffer, two times with Lithium-Chloride-buffer and two times with TE buffer. Next, 250 µl of freshly prepared elution buffer were added to the beads and samples were rotated for 20 minutes at RT, then the samples were centrifuged at 3000 rpm for 5 minutes at RT. 200 µl supernatant was transferred to a new tube and mixed with 250 µl freshly prepared cocktail buffer. In addition 50 µl input (unprocessed chromatin) were mixed with 200 µl elution buffer and 250 µl cocktail buffer. In order to digest the proteins the samples were incubated for 2 hours at 37° C. Subsequently, the samples were shaked over night at 65°C. DNA was isolated by phenol/chloroform extraction and ethanol precipitation. After centrifugation at 13000 rpm for 30 minutes, 4°C the pellet was dried and resuspended in 60 µl d H2O. The samples were either stored at -80°C or analysed by PCR.

3.6 Prokaryotic cells

3.6.1 Bacterial cultures on agar-plates

Bacteria were grown on agar-plates at 37°C. Therefore LB-medium was prepared and autoclaved with 15g/l agar. When the solution cooled down at approximately 50°C the antibiotics ampicillin or kanamycin were added at a concentration of 100mg/ml. The agar was poured over sterile plates and stored at 4°C. For bacterial cells cultivation 100 µl bacteria were plated and afterwards grown over night at 37°C.

3.6.2 Bacterial growth in solution

Bacteria were grown in LB medium with either ampicillin or kanamycin over night on a shaker at 37°C. Thereby the bacteria were inoculated from the agar-plates-colonies or glycerine stocks.

3.6.3 Bacteria conservation and reactivation

Bacteria stocks were harvested from expontialy growing cells and conserved in 1x volume 97% glycerine by shock-cooling using liquid nitrogen. Then the stocks were stored at -20°C. For reactivation cells from glycerine stocks were picked and inoculated into LB-media for subsequent growth at 37°C over night.

3.6.4 Cloning Techniques for generation of the Myc-H3.3-construct

3.6.4.1 Amplification of the H3.3 cDNA

For cloning the H3.3 cDNA into the pCMV-Myc-tag3B-vector, H3.3 cDNA was amplified form the pOZ/h3.3 plasmid (Dr.Nakatani) by PCR using primers containing restriction site sequences. The amplification product harbours the following recognitions sites: 5' BamHI and 3' XhoI. Following PCR the BamHI -XhoI fragment was used to further engineering the Myc-H3.3 construct.

3.6.4.2 Cloning of the PCR fragment into the pCR®4-TOPO® vector

The restriction digesting is known to function more efficiently, when the enzymes are able to bind to an integrated fragment rather than to a PCR product. Therefore the BamHI-H3.3 cDNA-XhoI fragment was cloned by the TOPO® Cloning Method into the pCR®4-TOPO® vector following the manufactors instructions (TOPO® cloning reaction). Subsequently, One Shot® TOP 10 chemically competent E.coli bacteria were transformed with the plasmid and plated on 100 µg/ml ampicilline-LB-agar-plates. In addition, for detection of the bacteria harbouring plasmids with the inserted fragment, the bacteria were selected by the blue/white-screening method (Ullmann, Perrin, 1970). Therefore 40 µl of 40

mg/ml X-Gal in DMF (dimethylformamide) was spread on the top of the agar. After growing on these plates the positive (colourless) bacteria were transferred into 5 ml LB-media and grown over night. Next, the plasmids were isolated by rapid mini preparations.

3.6.4.3 Rapid mini-preparation of plasmid DNA

For the rapid isolation and subsequent analysis of insertion of the desired fragment into the plasmid bacteria cells were transformed and cultured in 5 ml medium over night. Next day 1,5 ml were transferred to a new 2 ml tube and centrifuged for 30 seconds at 13.000 rpm. Then the pellet was resuspended in 100 µl reagent I. After five minutes incubation at room temperature the cells were lysed by adding 200 µl of reagent II. This solution was left for five minutes at 4°C. 150 µl reagent III were added to the samples. The solution was incubated for five minutes at 4°C and then centrifuged at 13000 rpm for 2 minutes and 4°C. To precipitate the plasmid DNA the supernatant was transferred to a new tube containing 10% final volume sodium acetate (3M), two times sample volume of pure ethanol and 1 µl glycogen. The solution was mixed, incubated at 4°C for 20 minutes and centrifuged at 14000 rpm for 30 seconds at 4°C. The pellet was washed two times with 1 ml 70 % EtOH, dried and resupended in 50 µl 0,1x TE. Subsequently, the isolated plasmids were digested with BamHI/XhoI. The digestion was visualized on agarose gel followed by recovery of the fragment using the QIAquick Gel Extraction Kit. The isolation was carried out following the manufactures instructions.

3.6.4.4 Ligation of the BamHI-H3.3-cDNA-XhoI fragment into the pCMV-Myc-tag3B vector.

5 µg of the pCMV-Myc-tag3B vector were digested with 5U of the BamHI/XhoI enzymes for 2 hours and analysed on agarose gel. The backbone was isolated using the QIAquick Gel Extraction Kit. Afterwards the covalent ligation of the fragment with the sticky BamHI/XhoI ends into the BamHI/XhoI-pCMV-Myc-tag3B-backbone was catalysed by T_4DNA-ligase. This enzyme is able toestablish a phosphodiester bound between a free 5'-phoshate-group and one 3' hydroxyl-group. Thereby the fragment was integrated into the pCMV-Myc-tag3B backbone. The ligation was carried out incubating 200 ng vector and the 3 times molar amount of fragment in 20 µl reaction volume at 16°C over night. The reaction was inactivated at 65°C for 10 minutes. 10 µl of the ligation was analysed in agarose gel and

the other half volume was used for the transformation of One Shot® TOP 10 chemically competent E.coli bacteria by heat shock following the manufactures instruction. After transformation bacteria were plated on 100 µg/ml Kanamycin-LB-agar-plates over night at 37°C. Plasmids from 10 different grown colonies were isolated by rapid mini-preparation and analysed by enzymatic digestion with BamHI/XhoI. Positive tested plasmids were sent for sequencing. Bacteria containing the correct fragment sequence were grown and the plasmids were isolated for transfection of CaSki and SiHa cells.

4 Results

4.1 The genome of high risk HPV-16.

HPV-16 has a circular, DNA double stranded genome with a size of 7904 bp, containing three major regions: early, late, and the upstream regulatory region (URR, also called long control region: LCR). These regions are divided by two polyadenylation (pA) sites: early pA (AE) and late pA (AL). The HPV-16 genome encodes eight open reading frames, six (E1, E2, E4, E5, E6 and E7) for the translation of the early viral proteins and two for the major (L1) and the minor (L2) capsid proteins (Zheng and Baker, 2006).

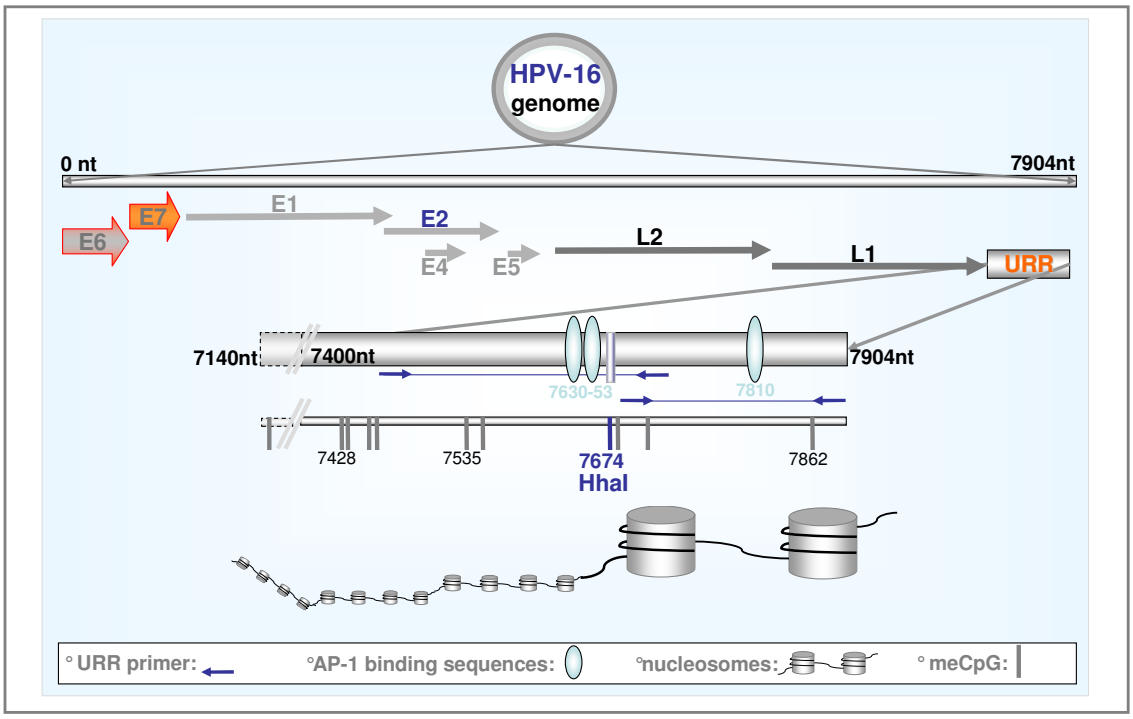

***Figure 4.1* The genome of the high-risk HPV-16.**
Schematic representation of the circular DNA genome of HPV-16. The arrows represent the different transcribed viral proteins. The URR is highlighted including AP-1 binding sites, potentially methylated CpGs and the HhaI restriction site. Other HPV-16 inducing transcription factors are not shown.

The URR, covers a 754 bp (10% of the HPV genome) long non-coding region, harbouring the origin of replication and multiple regulatory transcription factor binding sites for transcriptional initiation of the viral early and late promoters (Chong et al., 1991). The

data presented here deal with the analysis of demethylation of HPV-16 genomes and its influence on the transcription of the viral oncoprotein E7. Therefore it is important to take into consideration that eleven CpG are located within the URR (Rajeevan et al., 2006). Of specific interest in this study is the CpG site at nucleotide 7674, which is embedded within a HhaI recognition site and was used as an indicator for demethylation. In addition, for the transcriptional regulation of the oncogenes, the AP-1 transcription factor complex plays a major role (Offord and Beard, 1990; Butz and Hoppe-Seyler, 1993; Soto et al, 1999). There are three AP-1 binding sites located in the HPV-16 URR, the one located in the enhancer region has been further analysed in this study. Furthermore two particular histones were described to occupy defined regions on the enhancer and promoter (Stünkel and Bernard, 1999). All of these different factors, which together influence each other for initiating or silencing the oncogenes transcription, are summarized in the scheme on Figure 4.1.

4.2 Experimental approach to detect DNA demethylation

DNA methylation, which occurs only at cytosines located 5´ to guanosine (CpGs), plays a central role in epigenetical gene regulation by marking genes for transcriptional silencing. Besides a pivotal role in development, DNA methylation is also responsible for the X-chromosome inactivation in females and the silencing of tumor-suppressor genes in human cancers (Jones et al., 2002).

In promoter and enhancer regions CpGs are accumulated, whereas in the rest of the mammalian genome, only 20% of the statistically expected CpGs are present. This specific CpG distribution underlines the particular importance of gene regulation by methylation (Schorderet and Gartler, 1992).

Both CaSki and SiHa are polyploid HPV-16 positive cells (Patillo et al., 1977; Baker et al., 1987; Mincheva et al., 1987). CaSki cells possess approximately 600 mostly methylated and therefore transcriptionally silent copies of the HPV-16 genome. They are arranged mainly in tandem repeats and are localized over 11 chromosomal sites. In contrast, SiHa cells possess 2 unmethylated copies of the HPV genomes (Mincheva et al., 1987) To determine whether demethylation of the silenced HPV genomes is sufficient for the re-expression of the viral oncogenes, CaSki cells were treated with 5-Deoxyazacytidine (DAC).

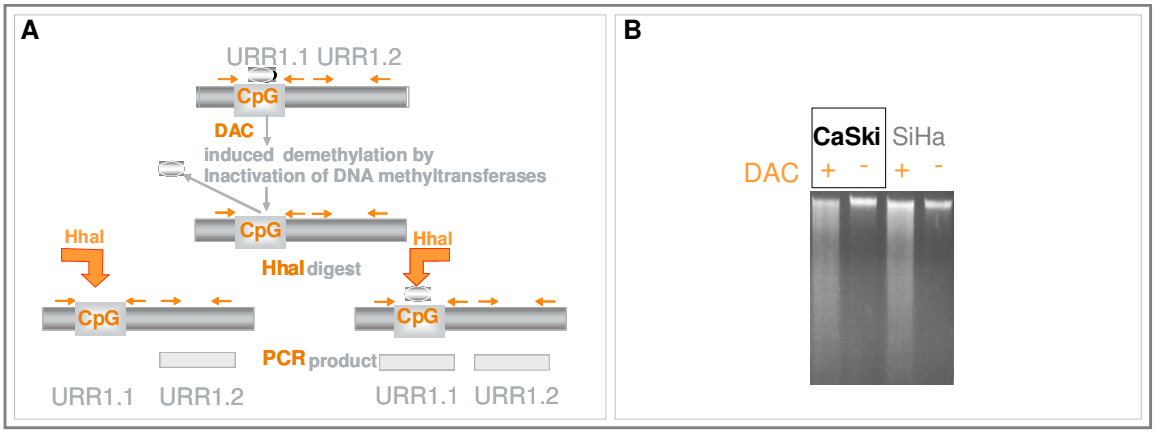

***Figure 4.2** Experimental approach and principle for detecting DNA demethylation by digesting genomic DNA with the methylation sensitive enzyme HhaI.*
(A) CaSki and SiHa cells were treated for four days with 5-Deoxyazacytidine (DAC). The isolated DNA was digested by the methylation sensitive enzyme HhaI. As an internal digest control pBluescript was added. After incubation overnight, the pBluescript samples were analyzed on agarose gel. Following isolation of the digested genomic DNA, PCR was performed with specific primers for the HPV-16 enhancer region (URR1.1 and URR1.2) for detection of demethylation. The demethylated and therefore HhaI digested DNA can not be amplified by the URR1.1 primers, that are specific for a region harboring the analysed HhaI restriction site. The URR1.2 primers are designed for a region without HhaI recognition sites and are used as DNA quantity control. (B) Agarose gel loaded with HhaI digested DNA isolated from cells treated for 4 days with 5 µM DAC and untreated controls.

In order to detect the demethylation of the HPV-16 genomes after DAC treatment, one can take advantage of the methylation sensitive enzyme HhaI, which can distinguish between methylated and unmethylated CpGs within its recognition site (5'-GCGC-3'). This property enables HhaI to cut DNA only when the embedded CpG is not methylated. Following successful DAC treatment, demethylated CpGs are located within HhaI recognition sites and the enzyme is able to digest the DNA (Figure 4.2A). Next, for detection of demethylation, the digested DNA was analysed by PCR. At sites where the enzyme digested the DNA, no PCR products were expected, whereas the uncleaved DNA was amplified. Specific primers for a sequence in the URR of the HPV-16 genome, which harbours a HhaI recognition site, were designed (URR1.1). Additionally, a primer set for another URR sequence (URR1.2), lacking a HhaI recognition site, was used as an experimental control (Figure 4.2A). SiHa cells harboring unmethylated HPV (Mincheva et al., 1987) were used as an additional positive control. DNA from these HPV genomes is always accessible for HhaI digestion at the URR1.1 sequence. From both cell lines, HhaI digested DNA from DAC treated samples and controls were visualized in an ethidiumbromide agarose gel (Figure 4.2B).

4.2.1 *Treatment of CaSki cells with 5-Deoxyazacytidine (DAC) induces demethylation of the HPV-16 URR*

The nucleotide analog DAC is incorporated into the DNA and induces demethylation by covalent irreversible binding to the catalytic core of DNA methyltransferases (Dmnt). This leads to both the functional inhibition and trapping of the Dmnt to DNA (Santi et al., 1984; Jüttermann et al., 1994; Schermelleh et al., 2005). DNA demethylation results in opening of heterochromatin followed by binding of transcription factors and the transcription initiation machinery. This leads to enhanced re-expression of silent genes (Magdinier et al., 2000; van der Velden et al., 2001). Previous experiments using DAC have shown that successful demethylation of HPV-16 genomes in CaSki cells requires treatment with 5 µM DAC for four days. Therefore all experiments have been performed under these conditions. Demethylation was detected by DNA digestion with the methylation sensitive enzyme HhaI and subsequent PCR (Figure 4.3). Using the HhaI site specific primers (URR1.1) for the HPV-16 URR, no PCR product was amplified in SiHa cells.

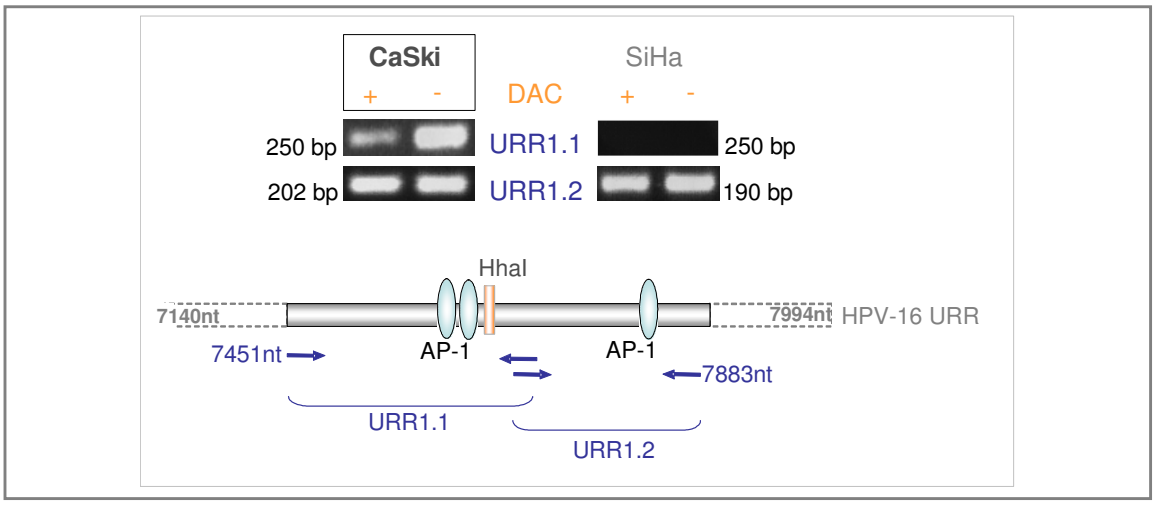

***Figure 4.3* Treatment of CaSki cells with 5-Deoxyazacytidine (DAC) for four days induces demethylation of the HPV-16 enhancer region.**
After treatment of CaSki and SiHa cells for four days with DAC, the isolated DNA was digested with the methylation sensitive enzyme HhaI. Subsequent PCR with the URR1.1 and URR1.2 primers was performed.

Untreated CaSki cells could not be cleaved at the same site by HhaI. The data supports previous reports that most of the HPV-16 genomes in CaSki cell are methylated (Kalantari et al., 2004; Rajeevan et al., 2006). After treatment of CaSki cells with DAC, a significant decrease in the specific PCR product using the URR1.1 primers was detected. These results

demonstrate that at least half of the 600 HPV-16 genomes in CaSki cells were demethylated (Figure 4.3), which was verified in different experiments. In order to ensure equal DNA amounts in all samples of the respective cell lines, PCR with the URR1.2 primers was performed. Here, no differences were detected (Figure 4.3).

Demethylation of the HPV-16 genomes in CaSki cells should result in an enhanced expression of the viral oncoproteins, because an increased amount of HPV-16 copies are accessible to transcription factors and the initiation complexes.

4.3 Expression analysis of the E7 protein after DAC induced demethylation

4.3.1 Enhanced proteasomal degradation of the oncoprotein E7 after DAC treatment in CaSki cells

To determine the level of the oncoprotein E7 expression after DAC treatment in CaSki and SiHa cells, Western blot analyses were performed. Since an effective demethylation of half of the HPV-16 genomes was detected in CaSki cells, an increase of the E7 expression was expected. Surprisingly, no up-regulation of the E7 protein could be detected in these cells. In contrast, the expression of the E7 protein was even diminished. SiHa cells did not show any down-modulation of E7 (Figure 4.4).

Previous reports have demonstrated that E7 is targeted for degradation by ubiquitination of N-terminal residues and subsequent proteolysis by the 26S proteasome (Kamio et al., 2004; Oh et al., 2005). In order to clarify whether E7 degradation after DAC treatment in CaSki cells was due to proteasomal degradation the cells were exposed to 20 µM of the proteasomal and calpain inhibitor MG132 for 8 hours.

The analysis of the cell lysates revealed an accumulation of E7 after incubation with MG132 in DAC treated CaSki cells, which reached the initial amount of the untreated sample. Treatment with the solvent DMSO alone did not rescue E7 from degradation. As controls, SiHa cells were treated under the same conditions and showed an E7 accumulation (Figure 4.4).

In order to prove the efficiency of MG132, p53 and pRb, which are known to be degraded by the proteasomal pathway, were analysed (Figure 4.4). In cervical carcinoma cells, p53, degradation is mediated by the oncoprotein E6 and its recruitment of the ubiquitin

ligase E6AP (Scheffner et al., 1990; Huibregtse et al., 1998). Exposure to MG132 in CaSki cells led to an accumulation of p53 in both DAC treated and control samples, which demonstrated that MG132 was functionally active. Interestingly, only SiHa cells do stabilize p53 already after DAC treatment alone. In contrast, this accumulation after DAC treatment was not detectable in CaSki cells. Nevertheless, an expected additional increase of p53 was observed after MG132 treatment in SiHa cells (Figure 4.4).

pRb is a key regulatory component for cell cycle progression from G1 to S-phase, since its degradation induces release of the transcription factor E2F, which leads to transcription of proteins required for this cell cycle transition (Weinberg, 1995). E7 binds to pRb and this interaction targets pRb to the proteasomal degradation (Münger et al, 1989; Gonzales et al., 2001). pRb can be rescued from degradation by MG132 treatment. DAC treatment in both CaSki and SiHa cells did not influence the pRb levels. An accumulation of pRb was observed after MG132 treatment in CaSki cells. No pronounced accumulation was detected in SiHa cells.

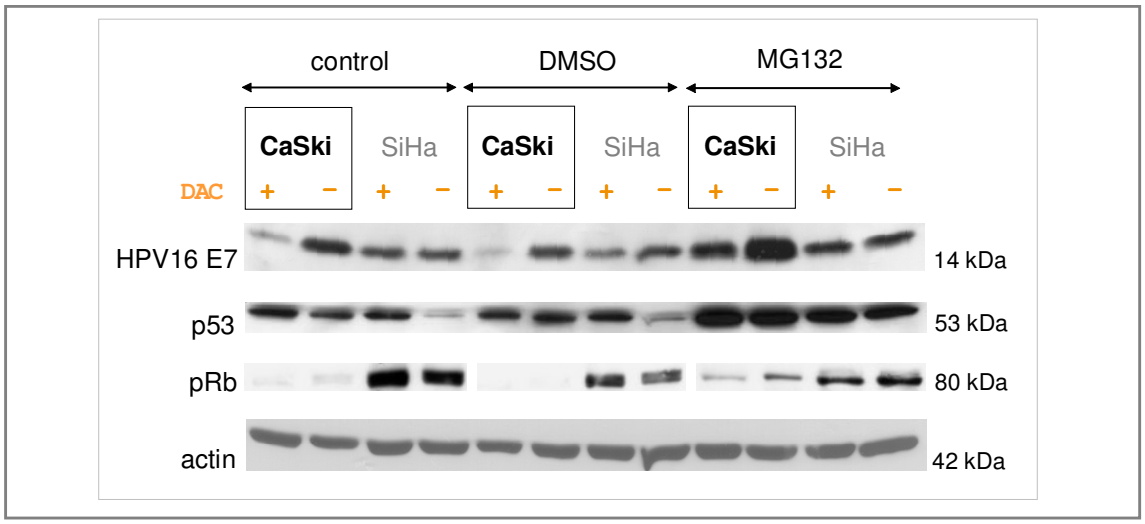

***Figure 4.4* Enhanced proteasomal degradation of the oncoprotein E7 after DAC treatment in CaSki cells.**
CaSki and SiHa cells were cultivated for 4 days in the presence or absence (control) of 5 µM DAC. After 88 hours incubation with DAC, the proteasomal and calpain inhibitor MG132 (20 µM) was added for additionally 8 hours to the DAC medium to ensure the total incubation time of 96 hours with DAC. 60 µg whole cells lysates (RIPA) were separated by 8 % / 12% SDS-PAGE and blotted on PVDF membranes followed by incubation with antibodies against HPV-16E7, p53, pRb, and actin.

These results imply that the observed E7 degradation after DAC treatment in CaSki cells is mediated by the proteasomal and/or calpain pathway. Furthermore, additional differences in the proteome activity of CaSki and SiHa cells after DAC treatment were detected. Notably, a stabilization of p53 was only observed in SiHa cells.

4.3.2 Analysis of the E7 stability after DAC treatment in HeLa (HPV-18) and somatic cell hybrid cell lines

Treatment of CaSki cells with DAC induces demethylation of HPV-16 genomes and is accompanied by down-regulation of E7 (Figure 4.4). In contrast, DAC treatment does not affect the E7 level in SiHa cells. The genetic background of every tumor cell line varies, since they were established from different patients and mutations, chromosomal aberrations, translocations and epigenetic marks occurred in each cell line. These differences and changes influence the composition of the proteome. Hence, the observed enhanced proteasomal E7 degradation might be, a dominant or recessive peculiarity particular for the genomic

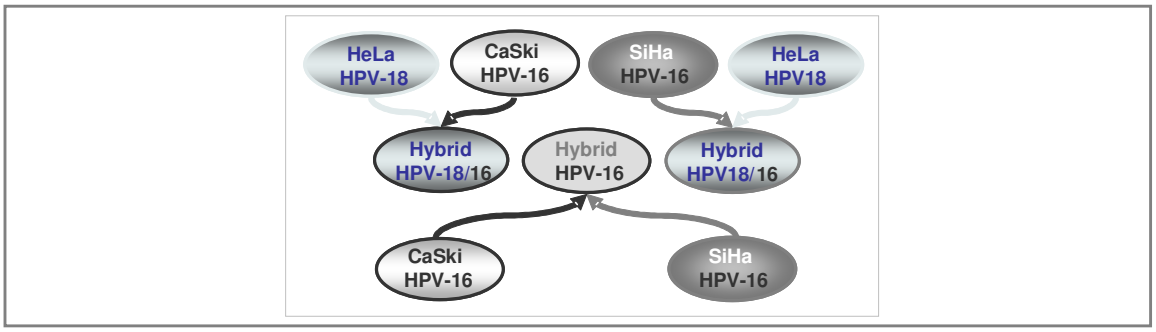

Figure 4.5 ***Schematic representation of the hybrid cell lines.***
Each hybrid cell line was generated by fusion of the parental cell lines CaSki, SiHa (HPV-16) and HeLa (HPV-18). Therefore these hybrids harbor mixed genomes derived from the parental cell lines and including viral genomes from HPV-18 and/or HPV-16 with their epigenetic modifications.

background in CaSki cells. In order to address this issue, we took advantage of somatic-cell hybrids (Soto et al., 2000). These hybrids harbour the HPV genomes from different parental cell lines: in case of HeLa X CaSki cells and HeLa X SiHa cells both HPV-18 and HPV-16 and in SiHa X CaSki cells only HPV-16 as depicted in Figure 4.5. As demonstrated in Fig. 3.6., all cell lines showed expression of the oncogene E7 from the incorporated HPV types, respectively. In addition, the HPV-18 positive cell line HeLa was also included in this approach.

4.3.3 *Enhanced degradation of the oncoprotein E7 after DAC treatment in hybrid cells*

The expression of E7 after DAC treatment in HeLa and hybrid cells was detected by Western blot analysis with HPV-16E7 and HPV-18E7 specific antibodies. The experiments showed that the amount of the expressed E7 oncoprotein varied among the untreated cell lines. HeLa X CaSki hybrid cells expressed the lowest HPV-18E7 amount. HeLa X SiHa hybrid cells expressed a higher amount, which was almost equal to HeLa cells (Figure 4.6). The HPV-16E7 levels of HeLa X CaSki and SiHa X HeLa hybrid cells were almost equal, but clearly less compared to the SiHa X CaSki hybrid cells, which expressed the highest amount. Analysis of HPV-16E7 and HPV18E7 levels after DAC treatment unravelled a down-regulation of both oncoproteins in all analysed cell lines, independently of the viral type. In SiHa X CaSki cells the HPV16E7 down-regulation was comparable to the decrease observed in CaSki cells. The amount of HPV-16E7 in HeLa X CaSki hybrid cells was also evidently reduced and furthermore HPV-18E7 was even not detectable anymore. In HeLa X SiHa

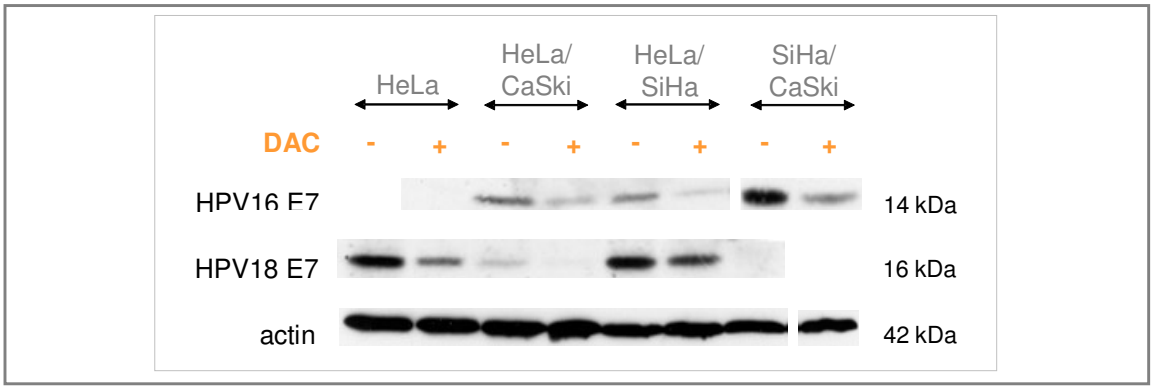

Figure 4.6 Enhanced degradation of the oncoprotein E7 after DAC treatment in hybrid cells.
Hybrid cells were cultivated for 4 days in the presence or absence (control) of 5 µM DAC. 60 µg whole cells lysates (RIPA) were separated by 12% SDS-PAGE and blotted on PVDF membranes followed by incubation with antibodies for HPV-16E7, HPV-18E7 and actin. The figure shows the same membrane for HPV-16E7. Due to higher expression of HPV-16E7 in SiHa/CaSki hybrids, the membrane was first exposed for 30 seconds. Subsequently, the same membrane was exposed for 90 seconds for detection of the other cell lines. For negative controls, either one sample containing HeLa lysate (for HPV-16E7) or SiHa/CaSki lysate (for HPV-18E7) were loaded.

hybrid cells, the expression of both proteins, HPV-16E7 and HPV-18E7, was also down-regulated. Analysis of the HPV-18E7 in HeLa cells showed a decreased level, even more pronounced in comparison to HeLa X SiHa hybrid cells. Taken together these experiments provide evidence that the DAC induced E7 degradation is not restricted to CaSki cells. It is a

common mechanism, independently of the HPV type, at least for the investigated HeLa and CaSki cells, and can be extended to hybrid cells containing the genomes of these cell lines.

4.3.4 Identification of E7 interacting proteins

4.3.4.1 Immunoprecipitation of the oncoprotein E7

Treatment of CaSki cells with the demethylating agent DAC might induce the expression of silent genes and therefore up-regulate proteins, which usually are not expressed in these cells. This has been shown in other experimental settings and is a general hallmark of DAC treatment (Jones at al, 1980). In order to identify novel potential targets of the E7 oncoprotein, which might induce the observed E7 degradation after DAC treatment in CaSki

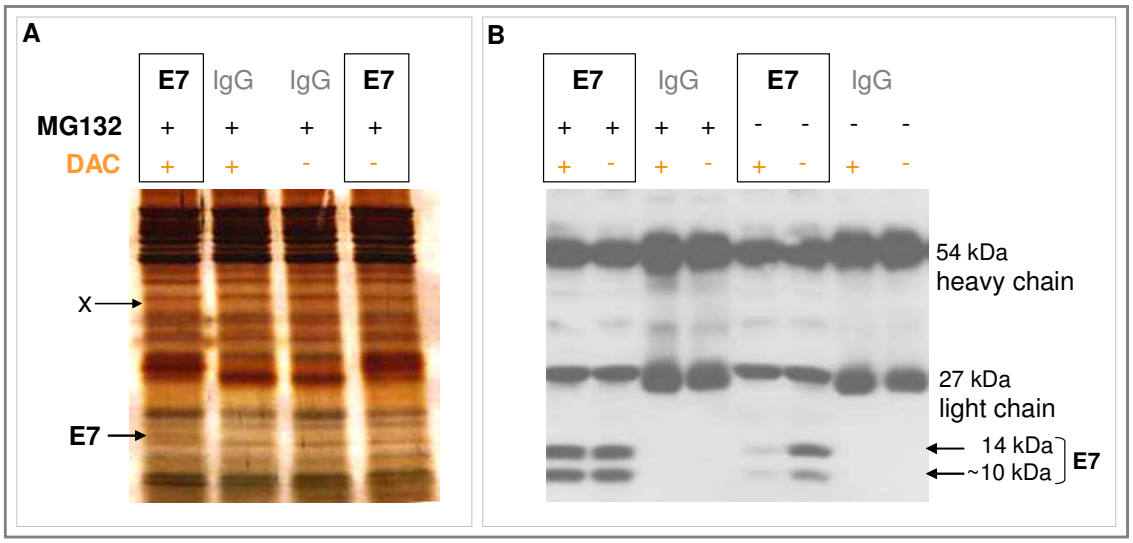

Figure 4.7 Immunoprecipitation of the oncoprotein E7.
(A) Silver staining of the E7 IP. CaSki cells were treated with DAC (5 µM, 4 days) and MG132 (20 µM, 8 hours) and harvested in IP-lysis buffer. After 1 hour lysis at 4°C, the extracts were centrifuged and incubated with 30 µl protein A agarose beads and 3 µg of the E7 antibody or 3 µg normal mouse IgG overnight. The precipitated proteins were visualized by silver staining of the 12% polyacrylamide gel. (B) Detection of the E7 oncoprotein by Western blot after IP as described in A.

cells, immunoprecipitation (IP) using a HPV-16E7 specific antibody was performed. First, it was tested whether the HPV-16E7 antibody was able to recognize the native epitope in an IP experiment. CaSki cells were treated with DAC alone or with DAC and the proteasomal inhibitor MG132 to stop E7 degradation. Cell lysates, from the different treatments and controls, were used for the IP. Subsequent Western blot analysis showed that the native E7

was specifically immunoprecipitated by the antibody (Figure 4.7B). As a negative control, cell lysates were also incubated with non-specific murine IgGs. In these samples E7 could not be detected by Western blot, verifying the specificity of the E7 antibody. The heavy and light chain of the IP antibodies were also recognized by the specific secondary antibody, because the same murine antibody directed against E7 was used for both IP and the Western blot. Importantly, an E7 decrease after DAC treatment (Figure 4.4), was confirmed by IP, as shown in Figure 4.7 B, where only a weak E7 signal was detected in the DAC treated sample.

In order to identify differences after the IP between the DAC treated and untreated samples, the co-precipitated proteins were visualized on a silver stained polyacryamide gel. A band can be detected at the expected size for E7 at approximately 14 kDa (Figure 4.7A). Additionally, there were slightly visible differences between the band patterns from DAC treated versus untreated samples. For example one band was detected at ca 40 kDa, which is only visible in the DAC sample, but neither in the control nor in the IgG samples, indicating that different proteins may interact with E7 in the presence of DAC. Nevertheless, unspecific bands were also detected by using this staining method. These are usually proteins that bind unspecifically to the agarose-protein-A-beads.

4.3.4.2 Mass spectrometry analysis of E7 co-precipitated proteins

In order to identify novel proteins responsible for targeting E7 to degradation after DAC treatment, mass spectrometry (MS) analysis was performed. CaSki cells were exposed to DAC and MG132 and harvested in IP lysis buffer. Untreated controls were equally processed. Next, the samples were incubated with the antibody against E7. IgG controls were not analyzed by mass spectrometry due to the complexity of the method.

Interaction with E7	author/ reference	MS data
TBP	Phillips et al., 1997 Lee et al., 2005	TBP associated factor 15
S4 subunit of the 26S proteasome	Berezutskaya and Bagchi, 1997	S2 subunit of the 26S proteasome (CA only)
MPP2	Lüscher - Firzlaff et al., 1999	MPP4
BRCA1	Avvakumov et al., 2003; Zhang et al, 2006	BRCA1
p600	Huh et al., 2005	p600
c-Myc	Wang et al., 2007	c-Myc
Dnmt1	Burgers et al., 2007	putative Dnmt
pRb	Dyson et al., 1989	pRb binding protein 1 and 7
		keratins
		Calpain1, calpain 12 (CA only)
		CARM1

***Figure 4.8* E7 interaction partners identified by MS.**
Immunoprecipitations were carried out with 3 µg E7 antibody in DAC plus MG132 and untreated samples. For MS E7 co-immunoprecipitated proteins were separated on a polyacrylamide gel. The one-dimensional gel was cut into 14 slices per sample. Each individual slice was digested by trypsin and further analyzed by MS. The identified peptides were submitted to a data base search for the corresponding protein. Only proteins representing a high detection score were further included as potential E7 interacting partners. Figure 4.8 shows an overview of high scored proteins. Additionally, the proteins already known to interact with E7, which were also detected here, are summarized (Figure 4.8) and confirm the specifity of our method. The oncoprotein E7 itself could not be detected, since after the digest by trypsin the remaining peptides are too small for detection by MS.

4.3.5 The degradation of the oncoprotein E7 is partially mediated by calpains

Analysis by mass spectrometry of proteins co-immunoprecipitated with E7 showed the presence of the non-ubiquitous calpain 12 only in the DAC treated sample (Figure 4.8). The well-conserved family of calcium dependent cystein proteases consists of several ubiquitous and cell specific isoforms. Calpains influence a variety of biological processes like migration, proliferation and apoptosis (Saez et al., 2006). One domain by which calpains identify their potentially targets is the PEST (Pro, Glu, Ser/Thr-rich) domain. E7 possesses a PEST domain localized close to the pRb binding site (Antonsson et al., 2006), which might be recognized by calpains.

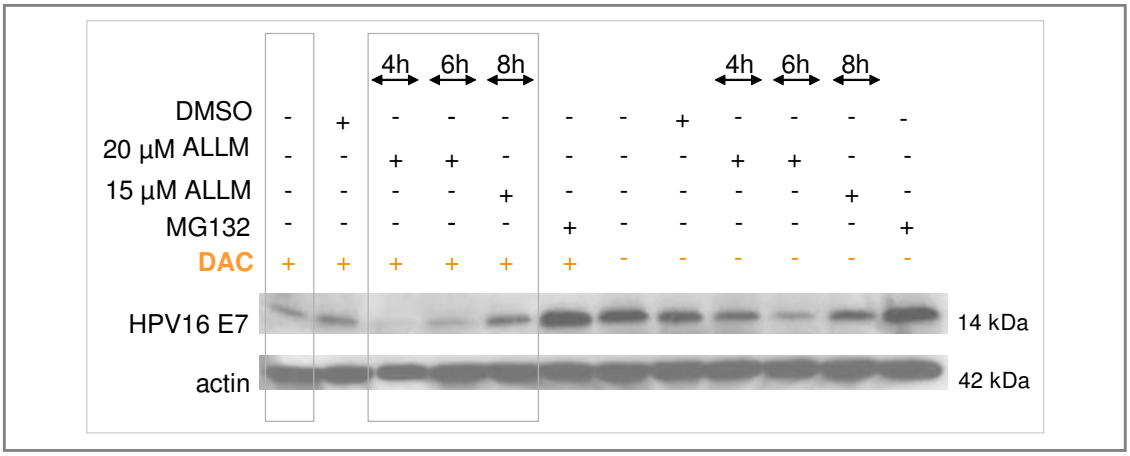

***Figure 4.9** The degradation of the oncoprotein E7 is partially mediated by calpains.*
CaSki were cultivated for 4 days in the presence or absence of 5 µM DAC. After 88 hours incubation with DAC the proteasomal and calpain inhibitor MG132 (20 µM) was added for an additional 8 hours to the DAC medium to ensure the total incubation of 96 hours with DAC. The calpain inhibitor ALLM was added for 4, 6 and 8 hours in concentrations of 15 µM and 20 µM. 50 µg whole cell lysates (RIPA) were separated in 12% SDS-PAGE and blotted on PVDF membranes followed by incubation with the antibody against HPV-16E7 and actin.

In previous experiments (Figure 4.4), MG132 treatment was shown to rescue E7 from DAC induced degradation. MG132 is a strong inhibitor of the proteasome, but also inhibits calpains. In order to verify the hypothesis that E7 was degraded by calpains after DAC treatment, CaSki cells were incubated for different time periods with different concentrations of the pan-calpain inhibitor ALLM (N-acetyl-leucyl-leucyl-methionine) (Stangl et al., 2004). As shown in Figure 4.9, this treatment partially rescued E7 from degradation, although it was not as efficient as the treatment with MG132 (Figure 4.9). Despite this observation, treatment with 20 µM ALLM for more than 4 hours was cytotoxic and induced apoptosis accompanied by reduced levels of E7 (Figure 4.9). Therefore, in order to monitor only the calpain inhibitory effect, concentration should not exceed 15 µM ALLM in CaSki cells. In summary, the exposure of DAC treated cells to 15 µM ALLM for 8 hours led to an accumulation of E7 which is less pronounced than the accumulation induced by MG132.

4.4 Interaction of CARM1 with the oncoprotein E7 after E7 IP

Mass spectrometry analysis unravelled another interesting protein co-precipitating with E7, namely the protein co-activator-associated arginine methyltransferase 1 (CARM1, PRMT4) (Figure 4.8). The detected signal was significant and reached a score of 39 with 7 matched peptides in both DAC treated and untreated samples. CARM1 has been described as

a positive regulator of transcriptional promoters by its ability to methylate proteins involved in chromatin remodelling, specifically the histone H3 at arginine 17 (Chen et al., 2000) and the histone acetyltransferases p300/CBP (Lee et al., 2005). Additional substrates of CARM1 are the RNA-binding-proteins HuR and HuD (Fujiwara et al.., 2006).

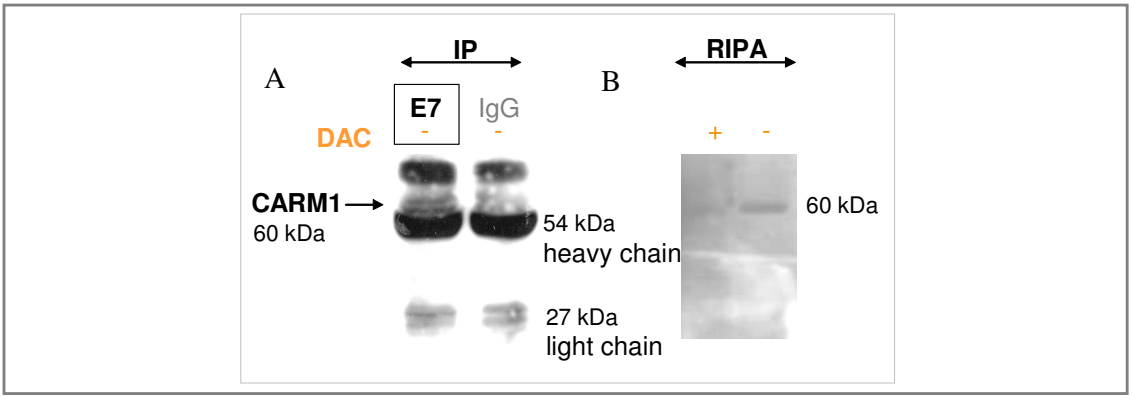

***Figure 4.10* Interaction of CARM1 with the oncoprotein E7 was detected after E7 IP.**
(A) CaSki cells were harvested in IP-lysis buffer. After 1 hour lysis at 4°C the extracts were centrifugated and incubated with 30 µl protein A agarose beads and 4 µg of the E7 antibody or 4 µg normal mouse IgG over night. The precipitated CARM1 proteins were detected by Western blot with a CARM1 specific antibody. (B) CaSki cells were harvested in RIPA buffer. 50 µg whole cell lysates (RIPA) were separated in the same 8% SDS-PAGE gel like the IP in A and blotted on a PVDF membrane, followed by incubation with the antibody against CARM1. Due to strong IgG and CARM1 signals in the IP, the membrane was first exposed for 20 seconds. Subsequently, the same membrane was exposed for 60 seconds for detection of CARM1 in the RIPA extracts.

In order to verify the MS data, IP were performed with the E7 antibody. Subsequently, Western blot experiments using an antibody against CARM1 were carried out. After the IP a specific band corresponding to the size of endogenous CARM1 was detected (Figure 4.10A). This signal was not visible in the IgG control samples. These data were obtained in two independent experiments suggesting that CARM1 binds to E7. For monitoring the specifity of the CARM1 antibody, RIPA extracts from CaSki cells were loaded with the IP on the same gel. Specific bands localized at ca 60 kDa were detected (Figure 4.10B). The different signal intensity is due to different exposure times.

4.4.1 Down-regulation of CARM-1 in DAC treated CaSki cells

To further investigate the E7-CARM1 interaction, CARM1 levels after DAC treatment were analysed by Western blot. Both CaSki and SiHa RIPA extracts were loaded on the gel for direct comparison of the two cell lines. CARM1 was expressed in both CaSki and SiHa cells to a similar extent. For the previous MS experiment the cells were treated with DAC and MG132. This was necessary in order to stop E7 degradation. Therefore, no differences in the CARM1 detection levels were observed between the treated and untreated MS samples (Figure 4.8). Interestingly, when CaSki cells were treated with DAC alone and analysed by Western blot, CARM1, like E7, was also down-regulated. SiHa cells did not show any effect (Figure 4.11). This result implies that an important positive regulator of transcription, CARM1 deregulated after DAC treatment.

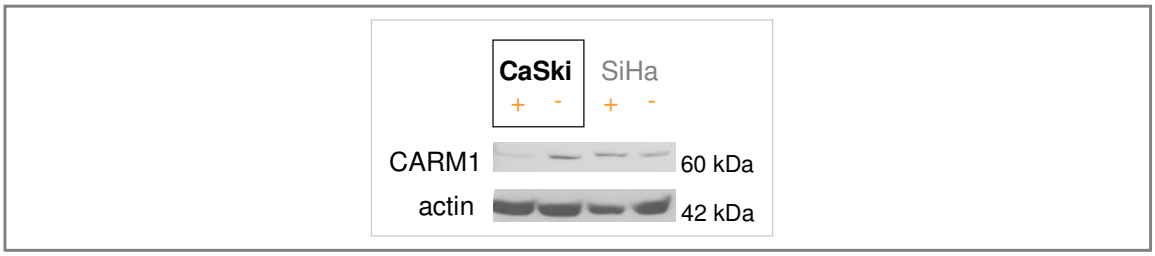

Figure 4.11 ***Down-regulation of CARM-1 in DAC treated CaSki cells.***
CaSki and SiHa cells were cultivated for 4 days in the presence or absence (control) of 5 µM DAC. 60 µg whole cells lysates (RIPA) were separated by 10% SDS-PAGE and blotted on PVDF membranes followed by incubation with antibodies for CARM1.

4.5 Analysis of E7 oncogene transcription after DAC induced demethylation in CaSki cells

4.5.1 Treatment with DAC induces a reduction of the HPV-16E7 transcription

After detection of the E7 degradation on protein level in CaSki cells the transcriptional expression levels of the E7 oncogene after DAC treatment were analysed in CaSki and SiHa cells. Therefore RT-PCR with HPV-16E7 specific primers was performed. After demethylation of the HPV-16 genomes in CaSki cells, an increase of E7 expression was

expected. However, this was not the case. In contrast, even a slight, but reproducible down-regulation of E7 transcription was observed in CaSki cells, whereas in SiHa cells no changes in the E7 expression levels were detectable by RT-PCR (Figure 4.12A).

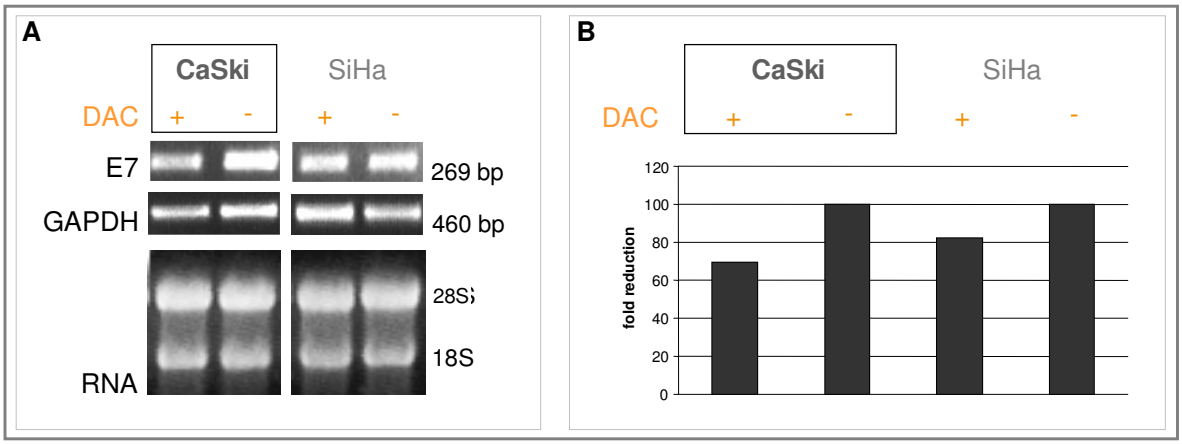

***Figure 4.12* Treatment with DAC induces a slightly reduction of the HPV-16E7 transcription.**
(A) RT-PCR. CaSki and SiHa cells were cultivated for 4 days in the presence or absence (control) of 5 µM DAC. RT-reaction with 1 µg RNA (in 20 µl volume) and random primers followed by PCR using 1 µl from the RT and HPV-16E7 and GAPDH primers. (B) Q-PCR with 1 µl from the RT-sample and HPV-16E7 primers. The results were first normalized to the housekeeping gene GAPDH and the adequate control sample.

To further verify these findings, quantitative real time PCR was performed with cDNA of DAC treated CaSki and SiHa cells. This approach confirmed the RT-PCR data and an approximately 30% reduction in the E7 transcription was determined for the cell line CaSki after DAC treatment. In contrast, for SiHa cells 10 % reduction was detected (Figure 4.12B). This result indicates that demethylation of the HPV-16 copies in CaSki was not sufficient for the transcriptional induction of E7. However, besides DNA demethylation, there are other requirements for induction and maintenance of transcription. The most important one is the accessibility of transcription factors and the transcription initiation complex to the DNA. Demethylation favours the opening of the chromatin but post-translational histone modifications and the incorporation of histone variants mark chromatin for activation and active transcription (Ahmand K. et al., 2002; McKittrick et al., 2004; Johnson et al., 2004; Chow et al. 2005). In addition the composition of transcription factors from the AP-1 family is decisive for the initiation of the HPV oncogene expression (Soto et al., 1999). Consequently, further experiments were designed to investigate these aspects.

4.5.2 Treatment with DAC for four days induces up-regulation of AP-1 family members in CaSki cells

Previous studies emphasized the essential role of AP-1 composition in transcriptional initiation from the HPV enhancer and promoter (Butz and Hoppe-Seyler, 1993; Kyo et al.,1997; Rösl et al., 1997). The upstream regulatory region (URR), including the enhancer of HPV-16 harbours three AP-1 binding sites (Figure 4.1). It has been demonstrated that a successful transcriptional induction is favoured by c-Jun/c-Fos dimers which are known as strong activators in contrast to c-Jun/Fra-1 dimers, which act like repressors or attenuators of the AP-1 signal (Rösl et al., 1997; Soto et al., 1999). Furthermore, an interaction between E7 and c-Jun, leading to an enhanced trans-activation by c-Jun, has been described (Antinore et al., 1996). CaSki and SiHa cells vary in the copy numbers of their integrated HPV-16 genomes, namely 600 versus 2-3 copies, respectively (Mincheva et al., 1987). Although most of the copies in CaSki cells are transcriptionally silenced by methylation, only one locus of the HPV tandem repeats is known to be active (Van Tine et al., 2001). Besides the difference in transcriptionally active HPV genomes, another important distinction is their AP-1 expression pattern. SiHa cells express the AP-1 family members c-Jun, JunD, JunB and c-Fos, whereas CaSki cells express c-Jun, Fra-1 and JunB (Soto et al., 2000, Figure 4.13). Therefore they exhibit an advantage expressing the strong activators of HPV transcription c-Jun and c-Fos, whereas CaSki cells are deficient in c-Fos expression. Additionally, due to a deletion, SiHa cells lack the binding site for the repressor Yin Yang 1 (YY1), which has been described to reduce the AP-1 binding to their recognition sites (O´Connor et al., 1996). These are important issues that enable the cells to achieve equal levels of the oncogenes E6 and E7 expression, besides the extreme difference in HPV copy number.

The E7 transcription is slightly down-regulated after DAC treatment in CaSki cells, despite the expected up-regulation after demethylation of the HPV genomes (Figure 4.12). Hence, the composition of the AP-1 transcription factors may play a decisive function for transcriptional initiation of HPV. To investigate a potential role of the AP-1 composition, the levels of the AP-1 family members, namely c-Jun, c-Fos, Fra-1, JunD and JunB were analysed by RT-PCR (Figure 4.13A).

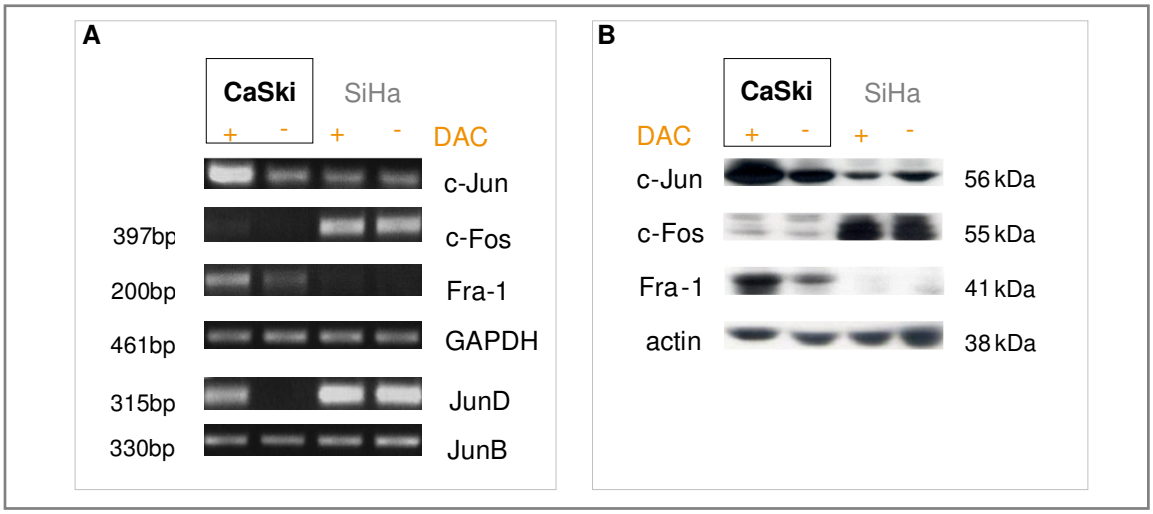

Figure 4.13 Treatment with DAC for four days induces up-regulation of AP-1 family members in CaSki cells.
RT-PCR and Western blot analysis of AP-1 family members. (A) CaSki and SiHa cells were cultured for 4 days in the presence or absence (control) of 5 µM DAC. RT-reaction was performed with 1 µg RNA (20 µl volume) using random primers. For the following PCR 1 µl from the RT-reaction and primers for the analysed cDNAs were used. (B) Western blot analysis of AP-1 family members. CaSki and SiHa cells were cultivated for 4 days in the presence or absence (control) of 5 µM DAC. 75 µg whole cells lysates (RIPA) were separated by 10 % SDS-PAGE and blotted on PVDF membranes followed by incubation with antibodies against the different AP-1 family members.

After DAC exposure, a strong up-regulation of c-Jun, Fra-1 and JunD was observed in CaSki cells, whereas no induction of c-Fos was detectable. In contrast, SiHa cells did not show any changes for the examined AP-1 factors (Figure 4.13A). To further validate the RT-PCR data, Western blot analyses of the AP-1 family members, specifically for c-Jun, Fra-1 and c-Fos were performed. JunD and JunB were not analysed by Western blot, since no qualitatively good antibodies were available. The expression levels of both c-Jun and Fra-1 were increased in CaSki cells after DAC treatment, whereas no differences were detected in SiHa cells (Figure 4.13B). Taking into consideration both the RT-PCR and the Western blot data, these results imply that the observed increase of c-Jun and Fra-1 in CaSki cells might negatively influence the transcriptional initiation from demethylated HPV genomes. Therefore, no up-regulation, but even a slightly down-regulation of the E7 transcription was detected after DAC treatment.

4.5.3 The up-regulation of AP-1 family members in CaSki cells is independent of the activation of the p38 and JNK signalling pathways

Mitogen activated protein kinases (MAPK), p38, Jun amino-terminal kinase (JNK) and extracellular-signal regulated (ERK) are activated by external signals like growth factors, cytokines and stress (Hazzalin and Mahadevan, 2001). MAPK phosphorylate diverse transcription factors that initiate the transcription of fos and jun genes, and additionally stabilize these proteins by phosphorylation resulting in an enhanced amount of AP-1 complexes (Hibe et al., 1993; Musti et al., 1997; Hess et al., 2004). Furthermore, the post translational phosphorylation, regulated by MAPK, alters the AP-1 activity in its transactivating potential and DNA-binding capacity (Morrison, 2003; Hess et al., 2004).

To exclude that DAC may induce the MAP Kinase pathway, Western blot analyses were carried out using phosphorylation specific antibodies for p38 and JNK.

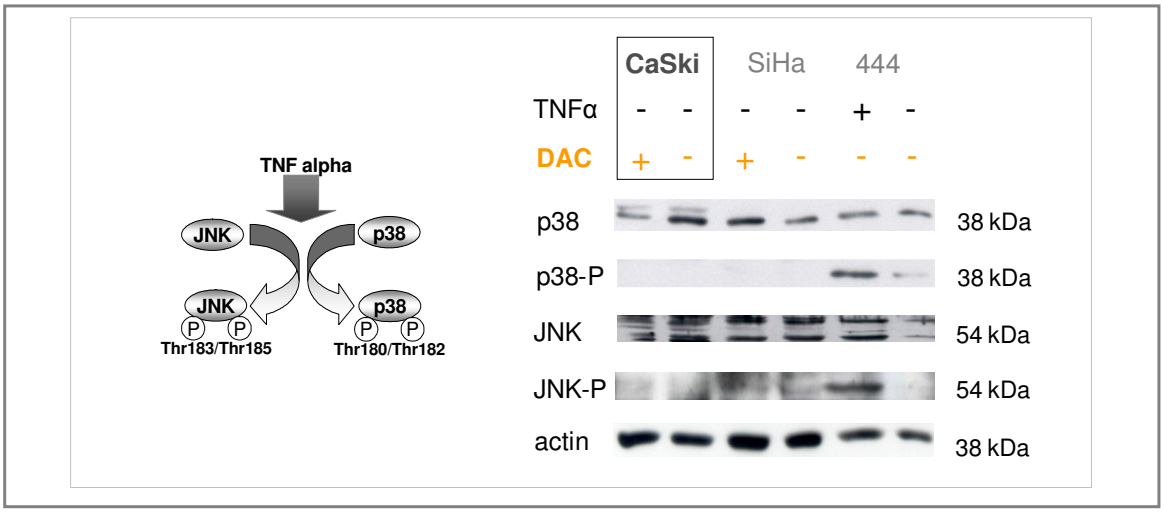

***Figure 4.14* Up-regulation of AP-1 family members in CaSki cells is independent of the activation of the p38 and JNK signalling pathways.**
CaSki and SiHa cells were treated with 5 µM DAC for 4 days. Western blot with 40 µg whole cell lysate from SDS lysis buffer. Incubation of the membrane with antibodies against unphosphorylated and phosphorylated p38 and JNK. 444 cells were stimulated for 30 min with 100U/ml TNF alpha as positive control for MAPK activities.

In order to prove the specificity of the used antibodies against phosphorylated p38 and JNK, 444 cells were included as positive controls. These cells were incubated for 30 minutes with 100U TNF to induce MAPK phosphorylation. All cell lines analysed showed almost the

same levels of unphosphorylated p38 and JNK. Nevertheless, the phosphorylated forms of these MAPKs were only detectable in the TNF alpha treated 444 cells. Neither CaSki nor SiHa cells showed enhanced phosphorylation of MAPKs after incubation with DAC (Figure 4.14). Consequently, the detected up-regulation of the AP-1 family members c-Jun, Fra-1 and JunD in CaSki cells after DAC treatment is not induced by MAPKs.

4.5.4 Enhanced binding of c-Jun-Fra-1 dimers to AP-1 consensus and AP-1 HPV-16 enhancer sequences after DAC treatment in CaSki cells

The previous experiments demonstrated that c-Jun and Fra-1 are up-regulated in CaSki cells after DAC treatment. In order to verify that the enhanced c-Jun and Fra-1 expression is paralleled by enhanced binding of these transcription factors to their recognition sequences electrophoretic mobility shift assays (EMSA) were carried out.

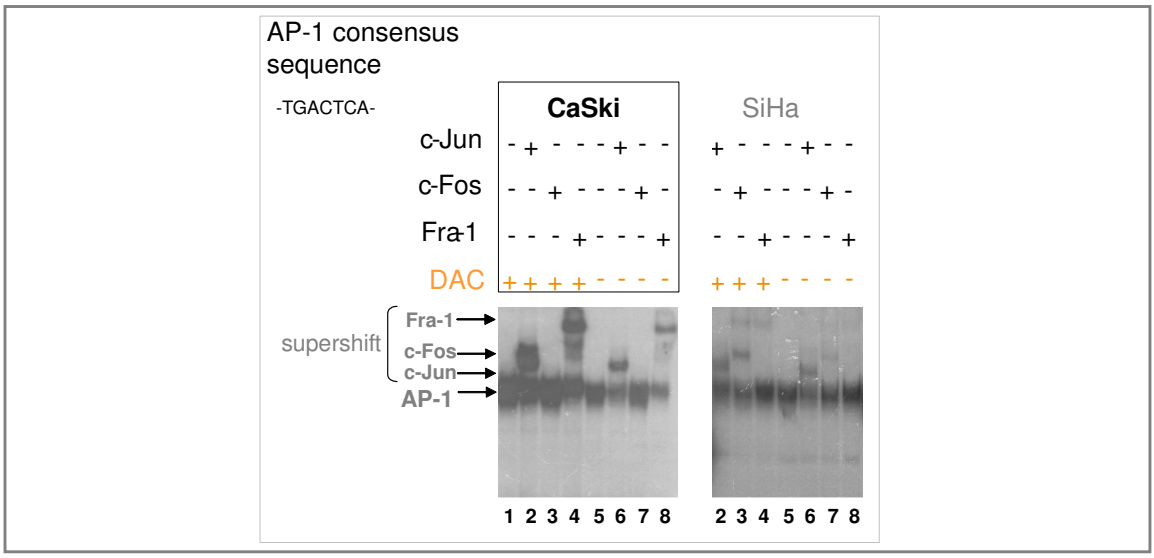

***Figure 4.15* Enhanced binding of c-Jun-Fra-1 dimers to AP-1 consensus sequence after DAC treatment in CaSki cells.**
Dissection of the AP-1 dimerisation patterns by supershift EMSA. CaSki and SiHa cells were cultivated for 4 days in the presence or absence (control) of 5 µM DAC. 2 µg nuclear extracts were incubated with ^{32}P labeled AP-1 consensus oligonucleotides. c-Jun, c-Fos and Fra-1 antibodies were added to the samples and they were subsequently separated by electrophoresis.

EMSA is a DNA-binding assay that allows binding analysis of transcription factors from nuclear extracts to their specific DNA recognition sequence *in vitro* (Lewis and Conradi, 1996). The binding of proteins to a synthetic radioactive labelled DNA-oligonuclotide is

detected. EMSA takes advantage of the observation that an unbound DNA fragment migrates rapidly during electrophoresis through a polyacrylamide gel, whereas DNA/protein complexes significantly slow the migration, causing the phenomenon called gel retardation. Additionally by using specific antibodies for the transcription factors of interest, this method allows the detection of the complex composition in a qualitative and quantitative manner.

For EMSA experiments, different oligonucleotides were used: the AP-1 consensus sequence, the AP-1 HPV-16 enhancer sequence and the Oct-1 sequence as binding control. (see Figure 4.15 lane 1-8/ Figure 4.16 lane 1-8, Oct-1). An increased binding of the proteins c-Jun and Fra-1, which had elevated levels, were detected in nuclear extracts from the DAC treated CaSki cells using AP-1 consensus and AP-1 HPV-16 enhancer sequences. Moreover, for these oligonucleotides, no binding of c-Fos was observed in CaSki cells, neither in the

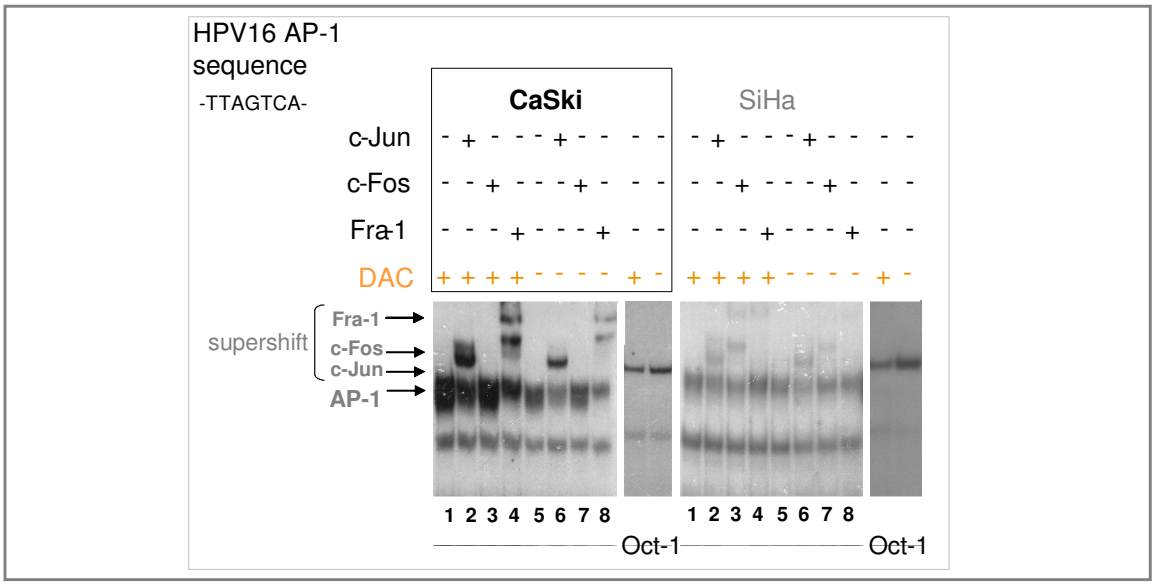

***Figure 4.16* Enhanced binding of c-Jun-Fra-1-dimers to the AP-1 HPV-16 enhancer sequence after DAC treatment in CaSki cells.**
Dissection of the AP-1 dimerisation patterns by supershift EMSA. CaSki and SiHa cells were cultivated for 4 days in the presence or absence (control) of 5 µM DAC. 2 µg nuclear extracts were incubated with ^{32}P labeled AP-1 HPV-16 enhancer oligonucleotides. c-Jun, c-Fos and Fra-1 antibodies were added to the samples and they were subsequently separated by electrophoresis.

control samples nor after DAC treatment. SiHa cells showed only a faintly increased binding of c-Fos after DAC treatment for both AP-1 consensus and AP-1 HPV-1 enhancer sequences. In comparison to a significant increase in CaSki cells (comparing lane 2 to lane 6 for c-Jun and lane 4 to lane 8 for Fra-1). The c-Fos increase in SiHa cells (compare lane 3 to lane 7) is not significant (Figure 4.15/Figure 4.16). Additionally, no increase in the c-Jun binding for

the investigated sequences was detectable, as shown by the comparison between the bands in lane 2 to lane 6. Furthermore, Fra-1 was not detectable as AP-1 component in SiHa cells, which again confirms the Western blot data. These results reveal a strong increase of c-Jun and Fra-1 binding upon DAC treatment in CaSki cells, however the treatment did not induce any changes of the AP-1 composition (Figure 4.15/Figure 4.16).

4.5.5 Treatment with DAC does not affect the HPV-16E7 transcription, while it induces changes in HPV-18 transcription.

For the analysis of the E7 protein levels after DAC treatment, somatic cells hybrids between SiHa, CaSki and HeLa were investigated. The advantage of such cell hybrids is that they allow a direct comparison to the parental cell lines and the involvement of trans-dominant or trans-recessive cell specific factors. To analyse E7 transcription after DAC treatment of the hybrids, RNA was isolated and analysed by RT-PCR for HPV-16E7 or Northern blot for HPV-18. On protein level, hybrids and HeLa cells exhibit a degradation of E7 after incubation with DAC (Figure 4.6). However, on transcriptional level, the cells differ from CaSki cells. A significant change of E7 transcription was not detectable, neither for the hybrids nor for HeLa cells. An exception is the hybrid cell line HeLa/SiHa, which did not show any changes in HPV-16E7, but a drastically decrease of HPV-18 mRNA expression after DAC treatment. HPV-16E7 primers do not recognize the HPV-18 cDNA, therefore no amplification was detected in HeLa cells. All analysed hybrids do express HPV-16E7, interestingly in comparable amounts (Figure 4.17). In contrast to HPV-16E7, HPV-18 mRNA is expressed differently among the hybrids. Under control conditions, HPV-18mRNA expression is very low in HeLa/CaSki hybrids, which corresponds to the Western blot results, where the HPV-18E7 protein expression was also the lowest (Figure 4.6). While HeLa cells and HeLa/SiHa hybrids show almost the same high amounts. After DAC treatment a slight down-regulation of HPV-18 mRNA was detected in HeLa cells and a strong in HeLa/SiHa cells. In contrast DAC treatment did not change the expression levels in HeLa/CaSki hybrids (Figure 4.17).

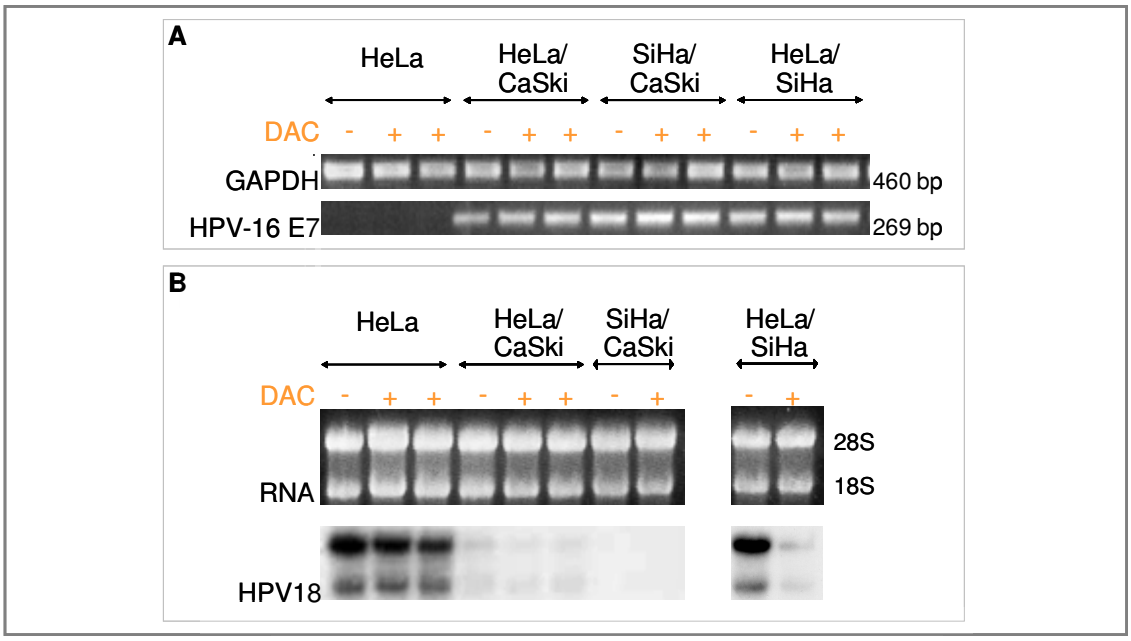

***Figure 4.17* Treatment with DAC does not affect the HPV-16E7 transcription in all analysed cell lines, while it induces changes in HPV-18 transcription.**
Cell lines were cultivated for 4 days in the presence or absence (control) of 5 µM DAC. (A) RT-reaction with 1 µg RNA (in 20 µl reaction volume) and random primers followed by PCR using 1 µl from the RT-reaction and HPV-16E7 and GAPDH primers. (B) Northern Blot with 5µg RNA, hybridized with ^{32}P radioactive labelled HPV-18 specific probe.

4.6 Generation of stable clones expressing the histone 3 variants H3.1 and H3.3 in the cell lines CaSki and SiHa

New exciting insights into the biology of histones have been established in recent years. First, the histone code theory, which underlines an important role of post-translational modifications of histones for the organization of chromatin conformation and therefore transcriptional regulation, was established (Allis et al, 2000). Now another crucial mechanism for marking transcriptional states has been discovered, namely the insertion of histone variants (Ahmand et al., 2002). Five different variants have been discovered so far for histone H3, H3.1, H3.2, H3.3, CENP-A and H3.1t (Allis et al, 2000). Although the histone H3 variants H3.1 and H3.3 differ only in 4 amino acids (Figure 4.18A), their incorporation into the chromatin is highly regulated and influences the differences between transcriptionally active and silenced sites (Ahmand et al., 2002; McKittrick et al., 2004; Johnson et al., 2004; Chow et al. 2005). In previous experiments, no up-regulation of E7 could be detected in CaSki cells after DNA demethylation. Therefore, demethylation of silenced HPV genomes

alone is not sufficient for induction of viral transcription. Thus, it was supposed that the incorporation of a stably expressed H3.3 variant, which marks transcriptionally active sequences, may alter the chromatin composition of the DAC demethylated HPV genomes towards a favorable state for transcription.

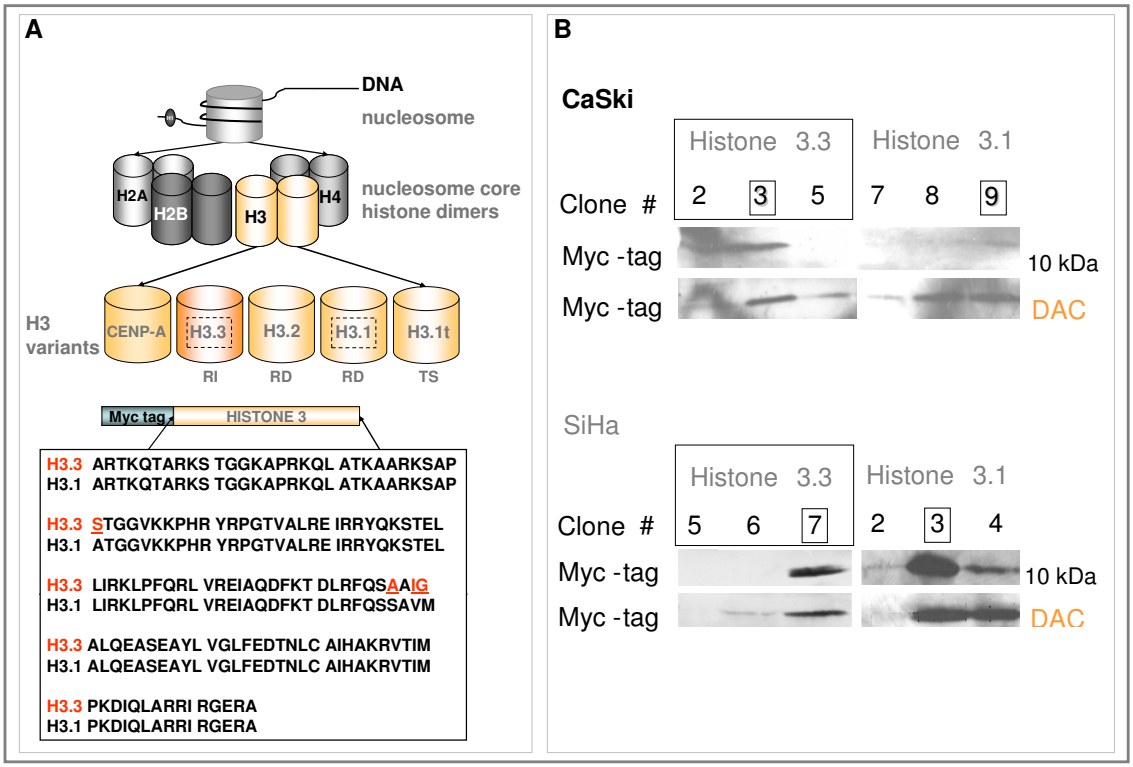

***Figure 4.18* Generation of stable clones expressing the histone 3 variants H3.1 and H3.3 in the cell lines CaSki and SiHa.**
(A) Schematic overview of the nucleosome core, the different H3 variants and the structure of the Myc-tagged histone 3.1 and histone 3.3 constructs (RI: replication independent; RD replication dependent, TS testis specific). (B) CaSki and SiHa stable clones were cultivated for 4 days in the presence or absence (control) of 5 µM DAC. Histones were isolated incubating nuclear extracts in 0.2M sulfuric acid and subsequently in acetone. Then samples were dissolved in SDS sample buffer and analysed by Western blot. The clones expressing the Myc-H3.1/H3.3 proteins were detected using a Myc-specific antibody.

To investigate this hypothesis, stable clones from CaSki and SiHa cells expressing Myc-tagged-H3.1 or H3.3 variants under the control of a CMV promoter were generated. The sub-cloned cells were tested for Myc-H3.1 or Myc-H3.1 expression. Thereby, the histones were isolated by incubation of nuclear extracts in 0.2M sulfuric acid and precipitated in acetone. Subsequently, the Myc-tagged histones were detected in Western blot using an antibody against the Myc-tag. Different clones were analysed in both cell lines. Since the CMV promoter can be *de novo* methylated in stable cells, treatment with DAC was necessary in some clones to induce Myc-H3.3 protein expression. In order ensure equal Myc- H3.3

expression, independently of DAC treatment, the CaSki clone 3 (C-c3:H3.3) was selected (Figure 4.18B) for further experiments.

4.6.1 Incorporation of Myc-H3.3 into the URR of HPV-16 after DAC treatment

Clone 3 (C-c3:H3.3) expresses Myc-H3.3 independently of DAC treatment. In order to verify the functionality, and incorporation of the expressed histone variant into the HPV genomes, Chromatin Immunoprecipitations (ChIP) were performed.

ChIP is a powerful method to identify *in vivo* interactions between proteins and specific DNA sequences (Orlando et al., 1997). Any chromatin-associated or DNA binding protein, including histones, can be analyzed, depending on which antibody is used. The initial step of this technique is the cross-linking of protein-protein and protein-DNA complexes in

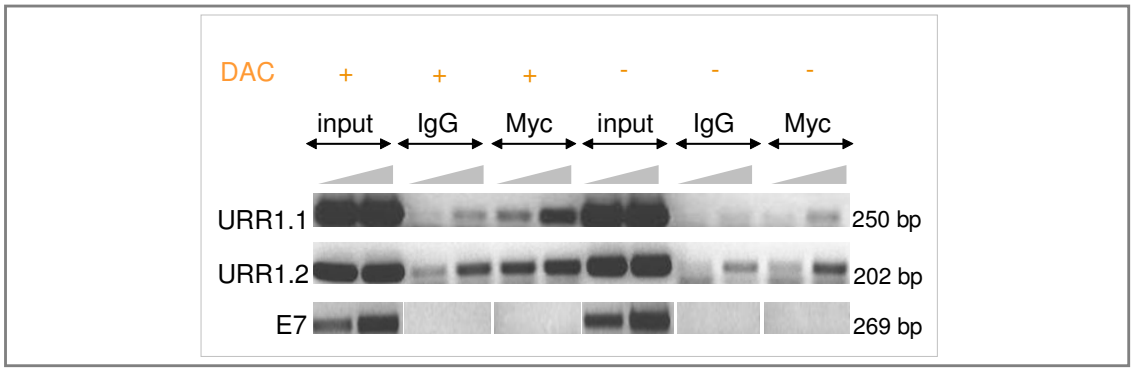

***Figure 4.19* Incorporation of histone Myc-H3.3 variants into the URR of HPV-16 after DAC treatment in CaSki stable Myc-H3.3 clone (C-c3:H3.3).**
C-c3:H3.3 cells were cultivated for 4 days in the presence or absence (control) of 5 µM DAC. ChIP was carried out with 20 µg chromatin using an anti-Myc antibody and IgG control antibodies. The precipitated DNA was analysed by PCR using primers for the HPV-16 URR and E7. As a control (input) 20 µg chromatin was de-cross-linked and this 1:100 diluted DNA was also used for PCR with HPV-16 URR and E7 primers. Input: control: DNA without immunoprecipitation from the same sample, IgG: control: immunoprecipitation with an unspecific antibody, Myc: immunoprecipitaion with the Myc-specific antibody.

live cells with formaldehyde (Orlando et al., 1997). Thereby, C-c3:H3.3 cells treated with DAC and untreated controls were used.

After cross-linking, the cells were lysed and subsequently the crude extracts were sonicated for shearing the DNA. DNA fragments up to 500 bp length were prepared. Myc-H3.3 histones cross-linked to DNA were immunoprecipitated by an antibody recognizing the Myc-tagged part of the H3.3. Subsequently, the cross-linking in the presence of high salt was

reversed by incubation of the samples at 65°C over night. The purified DNA fragments were amplified by PCR using the HPV-16 URR1.1, URR1.2 and E7 primers.

DAC treatment of C-c3:H3.3 cells induced a strong increase of incorporated Myc-H3.3 into demethylated HPV genomes. The histone variants were detected in the URR region of the HPV-16. Comparison of the IgG control samples to the Myc-IP samples after DAC treatment unravels a clear incorporation of Myc-H3.3 to both the URR1.1 and URR1.2 sequences. In control samples, Myc-H3.3, is incorporated only at lower levels (Figure 4.19). Interestingly, no incorporation was detectable for the E7 sequence. These results imply that Myc-H3.3 is predominantly incorporated into the HPV genome region that is important for transcriptional regulation (URR). The coding regions, in this case of E7, lack Myc-H3.3.

Consequently, the inclusion of Myc-H3.3 in the URR of HPV-16 after DAC treatment in C-c3:H3.3 cells might induce the transcription of the oncogene E7. Therefore, E7 expression in C-c3:H3.3 cells after DAC treatment was analysed.

4.6.2 Expression of the histone variant Myc-H3.3 in CaSki cells increases E7 transcription after DAC treatment

ChIP experiments with the stable CaSki clone:C-c3:H3.3 showed, that Myc-H3.3 is incorporated after DAC treatment in the HPV-16 URR. This incorporation may influence the transcriptional activity of demethylated HPV-16 copies. Gene activation was expected, since H3.3 is known to carry N-terminal modifications, which correlate with actively transcribed regions (Ahmand et al., 2002; McKittrick et al., 2004; Johnson et al., 2004; Chow et al., 2005).

In order to verify that H3.3 incorporation in the HPV-16 URR after DAC treatment indeed leads to an up-regulation of E7 transcription, Northern blot and quantitative PCR experiments were performed with both, parental CaSki cells and Myc-H3.3 expressing cells: C-c3:H3.3. A decrease of E7 expression was detected in CaSki cells after DAC treatment, as was shown in previous RT-PCR and qPCR data (Figure 4.12). In contrast, DAC treatment of C-c3:H3.3 cells leads to an up-regulation of E7. This was detected by qPCR and Northern blot. The qPCR data revealed approximately three fold induction of the oncogene E7 expression in DAC treated compared to untreated clones. Analysis by Northern blot, using the whole HPV-16 genome for hybridisation, further verified the observed up-regulation.

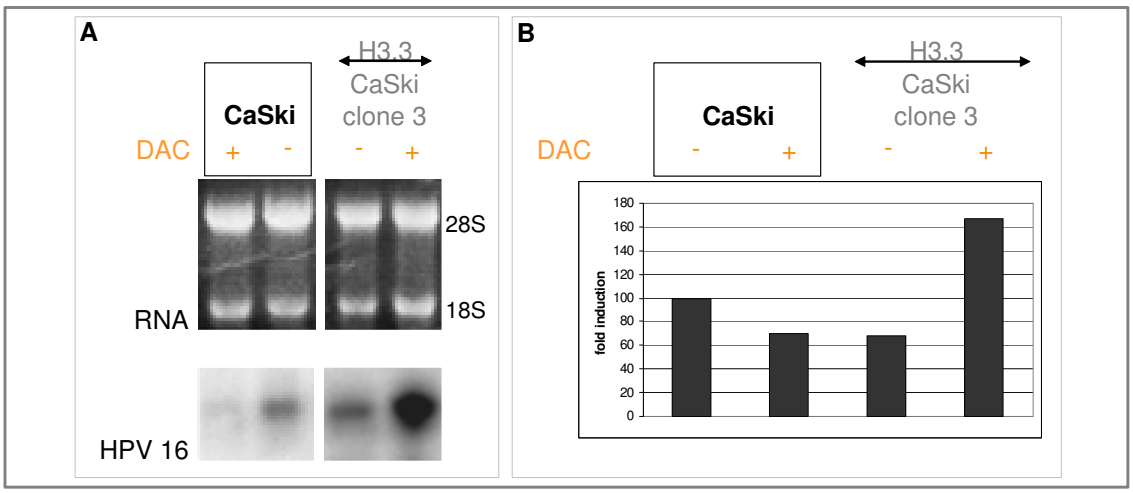

***Figure 4.20* Expression of the histone variant Myc-H3.3 in CaSki cells increases the E7 transcription after DAC treatment.**
CaSki cells stable clones expressing Myc-H3.3 were cultivated for 5 days in the presence or absence (control) of 5 µM DAC. (A) Northern Blot with radio labelled HPV-16 specific probe. (B) Q-PCR after RT-reaction with 1 µg RNA (in 20 µl volume) and random primers followed by PCR using 1 µl from the RT-reaction and HPV-16E7 and GAPDH primers. The results were normalized to GAPDH housekeeping amplification.

(Figure 4.20). These results, from two different experimental approaches qPCR and Northern blot, showed that up-regulation of E7 in CaSki depends on the interplay of two epigenetic mechanisms. Demethylation of the HPV genomes, and incorporation of H3.3, both are required for HPV-16 transcriptional activation.

5 Discussion

5.1 Effects of DAC treatment in CaSki cells at protein level

5.1.1 Treatment of CaSki cells with DAC induces demethylation of the HPV-16 URR

In order to investigate the mechanisms and factors involved in the re-activation of inserted HPV genomes during progression to cervical cancer, we used the cervical carcinoma cell line CaSki, which contains approximately 600 integrated HPV-16 genomes (Mincheva et al., 1987). Besides one locus on chromosome 14, where nascent HPV RNA was detected by tyramide-FISH, the other HPV copies are transcriptionally silenced by methylation (Van Tine et al., 2001). Therefore, DNA demethylation of the silent HPV-16 copies by DAC may mimic demethylation observed during progression to cervical cancer and provides an opportunity for the analysis of the epigenetic factors involved in the regulation of HPV-16 genomes.

The methylation status the of HPV genomes was assessed by digestion of the genomic DNA with the methylation sensitive enzyme HhaI and subsequent PCR with primers specific for the CpG site at the nucleotide 7674, which is located 40 bp upstream from two binding sites of the transcription factor AP-1 (Figure 4.3.). This site was of particular interest since it is located within the enhancer region of the HPV-16 URR. Analysis of demethylation demonstrated a decrease of DNA methylation after DAC treatment of approximately 50% compared to the untreated samples (Figure 4.3.). Besides this CpG site the HPV-16 genome possesses in its URR altogether 11 CpG sites (Figure 4.1.). Previous analysis of DNA methylation of the HPV-16 URR by bisulphite treatment revealed that these CpG sites in CaSki cells are heavily methylated (Kalantari et al., 2004). In 2006, Rajeevan et al. performed a more sensitive analysis by pyrosequencing. This method enables the direct quantification of DNA methylation. This approach demonstrated that there are differences between the methylation status of each CpG. While the CpGs in the promoter region are methylated in 96% of the integrated copies in CaSki cells, the specific CpG at the nucleotide 7674, which was analysed in our experimental setting, was methylated in 75% of the viral copies (Rajeevan et al., 2006).

5.1.2 DAC treatment in CaSki cells induces the down-regulation of the oncoprotein E7, which is mediated by calpains and proteasomal pathway

Treatment of CaSki cells with DAC induced demethylation of HPV-16 copies (Figure 4.3). DNA demethylation has been shown to re-induce transcription of silent genes (Magdinier et al., 2000; van der Velden et al., 2001). Hence, for detection of transcriptional re-activation, previous studies have focused on the detection of induced expression on mRNA level. Nevertheless, the actual amount of E7 oncoprotein after DAC treatment has never been analysed.

Surprisingly, in the case of CaSki cells DAC treatment leads to a down-regulation of the E7 oncoprotein expression (Figure 4.4). Analysis of the mRNA amount revealed only a slight down-regulation after DAC treatment which cannot, however, fully account for the observed down-regulation on protein level (Figure 4.12). Hence, other mechanisms must be involved. This leads to the assumption that different effects are responsible for the E7 down-regulation on protein level. One possible explanation for the E7 down-regulation is the involvement of previously methylated miRNA. In fact specific methylation of miRNA genes in tumor cells has been described recently (Saito et al., 2006). According to this report, DAC treatment led to an up-regulation of 17 out of 313 miRNAs. Hence, DAC treatment could have changed the miRNA expression pattern in CaSki cells. The new expressed miRNA may recognize E6/E7 mRNA in a not completely homologous manner, leading to translational inhibition and E7 protein down-regulation. However, also additional mechanisms may be involved in this process, because treatment of CaSki cells with DAC in presence of proteasomal and calpain inhibitor MG132 rescued E7 from degradation (Figure 4.4). Two different proteins have been described in the literature as involved in E7 proteasomal degradation: the ubiquitin ligase Skp2 (Oh et al., 2005) and, after IFN-γ induction, SOCS-1 (Kamio et al., 2004). SOCS-1 has been described to be methylated in 50% of cervical carcinomas (Widschwendter et al., 2004). However, no increase of SOCS-1 in CaSki cells after DAC treatment was observed. These experiments imply that Skp2 or other proteins might be involved in the E7 proteasomal degradation after DAC treatment (Kamio et al., 2004). In order to unravel new E7 interaction partners, mass spectrometry analyses were carried out after immunoprecipititaion with a highly specific mouse monoclonal E7 antibody (Figure 4.7.). Using this approach, calpains were identified as new potential interaction

partners (Figure 4.8.). While the ubiquitous calpain 1 was co-immunoprecipitated with E7 in both DAC treated and untreated samples, calpain 12 was only detected after DAC treatment. E7 has already been described to harbor a (Pro, Glu, Ser/Thr-rich) PEST domain, which is necessary for the interaction with IRF-9 (Antonsson et al., 2006). Since calpains are known to recognize their substrates by PEST domains as well as other regions (Saez et al., 2006), it was reasonable to assume that E7 could interact with calpains leading to its degradation. Although MG132 is a strong inhibitor of the proteasome, it olso blocks calpains. This suggests that calpains may be involved. To definitely clarify whether E7 is also degraded by calpains CaSki cells were treated with the specific calpain inhibitor ALLM. Under this conditions an accumulation of E7 was observed in the DAC exposed cells (Figure 4.9.), supporting the assumption that calpains are involved in E7 degradation. Nevertheless, inhibition of E7 degradation was not as efficient as with MG132, indicating that both pathways may act together in this process. This is in agreement with a recent publication, where calpains can interact with E7 and thus contribute to E7 mediated pRb degradation (Suhrbier et al., 2007).

5.1.3 E7 degradation after DAC treatment is also induced in HeLa and somatic hybrid cell lines

Treatment with DAC did not change the E7 levels in SiHa cells, but led to a down-regulation of the E7 oncoprotein in CaSki cells. In order to clarify, if this CaSki related phenotype is linked to a dominant or recessive genotype, hybrid cell lines were analysed. Furthermore, using these hybrids, it was possible to determine, if the observed degradation is specific only for HPV-16. Since CaSki cells have been established from a small intestine metastasis (Pattillo et al., 1977), these cells might harbor a specific genotype, which provides a background for the observed E7 down-regulation. To further investigate this hypothesis HeLa cells and somatic cell hybrids (Figure 4.5) were incubated with DAC. Subsequent Western blots revealed E7 protein down-regulation after DAC treatment in both parental lines and all somatic hybrid lines derived thereof (Figure 4.6). Interestingly, in CaSki X SiHa hybrids E7 was also degraded after DAC treatment suggesting that the CaSki genotype is dominant (Figure 4.6). Hence, these experiments demonstrate that the DAC induced E7 degradation is a dominant mechanism and is independent of the virus type, since HPV-18E7 was also down-regulated in HPV-18 positive HeLa cells after DAC treatment (Figure 4.6).

5.2 Identification of a novel interaction partner of E7: CARM1

Besides the E7-calpain interaction, mass spectrometery (MS), also provided evidence that E7 can associate with CARM1 (Figure 4.8). E7 is known to interact with a variety of proteins in order to alter cellular mechanisms for ensuring progression through the cell cycle and proliferation (Figure 2.4). So far, the best studied interaction partner is pRb (Dyson et al., 1998) but interactions with other proteins such as the transcription factor c-Jun (Antinore et al., 1996), Mi2 (Brehm et al., 1999), a member of the NURD complex and histone deacethylases (HDACs) (Brehm et al., 1999), have also been described.

In order to further validate the MS results endogenous E7-CARM1 interaction was verified by immunoprecipitating E7 and probing against CARM1 by Western blot. Following the E7-IP, a distinct band migrating near the heavy chain of E7 antibody was visualized corresponding to the size expected for CARM1 (Figure 4.10). This result was reproducible strengthening the conclusion that the identified interaction between endogenous E7 and CARM1 proteins is valid.

Nevertheless, the functional role of the E7-CARM1 interaction needs to be determined. CARM1 is a strong activator of transcription (Bauer et al., 2003, El Messaoudi et al., 2006). This is due to its ability to methylate Lsy17 on histone H3 (Bauer et al., 2003). CARM1 co-activates several genes like cycline E1 (El Messaoudi et al., 2006) and nuclear receptor regulated genes (Chen et al., 1999). In addition to gene activation, a crosstalk between CBP histone acetyltransferase transcription factors and CARM1 was reported (Daujat et al., 2002). Besides chromatin modification, CARM1 is also involved in methylation with concomitant activation and stabilisation of proteins such as the transcriptional coactivator p/CIP (Naeem et al., 2006). The E7-CARM1 interaction might lead to recruitment of CARM1 to E7 regulated genes, for example Cdc25, resulting in their activation. Of particular interest is the idea that E7 might recruit CARM1 to the HPV enhancer for inducing its own transcription. This hypothesis can be analysed by ChIP for the CARM1 substrate Lys17me with primers specific for the HPV enhancer. Interestingly CARM1 is down-regulated after DAC treatment of CaSki cells (Figure 4.11). This may explain the slight E7 down-regulation at transcriptional level after DAC treatment (Figure 4.12).

5.3 Effects of DAC treatment at transcriptional level in CaSki cells

5.3.1 Treatment with DAC induces a slight reduction of HPV-16E7 transcription

Analysis of the E7 oncoprotein expression on protein level showed that E7 is down-regulated after DAC treatment of CaSki cells. Additional analysis of the E7 transcription also revealed a slight reduction after DAC treatment in CaSki cells on mRNA level (Figure 4.12.). This was an unforeseen result, since the enhancer region of at least half of the HPV genomes was demethylated and therefore re-activation of viral transcription was expected. In accordance with our data a recently published report described only a minimal induction of E7 transcription after DNA demethylation by hydralyzine, in CaSki cells. Furthermore, analysis of E7 transcription in cervical cancer patients treated with hydralazine revealed a down-regulation of the oncogene expression (de la Cruz-Hernández et al., 2007). This was also observed in the somatic cell hybrids and parental HeLa cells (Fig 3.17).

In order to investigate the reason for lack of viral transcriptional induction, the AP-1 composition in CaSki and SiHa cells after DAC treatment was analysed. AP-1 is an essential transcription factor for the initiation of HPV transcription (Butz and Hoppe-Seyler, 1993). Here the composition of the AP-1 family members is known to play a crucial role for the induction of HPV transcription. c-Jun/c-Fos are known as strong activators, in contrast to c-Jun/Fra-1 dimers (Rösl et al., 1997; Soto et al., 1999). Three different AP-1 binding sites are present in the upstream regulatory region (URR) of the HPV-16. Interestingly, monitoring AP-1 expression by RT-PCR and Western blot, a strong up-regulation of c-Jun, Fra-1 and JunD was detected in CaSki, but not in SiHa cells after DAC treatment (Figure 4.13). In addition, expression of c-Fos, the activator of HPV transcription, could still not be detected in CaSki cells. To further characterize the AP-1 composition in CaSki cells after DAC treatment EMSA experiments were carried out. The results revealed that the predominant AP-1 complexes in CaSki cells consist of c-Jun/Fra-1 dimers, which bind both to AP-1 consensus and HPV-16AP-1 sequences (Figures 3.15/3.16). This AP-1 composition has been shown to attenuate AP-1 activity leading to a reduction in the transcriptional activity (Soto et al., 1999). Therefore, this kind of AP-1 complex may account for the absence of gene induction, leading even to a slight down-regulation in the E6/E7 transcription. The observed increase of c-Jun, JunD and Fra-1 in DAC treated CaSki cells was not due to activation of the stress signalling pathway mediated by JNK or p38, since no phophorylation of these MAPK could be detected

(Figure 4.14.). However, the up-regulation may be due to a positive feedback loop since Fra-1 and c-Jun harbour AP-1 sites in their enhancer regions (Angel et al., 1988, Adiseshaiah et al., 2005). Another interesting possibility is the induced up-regulation of these AP-1 family members by demethylation. The transcriptional initiation regions of all three up-regulated genes harbor a high number of CpG sites. In normal cells, these CpGs are unmethylated, ensuring the typical rapid transcriptional initiation of immediate-early genes. CaSki cells are polyploid harbouring 3 copies of chromosomes 1 and 11 where the genes for *c-jun* and *Fra-1*, respectively are located (Jesudasan et al., 1995). Therefore, it would be interesting to find out if these additional chromosomes are transcriptionally active or if the additional *c-jun* or *fra-1* genes are silenced by methylation. In the latter case treatment with DAC might induce gene re-expression leading to up-regulation of the proteins.

5.4 Demethylation of the HPV genomes serves as a prerequisite for transcriptional activation, but additionally requires the incorporation of H3.3 into the URR of HPV-16

Chromatin modifications are essential epigenetic marks for transcriptional control. (Kouzarides, 2007). In the last few years another level of regulation has become evident, namely the importance of histone variants in the acquisition of different histone marks. The histone variants H3.1 and H3.3 have been the particular focus of investigation (Ahmand et al., 2002; McKittrick et al., 2004; Johnson et al., 2004). In order to analyse the influence of these histone variants in the transcriptional regulation of HPV genomes, stable cell lines from CaSki cells expressing Myc-tagged H3.1 or H3.3 have been generated and analysed (Figure 4.18). This experimental approach provides the first demonstration that in CaSki cells, despite demethylation of HPV genomes by DAC, the incorporation of Myc-H3.3 into the nucleosomes of HPV-16 URR was necessary for re-activation of silent HPV copies. Only in combination these epigenetic changes, led to a strong activation of the HPV-16E7 expression (Fig. 3.20).

Three major differences have been characterized between the histone 3 variants H3.1 and H3.3. Firstly, H3.3 is incorporated into nuclesomes at transcriptionally active regions and is thought to exchange H3.1 at these sites after gene activation (McKittrick et al., 2004, Hake and Allis, 2006). Secondly, H3.3 is incorporated throughout the cell cycle in contrast to H3.1 which is strictly incorporated into chromatin only during replication (Ahmad and Henikoff

2002). Finally, the h3.3 gene is expressed throughout the cell cycle, whereas h3.1 is expressed only during the DNA synthesis phase (Sarma and Reinberg, 2005). Previous studies have concluded that H3.3 incorporation at active loci not only marks transcriptionally active genes, but deposition of H3.3 might maintain cellular memory of active transcription states during mitosis (McKittrick et al., 2004). Interestingly, euchromatin is synthesised early in the S-phase in contrast to heterochromatin which is replicated late (Gilbert, 2001; Gilbert, 2002). Furthermore, due to the delay in synthesis there is more H3.3 than H3.1, at the beginning of the S-phase where the euchromatin is replicating. Hence, more H3.3 is incorporated into the nucleosomes whereas predominantly H3.1 is incorporated during the late S-phase, when the heterochromatin is duplicated (Ahmad et al., 2002).

In order to analyse the Myc-H3.3 expression in the stable cell lines, histones were isolated and the amount of Myc-H3.3 was detected by Western blot using an anti-Myc antibody (Fig.3.18). This experiment revealed that not all stable CaSki clones expressed and incorporated the recombinant Myc-H3.3 in the absence of DAC. This was apparently due to the CMV promoter which drives the ectopic transcription of Myc-H3.1 or Myc-H3.3 and is known to potentially become silenced by methylation when inserted in the host genome. The experiments presented here were carried out with the Myc-H3.3 clone3 from CaSki cells, which expresses the tagged histone in the absence of DAC. This clone does not differ in its E7 expression levels from the CaSki parental cell line. This implicates that the expression of Myc-H3.3 alone is not sufficient for re-activation of the silenced HPV-16 genomes in CaSki cells (Fig 3. 20).

After quantification of the Myc-H3.3 amount in the CaSki cell clone 3 the incorporation of the Myc-H3.3 into the HPV genomes was analysed by ChIP. Interestingly, the recombinant Myc-H3.3 protein is only minimally incorporated into the chromatin in methylated HPV genomes in the absence of DAC (Fig. 3.19.). This might be due to the late replication of the methylated genomes, which implies preferential H3.1 incorporation (Hake and Allis, 2006). In addition, the methylated HPV genomes should have a close chromatin structure, which did not favour histone exchange.

For monitoring the effects of DNA demethylation in addition to Myc-H3.3 expression, the Myc-H3.3 clone 3 from CaSki cells was treated with DAC. Analysis of E7 transcription revealed that E7 mRNA was up-regulated after treatment with DAC (Figure 4.20.). Subsequent ChIP analysis for detection of incorporation of Myc-H3.3 at the promoter and enhancer regions of the HPV genomes showed an enhanced incorporation of the variant after DAC treatment compared to the untreated clone cells (Figure 4.19.). Taking together, these

experiments suggest that DAC treatment alone was not sufficient for re-activation of the silent HPV genomes. However, in combination with stable expression of the histone Myc-H3.3, gene silencing can be reverted and the HPV copies obtain the transcriptionally active state. The opening of the chromatin by demethylation favours Myc-H3.3 incorporation at the promoter and enhancer region. Hence, the particular combination of DNA demethylation and incorporation of a histone variant were able to establish a chromatin state which lacks repressive marks leading to the re-expression of the silent HPV copies in Myc-H3.3 clones. Both epigenetic marks, histone modifications and DNA methylation, are strongly influenced by and tightly regulate each other (Fahrner et al., 2002). Experiments in *Arabidopsis thaliana* have shown that a decrease in DNA methylation, as observed after DAC treatment in CaSki cells, does not directly cause loss of repressive marks, such as histone 3 lysine 9 methylation (H3K9me). Whereas reactivation of transcription is accompanied by loss of the H3K9 marks (Johnson et al., 2002). However, another study in bladder cancer cells reported a regional induction of chromatin remodelling after DAC treatment (Nguyen et al., 2002). The results of these studies, combined with the results presented here, lead to the proposal that although DNA methylation and chromatin remodelling influence each other, the outcome for a gene reactivation depends on the particular structure of the heterochromatin. Tumor-suppressor genes can be silenced in cancer, but they are usually located in euchromatin regions, which become silent by DNA methylation predominately at the promoters of the respective genes and are then referred to as facultative heterochromatin regions (Ehrlich, 2002; McGarvey et al., 2006). These regions can be reversed by DNA demethylation relatively easier than regions which are incorporated in late replicating heterochromatin or constitutive heterochromatin (Shafer and Priest, 1984). Our data suggest that the silent HPV genomes in CaSki cells could be predominantly located within late replicating highly condensed constitutive heterochromatin, which needs both DNA demethylation and repressive histone marks remission for its reactivation.

In conclusion, the results presented here can be summarized by the following model Figure 5.1): The silent HPV genomes in CaSki cells are integrated in late replicating constitutive heterochromatin domains. Therefore DNA demethylation by DAC is not sufficient for altering the histone repressive marks, which in turn does not allow the re-expression of the HPV-16 genomes. Furthermore, the up-regulation of the AP-1 family members such as Fra-1, c-Jun and JunD after DAC treatment leads to an accumulation and enhanced binding of AP-1 complexes, which conteract transcription from the already active HPV genomes. This may lead to a slight down-regulation of the E7 expression at mRNA

level. In contrast, the expression of Myc-H3.3 in CaSki clone 3 in the absence of DAC, led to a very inefficient incorporation of Myc-H3.3. Since under these conditions the repressive chromatin state generated by methylation and remaining histone marks. Upon demethylation of the HPV genomes by DAC and the enhanced exchange of Myc-H3.3 the repressive marks are significantly reduced. The incorporation of Myc-H3.3 recruits transcription initiation complexes. This leads to transcriptional initiation, which subsequently induces a positive feed-back loop leading to an enhanced incorporation of Myc-H3.3 which in turn harbours positive marks, and therefore increases transcriptional activity (Figure 5.1).

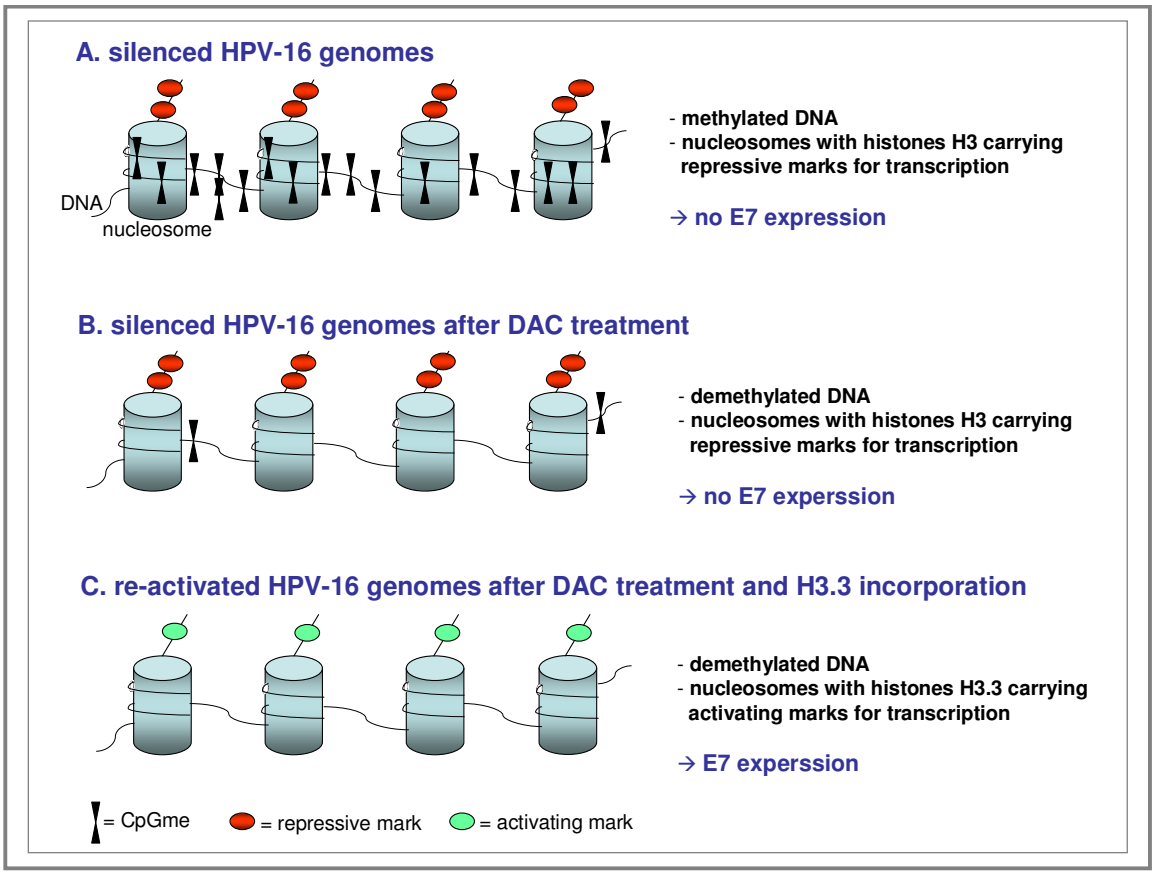

Figure 5.1 ***Schematic overview of the observed mechanism for the re-activation of silenced HPV-16 genomes***

CaSki cells harbour silenced HPV-16 genomes, with methylated DNA and histones (H3) with negative marks for repression of transcription. After DAC treatment, the DNA is demethylated but the nucleosomes still harbour histones with repressive marks. In CaSki clones expressing H3.3, this histone variant, is incorporated into the HPV URR after DAC treatment. Now the histones (Myc-H3.3) harbour positive marks and the transcritption of E7 is induced, leading to the up-regulation of E7 mRNA.

6 Abbreviations and Reference List

6.1 Abbreviations

5-azycytidine	AC
N-acetyl-leucyl-leucyl-methionine, calpain inhibitor	ALLM
activator protein 1	AP-1
activating transcription factor	ATF
antioxidant-response elements	AREs
basic leucin zipper domain	bZIP
breast cancer 1	BRCA1
Chromatin Immunoprecipitations	ChIP
cAMP-respose element	CRE
chromatin assembly factor	CAF-1
CaSki clone 3 expressing Myc-H3.3	C-c3:H3.3
coactivator-associated arginine methyltransferase 1	CARM-19
conserved region	CR
cytosines located 5´ to guanosine	CpGs
colony stimulating factor-1 receptor	CSFR
5-aza-2'-deoxycytidine	DAC
DNA methyltranferase1	DNMT1.
extracellular-regulated kinase	ERK
epidermal growth factor receptor	EGFR
electrophoretic mobility shift assays	EMSA
immediate early genes	IE
immunoprecipitation	IP
folate receptor	FR
glucocorticoid receptor	GR
heterochromatin associated protein 1	HP1
Histone acetyltransferases	HAT
Histone deacetylases	HDAC
H3 lysine 9 acetylation	H3K9ac
H3 lysine 36 methylation	H3K36me
H3 lysine 79 methylation	H3K79me

H3 lysine 9 methylation	H3K9me
phosphorylation of H3 at serine 10	H3S10phos
histone methyltransferase	HMT
Histone variant 3.3	H3.3
Histone 3.1	H3.1
Human papilloma viruses	HPV
human immunodeficiency virus	HIV
histone cell cycle regulation defective homolog A	HIRA
c-Jun N-terminal kinase	JNK
Mixed-lineage leukaemia	MLL1
methyl-CpG-binding –protein 2	MeCP2
MAF recognition elements	MAREs
Mitogen activated protein kinases	MAPK
Mass spectrometry	MS
MAP-Kinase-Kinase-Kinases	MAPKKK
MAP-Kinase-Kinases	MAPKK
musculoaponeurotic fibrosarcoma	MAF
proteasomal and calpain inhibitor	MG132
Recombinant Myc tagged Histone H3.3	Myc-H3.3
nuclear factor 1	NF1
octamer binding factor 1	OCT1
Polymerase chain reaction	PCR
antioxidant pyrrolidine-dithiocarbamate	PDTC
Papilloma viruses	PV
(Pro, Glu, Ser/Thr-rich) domain	PEST
plant homeo domain	PHD
quantitative PCR	qPCR
replication dependent	RD
retinoblastoma protein 1	pRb
Reverse Transcription	RT
Enhancer-of-zeste E(z), Trithorax (Trx)	SET
suppressor of cytokine signaling	SOCS-1
Suppressor of variegation 3-9	Su(var)3-9
steroidogenic factor 1	SF1

tumor necrosis factor alpha	TNF-alpha
12-O-tetradecanoylphorbol-13-acetate	TPA
TPA response element	TRE
trithorax	Trx
transcription enhancer factor 1	TF1
TATA-box binding protein	TBP
upstream regulatory region	URR
Vascular Endothelial Growth Factor-D	VEGF-D
Western blot	WB
Yin Yang 1	YY1

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