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UNIVERSITY OF TARTU
Press

Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

Dissertation was accepted for the commencement of the degree of Doctor of Philosophy in biomedical technology on November 9th, 2016 by the Council of the Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia.

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Commencement: Auditorium 121, Nooruse 1, Tartu, Estonia, at 10.15 on December 12th, 2016

Publication of this thesis is granted by the Institute of Technology, Faculty of Science and Technology, University of Tartu.

ISSN 2228-0855
ISBN 978-9949-77-288-9 (print)
ISBN 978-9949-77-289-6 (pdf)

University of Tartu Press
www.tyk.ee

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LIST OF ORIGINAL PUBLICATIONS

The current dissertation is based on the following publications referred to in the text by their Roman numbers:

I. Reet Kurg, **Piia Uusen**, Toomas Sepp, Mari Sepp, Aare Abroi, Mart Ustav. (2009) Bovine papillomavirus type 1 E2 protein heterodimer is functional in papillomavirus DNA replication in vivo. *Virology* 386(2):353–359.

II. Reet Kurg, **Piia Uusen**, Liisi Võsa, Mart Ustav. (2010) Human papillomavirus E2 protein with single activation domain initiates HPV18 genome replication, but is not sufficient for long-term maintenance of virus genome. *Virology* 408(2):159–166.

III. **Piia Kivipõld***, Liisi Võsa*, Mart Ustav and Reet Kurg. (2015) DAXX modulates human papillomavirus early gene expression and genome replication in U2OS cells. *Virology Journal* 12:104.
*Authors contributed equally to this work

My contribution to the papers is as follows:

Ref. I – I performed all the experiments involving the single-chain E2 heterodimer protein, analyzed the data and participated in writing the manuscript.

Ref. II – I participated in the experimental design, did all of the experiments, except for the DNA binding assay and replication assay of HPV18/E8$^-$ genomes, analyzed the data and wrote parts of the manuscript.

Ref. III – I designed, performed and analyzed the data of experiments determining the effect of DAXX down-regulation on HPV18 and HPV11 transcription and replication, and wrote parts of the paper.

LIST OF ABBREVIATIONS

ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
ATRX	X-linked mental retardation and α-thalassaemia syndrome protein
ASV	avian sarcoma virus
BPV1	bovine papillomavirus type 1
Brd4	bromodomain-containing protein 4
CBP	cAMP response element-binding (CREB) protein-binding protein
CENP-C	centromere protein C
Chk2	checkpoint kinase 2
co	codon-optimization
DAXX	death domain-associated protein
DBD	dimerization and DNA binding domain
DDR	DNA damage response
DNMT	DNA methyltransferase
E1BS	E1 binding site
E2BS	E2 binding site
E6-AP	E6-associated protein
EBV	Epstein Barr virus
EP400	E1A-binding protein P400
FISH	fluorescent *in situ* hybridization
hAd5	human adenovirus type 5
HCMV	human cytomegalovirus
HDAC	histone deacetylase
HIPK2	homeodomain-interacting protein kinase 2
HIV-1	human immunodeficiency virus type 1
HLA	human leukocyte antigen
hNAP1	human nucleosome assembly protein 1
HPV	human papillomavirus
HSV-1	herpes simplex virus type 1
IFN	interferon
JARID1C	Jumonji/ARID domain-containing protein 1C
MIEP	major immediate early promoter
MKlp2	mitotic kinesin-like protein 2
MME	minichromosome maintenance element
MNR	complex consisting of Mre11, Rad50 and Nbs1
NCoR1	nuclear receptor co-repressor 1
ND10	nuclear domain 10
NF-κB	nuclear factor-κB
ORF	open reading frame

p/CAF	p300/CREB-binding protein-associated factor
Pax3	paired box protein 3
PCNA	proliferating cell nuclear antigen
PDZ	post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PML	promyelocytic leukemia protein
pRb	retinoblastoma tumor suppressor protein
RFC	replication factor C
RNAi	RNA interference
RPA	replication protein A
scE2	single-chain E2 heterodimer
siRNA	small interfering RNA
Sp1	specificity protein 1
SP100	speckled protein of 100 kDa
SUMO-1	small ubiquitin-related modifier 1
SV40	Simian virus 40
SWI2/SNF2	Switch/Sucrose non-fermentable
TAD	transactivation domain
TBP	TATA binding protein
TFIIB	transcription factor II B
TopBP1	topoisomerase II β-binding protein 1
URR	upstream regulatory region
VLP	virus like particle

1. INTRODUCTION

Human papillomaviruses (HPVs) are highly prevalent pathogens of tremendous medical importance due to their association with several human cancers and because they are the most common sexually transmitted pathogens. The accumulation of scientific and epidemiologic evidence dating back to the early 1970s implicates an association between HPV infection and the development of cervical and other cancers. Professor Harald zur Hausen was awarded the Nobel Prize for Medicine or Physiology in 2008 for establishing HPV as the principal factor responsible for cervical cancer. Today, cervical cancer is the second most common cancer in women worldwide with approximately half a million cases per year worldwide, with approximately half of these being fatal. The available HPV vaccines effectively protect against new HPV infection, however, they are ineffective at eliminating ongoing infections. Rapidly accelerating advance in knowledge has increased our understanding of the biology of HPV and host responses to infection in considerable molecular detail. Nevertheless, there is no virus-specific treatment currently available. Thus, there is still a need for ongoing biomedical research into HPVs and their associated diseases to lead to the development of better strategies for disease treatment, which are necessary to complement current methods of disease management.

Papillomaviruses infect the epithelial cells of skin or mucosa of vertebrates, where they replicate their genomes as extrachromosomal elements. In order to support papillomavirus DNA replication, the virus encodes two early proteins, E1 and E2, all other replication proteins and enzymes are provided by the host cell. The viral helicase E1 is the primary replication initiator protein that functions in concert with the E2 protein. In addition to its role in viral DNA replication, the E2 protein is also required for proper partitioning of viral genomes in dividing cells to establish persistent infections, and can regulate transcription of viral genes, thus serving as the master regulator of papillomavirus life cycle. The virus also encodes truncated versions of the E2 protein which serve to regulate the function of the full-length E2 protein. The first part of my studies that formed the basis of this dissertation was focused on studying the functional activities of E2 heterodimers consisting of the full-length and truncated E2 proteins. Specifically, I determined the cellular localization and the replicative activity of the E2 heterodimer protein by using the bovine papillomavirus type 1 (BPV1) as a model, and studied the functions of E2 heterodimers of HPV18 and 11 in viral replication and regulation of papillomavirus early genes.

The incoming genomes of many DNA viruses are subjected to specific nuclear structures called the nuclear domain 10 (ND10) wherein they initiate their replicative program. The E1 and E2 proteins of HPV have also been shown to replicate viral genomes in replication centers that are formed in close association to ND10. In the second part of this thesis, I studied the HPV replication compartments in relation to ND10-associated proteins PML and DAXX, and the effect of the DAXX protein on viral early gene expression and replication of viral genomes in HPV replication permissive human osteosarcoma cell line U2OS, which serves as an useful tool to study different aspects of the HPV life cycle in a cost-effective manner.

2. LITERATURE REVIEW

2.1. General introduction to papillomaviruses

Papillomaviruses are small non-enveloped double-stranded DNA viruses that belong to the family *Papillomaviridae* and infect the epithelial tissue of a wide variety of vertebrates. The viruses are species-specific and tissue-tropic, with a predilection for infection of either cutaneous or internal mucosal epithelium. Papillomavirus infections are very common and mostly asymptomatic. Active viral infections lead to epithelial hyperproliferation, which varies in severity depending on the site of infection and the virus type involved, and are frequently cleared by the immune system in less than a year or two.

Papillomaviruses are classified by genotype (Bernard et al., 2010) and, according to the Papillomavirus Episteme database, more than 200 HPV types have been identified to date by sequencing the gene encoding the major capsid protein L1. HPV genotypes that infect anogenital epithelium belong to subgroup A (alpha-papillomaviruses) and they are divided into low-risk and high-risk categories based on the spectrum of lesions they are associated with and the potential of these lesions to progress to cancer. The low-risk types, such as type 6 and type 11, cause the majority of genital warts and virtually all laryngeal papillomatosis (Lacey, 2005). Persistent infections in the anogenital tracts by certain high-risk HPV genotypes, such as type 16 and type 18 and other closely related types, can at low frequency progress to high grade dysplasias and carcinomas in men and women, including cervical, vulvar, vaginal, penile and anal cancers. Virtually 100% of cervical cancers contain the high-risk genital HPV DNA sequences, with HPV16 found in ~50% of cases being the most important player (Walboomers et al., 1999). HPV16 also causes a subset of head and neck cancers (Gillison and Lowy, 2004). As anogenital HPV infections are one of the most common sexually transmitted diseases, the mucosotropic alpha-HPV types have commanded the attention of most of the basic research efforts and clinical translation into vaccine development, patient screening, and therapeutic strategies.

There is presently no cure for HPV, and prevention of HPV infection through vaccination is the most effective means of reducing the global burden of HPV-related diseases. Available prophylactic vaccines are based on virus-like particles (VLPs) consisting only of the major viral capsid protein, L1, of HPV. The quadrivalent HPV6, 11, 16 and 18 recombinant VLP vaccine (4vHPV), Gardasil®, was designed by Merck to protect against HPV16 and 18 that cause ~70% of cervical cancers and HPV6 and 11 that cause ~90% of genital warts. The other available vaccine is bivalent HPV16 and 18 vaccine (2vHPV) Cervavix by GlaxoSmithKline. In order to broaden the coverage against additional HPV types that cause cervical cancer, a next generation vaccine has been developed. The nine-valent HPV VLP vaccine (9vHPV), Gardasil 9, contains HPV31, 33, 45, 52 and 58 VLPs combined with the quadrivalent VLP vaccine HPV types (Bryan et al., 2016). The recently approved HPV vaccines, however, have several limitations, including incomplete coverage of high-risk HPV genotypes, high cost and limited availability in developing countries, which account for 80% of

the deaths due to cervical cancer. In addition, they cannot cure the millions of people that are already infected. Therefore, there is still an urgent need to understand the oncogenicity of papillomaviruses in more detail and to identify other approaches to prevent HPV infections.

The BPV1 has been the best studied of the papillomaviruses at molecular level. BPV1 normally infects fibroblasts and epithelial cells inducing cutaneous fibropapillomas in cattle. Because it is capable of replicating in and transforming mouse C127 fibroblasts, in which the viral DNA is maintained as a stable multicopy extrachromosomal plasmid (Law et al., 1981), the BPV1 has been used extensively as a model for studies of papillomavirus replication in mammalian cells.

2.2. The papillomavirus life cycle

Papillomaviruses are non-enveloped viruses, whose protein shell consists of 360 molecules of the major capsid protein, L1, assembled into 72 pentamers (Baker et al., 1991b). The minor capsid protein, L2, requires the pentameric L1 structure for interaction and is present in up to 72 molecules in the viral capsid (Buck et al., 2008; Finnen et al., 2003). The viral capsid with a diameter of 50–55 nm encloses the circular double-stranded DNA genome of ~8kb in complex with cellular histones.

The productive life cycle of papillomaviruses takes place in the epithelial tissue that they infect and absolutely depends on the terminal differentiation process of keratinocytes in the squamous epithelium (Doorbar et al., 2012). In order to produce infectious particles that are eventually secreted from the epithelial tissue surface, timely and coordinated expression of different viral gene products is required as the infected cell moves towards the epithelial tissue surface (Fig. 1). The productive replication cycle, the time from infection to release of the virus, takes at least 3 weeks as this is the time needed for the keratinocyte to undergo complete differentiation and desquamate. At most epithelial sites papillomavirus infection requires epithelial wounding or microwounding for virus particles to gain access to the epithelial basal layer, which contains mitotically active cells in uninfected epidermis. The initial steps of the infectious process, however, take place on the basement membrane (Kines et al., 2009). Binding to heparin sulphate proteoglycans in the basement membrane induces a conformational change of the capsids and results in L2 cleavage. Following an additional conformational change after cleavage, the capsids are then transferred to the epithelial cell surface (Kines et al., 2009). It is thought that papillomaviruses have adapted their life cycle to the wound-healing process because the transfer occurs preferentially to the basal cells as they migrate over the basement membrane into the site of trauma. As these cells undergo cell division to re-establish the epithelial layering, infection will ensue. The identity of the basal cell surface bound receptor needed for virus entry is still unclear. Following cell surface binding and endocytosis, papillomaviruses are trafficked through the endosomal system that results in partial uncoating of the virus (Bienkowska-Haba et al., 2012). Eventually, the complex of the minor capsid

protein, L2, and the viral genome escape from endosomes, the complex is transported into the nucleus. Early stages of mitosis are needed for establishing papillomavirus infection (Pyeon et al., 2009), providing one reason why papillomaviruses infect only undifferentiated, proliferating basal cells. In the nucleus, the L2 and viral genome complex associate with nuclear substructures known as ND10 (Day et al., 1998; Florin et al., 2002b) where viral transcription and replication has been demonstrated to take place (Swindle et al., 1999). Activation of promoter that initiates expression of viral early proteins results in initiation of replication from viral origin of replication and production of approximately 20 to 100 extrachromosomal copies of the viral DNA per cell. The viral proteins E1 and E2 are directly involved in and essential for initial amplification replication phase (Chiang, Ustav 1992, Ustav and Stenlund 1991). All other enzymes and proteins needed for viral DNA replication are supplied by the host cells.

After initial replication, or establishment phase, the basal cells are driven to proliferate in order to produce a sheet of infected basal cells. As infected basal cells divide, the viral genomes are stably maintained in their nucleus as extrachromosomal replicating elements that replicate in synchrony with the host cellular DNA replication (McBride, 2008). After basal cell division, one of the daughter cells withdraws from the cell cycle, migrates away from the basal layer towards the stratum granulosum and becomes committed to differentiation (Fig. 1). The other daughter cell continues to divide in the basal layer thereby providing a reservoir of infected cells during the usually long-lived and persistent papillomavirus infection. The viral early proteins E6 and E7 are essential in driving cell proliferation after establishment phase as well as in stimulating cells to re-enter the cell cycle as they are being pushed towards the epithelial surface by the division of the cells beneath. High-risk and low-risk HPV types differ substantially in their ability to drive cell cycle entry and cell proliferation in the basal and parabasal cell layers. In the case of the high-risk types that cause neoplasia, the viral E6 and E7 proteins promote basal and parabasal cell division, and as the infected cells leave the basal layer, they remain active in the cell cycle. Low-risk HPV types do not massively stimulate basal cell proliferation, and induce cell cycle re-entry and genome amplification only in the upper epithelial layers, this being one of the reasons why the low-risk HPV types do not generally cause neoplasia. This difference is determined by the different abilities of the high- and low-risk E6 and E7 proteins to modulate the acitivity of proteins involved in tumor suppression and cell cycle regulation (Doorbar et al., 2012). The excessive cell cycling and interference with the DNA damage control functions in the basal compartment induced by persistent over-expression of high-risk E6 and E7 proteins can lead to accumulation of deleterious host gene mutations selected for survival and growth. At low frequency, neoplasia caused by persistent infection of the high-risk HPV types can progress to high-grade lesions and to carcinomas, where the viral DNA is often found integrated into host genomic sequences. This integration usually disrupts the E1 or E2 open reading frame (ORF) and results in loss of negative feedback regulation of the early promoter leading to unchecked and invariably highly elevated levels of E6 and E7 (Chow et al., 2010).

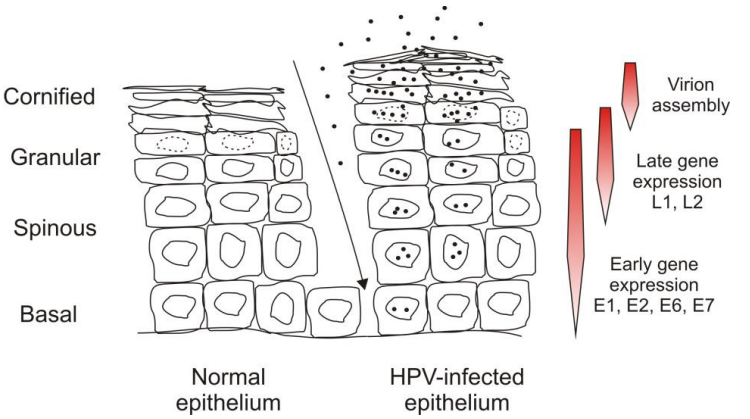

Figure 1. Schematic representation of the stratified squamous epithelium and viral gene expression throughout the life cycle of the papillomavirus.

During productive infection, the expression of E6 and E7 proteins in the differentiating compartment of the epithelium disrupts the normal differentiation process and allows the infected cell to re-enter S phase which re-establishes a replication-permissive milieu for viral genome amplification and packaging into infectious particles. Of key importance in the vegetative phase is the upregulation of the late promoter (located within the E7 gene) upon differentiation which leads to an increase in the levels of viral proteins necessary for replication, including E1, E2, E4 and E5 (Fig. 1) (Doorbar, 2007). Unlike the early promoter, the late promoter is not repressed by E2 protein at high concentrations, resulting in high levels of expression and triggering amplification of viral DNA (Steger and Corbach, 1997). The viral copy number rises to at least 1000 copies per cell. The papillomaviruses then switch to late gene expression, the expression of minor coat protein, L2, preceding the expression of major coat protein, L1 (Fig. 1). In addition to the virion structural proteins, the non-structural E2 protein is also thought to be required for viral genome packaging. For virus genome encapsidation, E2 protein recruits L2 to regions of replication at ND10 (Day et al., 1998). Virus assembly takes place when L1 capsomeres, pre-assembled in the cytoplasm, are translocated into the nucleus and recruited to ND10 by L2 (Florin et al., 2002a). While L1 can self-assemble into virus-like particles, L2 enhances their assembly and viral infectivity (Holmgren et al., 2005; Roden, 2001). During virus maturation in the most superficial, dying keratinocytes, L1 capsomer interactions are stabilized by disulphide cross-linking, leading to the production of extremely stable infectious virions (Buck et al., 2005; Finnen et al., 2003). Eventually the infectious particles are shed from the epithelial surface as cornified envelopes desquamate (Fig. 1). The strategy of restricting viral DNA replication and accumulation of virion structural proteins to high level to cells in the upper epithelial layers already destined for death by natural causes results in persistent chronic infection and is important for immune evasion by the virus.

2.3. Papillomavirus genome organization and encoded proteins

All papillomaviruses have a double-stranded circular DNA genome of approximately 7000–8000 bp in size. Only one strand of the double-stranded genome serves as a template for viral gene expression and the transcribed polycistronic RNA species undergo extensive alternative splicing. The coding region is divided into early (E) and late (L) regions. The early coding region contains ORFs E1-E8 which are required for regulation of viral DNA replication and viral gene expression, and for induction of cell proliferation. The late region ORFs, L1 and L2, encode the viral capsid proteins. The early and late regions are both followed by a poly-A addition site, pA_E and pA_L respectively. A non-coding upstream regulatory region (URR), also called the long control region (LCR), of approximately 500–1000 bp contains the origin of replication, binding sites for viral E1 and E2 proteins and cellular transcription factors, transcriptional enhancers and promoters (Fig. 2). For BPV1, the six promoters active in transformed cells are P_{89}, P_{890}, P_{2443}, P_{3080}, P_{7185} and P_{7940}. The major late promoter, P_{7250} or P_L, is active in productively infected keratinocytes (Howley and Lowy, 2001). Multiple promoters are also involved in generating the various transcripts for the anogenital tract HPVs (Fig. 2). The major early promoter initiates upstream of the E6 ORF, encodes early viral proteins, and is expressed in basal cells as well as throughout the stratifying epithelium prior to productive replication. In HPV18 this promoter is referred to as P_{105}, while in HPV16 and HPV31 it is referred to as P_{97} and P_{99}, respectively. The differentiation-dependent late promoter (P_{811} in HPV18, P_{670} in HPV16 and P_{742} in HPV31), located in the E7 ORF, is activated coincident with the induction of productive replication (Chow et al., 1987; Frattini et al., 1997; Grassmann et al., 1996; Hummel et al., 1992; Smotkin et al., 1989). Several additional minor promoters have been found to play important roles during the HPV life cycle (Ozbun and Meyers, 1998a).

E1 protein is encoded by the largest and most conserved ORF of the papillomavirus genome. The E1 is a 68-kDa replication protein, which binds specifically to the origin of replication and contains ATPase and DNA helicase activities (Chiang et al., 1992; Sedman and Stenlund, 1998; Ustav and Stenlund, 1991; Yang et al., 1993). E1 binding to viral E2 protein is required for initial recruitment of E1 to the origin (Mohr et al., 1990; Sedman et al., 1997). Unlike E2, E1 is needed for both initiation and elongation of papillomavirus replication (Liu et al., 1995). E1 interacts with and recruits several cellular replication factors for viral DNA synthesis, such as DNA polymerase α/primase, the single-stranded DNA-binding protein, replication protein A (RPA), and topoisomerase I (Clower et al., 2006; Hu et al., 2006; Melendy et al., 1995; Park et al., 1994).

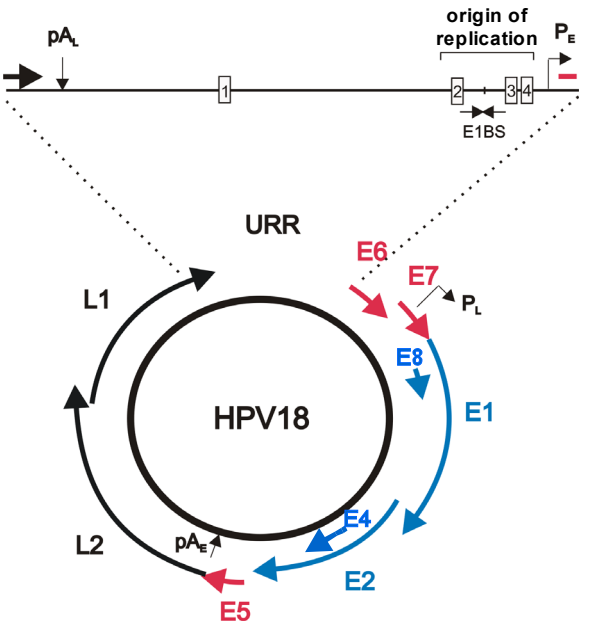

Figure 2. Schematic representation of the HPV18 genome. The early (E) and late (L) open reading frames are indicated as E1-E8 and L1-L2, respectively. The upstream regulatory region (URR) containing the origin of replication, E1 binding site (E1BS) and E2 binding sites (boxes with numbers) is shown on the top. The positions of the early (P_E) and late promoter (P_L) and polyadenylation sites (pA_E, pA_L) are marked with arrows.

The E2 protein is the master regulator of papillomaviruses that is required for the initiation of viral DNA replication, transcriptional regulation of viral genes and viral genome maintenace. In initiation of viral DNA synthesis, the role of E2 is to increase the sequence-specificity of E1 for the origin by binding cooperatively to adjacent binding sites in the origin through multiple protein-protein interactions (Berg and Stenlund, 1997; Mohr et al., 1990; Sedman and Stenlund, 1995; Sedman et al., 1997; Stenlund, 2003). Through binding to their specific binding sites in the viral genome, E2 proteins also act either as activators or repressors of viral transcription depending on the location of the E2 binding site and the recruited cellular factors (Soeda et al., 2006; Spalholz et al., 1985; Thierry and Yaniv, 1987). E2 functions in viral genome maintenance by tethering the genomes to mitotic apparatus in dividing cells (Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). E2 protein is approximately 50 kDa in size. In addition to full-lenght E2 protein, E2 gene encodes truncated E2 proteins by using either an internal promoter or alternative RNA splicing. These truncated E2 proteins act as transcriptional and replicational repressors. The full-length and truncated E2 proteins function as dimers which are formed through their common C-terminal DNA binding and dimerization domain (McBride, Byrne 1989). The structure and role of E2 proteins in viral life cycle is discussed in more depth in chapter 2.5.

E4 is the most divergent protein in sequence and length among different papillomavirus types. Although the E4 ORF is located in the early region of the viral genome, it is mainly expressed during the late stages of the virus life cycle. The E4, a small 10–20 kDa phosphoprotein, is primarily synthesized as a fusion with the first 5 amino acids of E1 (E1^E4) and expressed at high levels in cells of the differentiating layers of the epithelium in which vegetative viral DNA replication is ongoing (Doorbar et al., 1986; Doorbar et al., 1997; Doorbar et al., 1990; Nasseri et al., 1987). The E4 protein has been found associated with cytokeratins and induce the reorganization of the cytokeratin network (Doorbar et al., 1991; Roberts et al., 1993; Wang et al., 2004), and to cause important defects in the cornified cell envelope (Brown et al., 2006; Bryan and Brown, 2000), suggesting a role for E4 in facilitating viral egress from the cell and in person to person transmission. Due to its abundant expression, E4 is easily visualised in biopsy material by immunostaining and could serve as a biomarker of active virus infection (Middleton et al., 2003).

The E5 protein is a short membrane-associated hydrophobic protein. The 44-aa E5 protein of BPV1 and other fibropapillomaviruses (delta-papillomaviruses) acts as their primary oncogene. The transforming activity of BPV1 E5 protein is tightly linked to its ability to interact with and activate the platelet-derived growth factor (PDGF) β receptor (PDGFβ-R) in a ligand-independent manner (Drummond-Barbosa et al., 1995; Goldstein et al., 1994; Nilson and DiMaio, 1993; Petti et al., 1991). The ~80-aa HPV E5 proteins display weak transforming activity *in vitro* (Leechanachai et al., 1992; Pim et al., 1992; Straight et al., 1993), instead, the E6 and E7 proteins are the major HPV oncogenes. HPV E5 protein stimulates the transforming and mitogenic activity of the epidermal growth factor (EGF) receptor (EGFR) in a ligand-dependent manner (Crusius et al., 1998; Leechanachai et al., 1992; Pim et al., 1992; Straight et al., 1993). A failure to acidify endosomes and blockage of endocytic trafficking by E5 proteins have been suggested to be responsible for decreased growth factor receptor degradation and increased receptor recycling to the cell surface, resulting in enhanced receptor activity (Straight et al., 1993; Thomsen et al., 2000). In addition, E5 proteins contribute to immune evasion by down-regulating cell-surface expression of major histocompatibility complex class I (MHCI; HLA class I in humans) antigens (Ashrafi et al., 2006; Ashrafi et al., 2005; Ashrafi et al., 2002; Marchetti et al., 2002) and thereby potenitally inhibiting recognition of infected cells by cytotoxic T lymphocytes (Campo et al., 2010).

The ~150-aa E6 protein is a multifunctional oncoprotein that forms complexes with and modulates the activity of key cellular proteins that regulate cellular growth and differentiation. The best known feature of high-risk E6 protein is its ability to interact with and degrade the major tumor suppressor protein, p53, in conjuction with cellular E3 ubiquitin ligase, E6-AP, to overcome the proapoptotic activities of p53 and allow for cell cycle progression (Huibregtse et al., 1991; Scheffner et al., 1990; Werness et al., 1990). The E6 proteins from high-risk and low-risk HPVs bind p53, however, the binding of low-risk HPV E6 protein to p53 is weaker (Lechner and Laimins, 1994) and does not involve the p53 core domain needed for its degradation (Li and Coffino,

1996). In addition, both high-risk and low-risk E6 proteins interact with histone acetyltransferase p300 and inhibit the transcriptional activity of p53 (Patel et al., 1999; Thomas and Chiang, 2005). The high-risk E6 proteins also contain a PDZ binding motif and promote degradation of several PDZ family proteins associated with processes such as cell polarity, cell proliferation, maintaining cell-to-cell interactions, and signal transduction (Ganti et al., 2015). The high-risk E6 proteins are able to activate the telomerase (Galloway et al., 2005; Klingelhutz et al., 1996) and to maintain telomere integrity during repeated cell divisions.

The third papillomavirus oncoprotein is the E7 protein, a small protein of about 100 amino acids. The key function of E7 lies in its ability to bind retinoblastoma tumor suppressor protein, pRb (p105), and the related pocket proteins, p107 and p130 (Dyson et al., 1992; Dyson et al., 1989). The E7 protein binds to the hypophosphorylated form of pRb that results in the release of E2F family of transcription factors, allowing them to activate transcription of cellular genes involved in cellular DNA synthesis and progression of the cell cycle into S phase (Chellappan et al., 1992). The E7 proteins of low-risk HPV types bind pRb with lower efficiency compared to high-risk E7 (Munger et al., 1989). In addition, high-risk E7 protein destabilizes pRb by promoting its degradation via ubiquitin-proteasome-mediated proteolysis (Boyer et al., 1996). The E7 protein binds cyclin A and E and enhances their activities (McIntyre et al., 1996; Tommasino et al., 1993), and interacts with and abrogates the inhibitory activity of cyclin-dependent kinase inhibitors $p27^{kip1}$ (Zerfass-Thome et al., 1996) and $p21^{cip1}$ (Funk et al., 1997; Jones et al., 1997). In addition, E7 interacts with histone deacetylases (HDACs) (Brehm et al., 1999). The high-risk E7 protein also induces abnormal centrosome duplication which results in host genome instability and aneuploidy (Duensing et al., 2000).

The late viral proteins, L1 and L2, are the structural components of the viral capsid. The ~55-kDa L1 protein is the major protein in the viral capsid that is comprised of 72 pentameric L1 capsomers and up to 72 molecules of the ~70-kDa minor capsid protein, L2 (Baker et al., 1991a; Buck et al., 2008; Finnen, 2003). The ability of L1 to self-assemble into virus-like particles that closely mimic the natural surface of native papillomavirus virions serves as the basis of current HPV vaccines (Kirnbauer et al., 1992). L1 is responsible for the initial interaction of the papillomavirus capsid with the host by interacting with heparin sulphate proteoglycans which results in conformational changes exposing L2 for cleavage by cellular furin protease (Giroglou et al., 2001; Johnson et al., 2009; Joyce et al., 1999; Kines et al., 2009; Richards et al., 2006). The L2 protein plays an essential role in numerous steps of the viral infectious entry pathway, which include the induction of conformational changes in cell-bound virions, the egress of viral genomes from the endosomes and accompanying the viral genome into the nucleus to ND10 (Day et al., 1998; Day et al., 2004; Florin et al., 2002b; Kamper et al., 2006; Kines et al., 2009; Richards et al., 2006). The L2 protein enhances the assembly of virions and their infectivity, and participates in encapsidation of the viral genome (Buck et al., 2005; Day et al., 1998; Holmgren et al., 2005; Roden et al., 2001).

2.4. Papillomavirus DNA replication

Papillomavirus life cycle takes place in cutaneous or mucosal keratinocytes and is tightly linked to the normal differentiation process of the epithelium. The papillomavirus replicative cycle can be divided into three phases during which the virus genomes replicate as multicopy extrachromosomal genetic elements in the nuclei of host cells. After successful infection of a basal keratinocyte by the virus, initial amplification of papillomavirus DNA is triggered that increases the viral copy number to a few hundred copies per cell. This is followed by stable maintenance phase during which the viral copy number is kept constant during several rounds of cell division. The third phase of DNA replication is vegetative DNA replication that results in second increase in the viral copy number (Doorbar et al., 2012; Kadaja et al., 2009b). The initial establishment amplification replication and stable maintenance replication can be modelled in simplified cell culture systems. Keratinocyte differentiation dependent DNA amplification and assembly of virus particles can be analyzed in organotypic raft cultures or, alternatively with limitations in production of progeny virus particles, by suspension in methylcellulose or in the presence of high Ca^{2+} (Chow, 2015).

2.4.1. Initial amplificational replication

After viral entry into the cell nucleus, papillomavirus genomes replicate more frequently than the cellular genome to quickly reach an optimal copy number. Significant part of the knowledge in the early steps of papillomavirus replication cycle has been gained by studying the BPV1 replication and maintenance in mouse fibroblast cell line C127. The initiation of replication requires the origin of replication and expression of viral proteins E1 and E2, all other proteins and enzymes needed for replication are provided by the host cell replication machinery. The origin of replication is located within the URR of the viral genome and consists of binding sites for E2, from which only one is absolutely required for replication, and an A/T-rich region containing an array of binding sites for E1 (Fig. 2, 3) (Del Vecchio et al., 1992; Remm et al., 1992; Ustav et al., 1993; Ustav and Stenlund, 1991; Ustav et al., 1991). Thus, the initial amplificational replication can be modelled in transient cell culture assay by co-transfecting origin-containing plasmid together with E1 and E2 expression plasmids (Del Vecchio et al., 1992; Remm et al., 1992; Ustav and Stenlund, 1991). The overall structure of BPV1 and HPV origins and the interactions among viral *cis*-elements and *trans*-factors required for viral DNA replication are functionally conserved as mixed combinations of E1 and E2 proteins from different papillomaviruses can initiate DNA replication from different origins (Chiang et al., 1992; Del Vecchio et al., 1992; Kadaja et al., 2007; Sverdrup and Khan, 1994). Papillomavirus DNA replication is not cell type specific (Chiang et al., 1992; Del Vecchio et al., 1992; Geimanen et al., 2011), rather, transcriptional control of viral gene expression exhibits stringent cell type specificity.

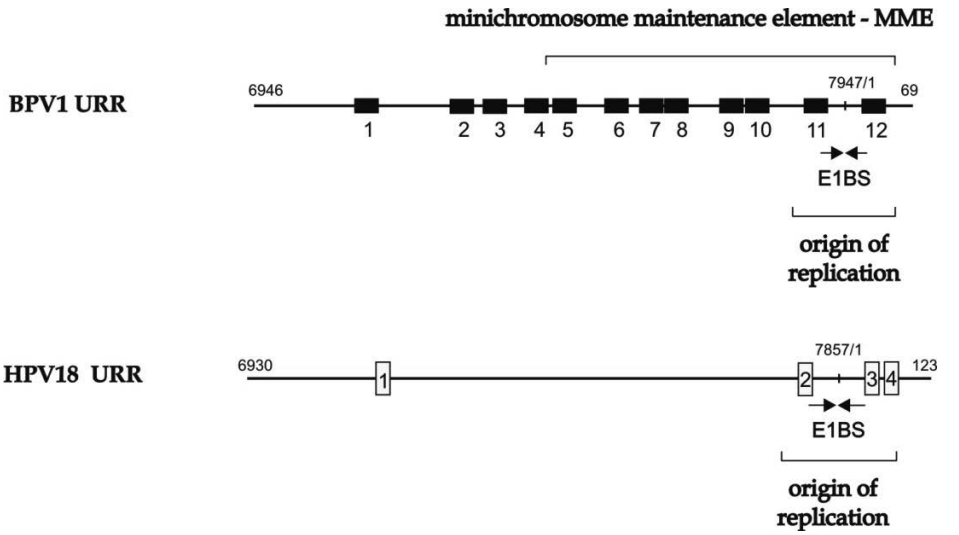

Figure 3. The upstream regulatory region (URR) of BPV1 and HPV18. E1 binding sites (E1BS) are marked with arrows and E2 binding sites with boxes. The minimal origin of replication consists of E1BS and E2 binding sites. The minichromosome maintenance element (MME) of BPV1 is required for maintenance and segregation of viral DNA. (Kurg, 2011)

Initiation of the replication of viral genome starts with cooperative binding of E1 and E2 proteins to their adjacent binding sites in the viral origin (Berg and Stenlund, 1997; Mohr et al., 1990; Ustav et al., 1993; Ustav and Stenlund, 1991). The E1 protein is a hexameric ATP-dependent DNA helicase that participates directly in replication initiation by melting the DNA at the viral origin and in subsequent elongation by unwinding the DNA double helix during replication fork progression (Sedman and Stenlund, 1998; Yang et al., 1993). E1 by itself has low sequence specificity, it can initiate DNA replication from non-specific DNA sequences *in vitro* (Bonne-Andrea et al., 1995). In the presence of E2, the E1 sequence-specificity is increased (Sedman and Stenlund, 1995; Sedman et al., 1997). E2 also enhances E1 binding to DNA through the DNA binding domain by blocking the non-specific DNA-binding activity of the E1 helicase domain (Stenlund, 2003). The binding of E1 and E2 complex is followed by formation of E1 hexameric complex with DNA helicase acitivity (Fig. 4) (Fouts et al., 1999; Sanders and Stenlund, 1998; Sanders and Stenlund, 2000; Schuck and Stenlund, 2005; Sedman and Stenlund, 1998) and recruitment of cellular replication factors to viral origin that include RPA, RFC, PCNA, DNA polymerase α/primase, topoisomerase I (Clower et al., 2006; Hu et al., 2006; Melendy et al., 1995; Park et al., 1994).

During initial amplification, viral DNA replication is initiated during S phase and extended to G2 phase (Reinson et al., 2015; Reinson et al., 2013), and has been considered to use both bi-directional theta-type and recombination-dependent replication modes (Orav et al., 2015). Viral DNA replication takes place in replication compartments which are often associated with components of

ND10 (Fradet-Turcotte et al., 2011; Reinson et al., 2013; Sakakibara et al., 2011; Swindle et al., 1999). In addition, viral proteins and viral DNA replication activate the cellular DNA damage respone (DDR) and relocalize the DNA repair and recombination proteins to viral replication centers to facilitate viral DNA amplification (Fradet-Turcotte et al., 2011; Gillespie et al., 2012; Kadaja et al., 2009a; Moody and Laimins, 2009; Reinson et al., 2013; Sakakibara et al., 2011).

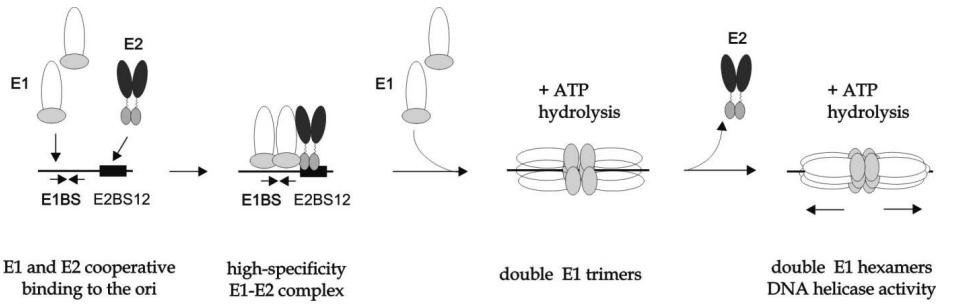

Figure 4. Assembly of the replication initiation complex. In the first step, dimers of the E1 and E2 proteins bind to their specific binding sites in the origin of replication in a cooperative manner forming a highly sequence-specific complex. The second step involves the recruitment of additional E1 molecules into this complex and the displacement of E2 in the presence of ATP. ATP hydrolysis is also required for the formation of double E1 hexameric complex with DNA helicase acitivity. (Kurg, 2011)

2.4.2. Stable maintenance replication

After the rapid initial amplification, the viral genomes are stably maintained at an almost constant copy number in the proliferating basal layer of the epithelium to sustain a persistent infection. In the maintenance phase, replication of extra-chromosomal viral DNA proceeds at a moderate level and is synchronized to cellular proliferation. In order to be successfully maintained in host cells during latent infection, these viruses associate their genomes to cellular chromatin which provides partitioning of viral genomes to daughter cells in approximately equal numbers and ensures that they are retained in the nucleus after cell division. The partitioning and extrachromosomal maintenance of viral genomes in the stable maintenance phase is dependent on the viral E2 protein (Ilves et al., 1999; Skiadopoulos and McBride, 1998). The cellular partners needed for efficient segregation and the segregation mechanism are discussed in chapter 2.5.4 of this thesis.

The origin of replication used during stable maintenance replication of viral genomes has been mapped to the same region in the URR that is also used for E1/E2-dependent initiation of DNA replication (Auborn et al., 1994; Flores and Lambert, 1997; Schvartzman et al., 1990; Yang and Botchan, 1990). Both E1 and E2 are required for replication in the establishment of papillomavirus genomes as episomes in infected cells. However, the E1 protein has been shown to be dispensable for the maintenance stage of viral genome replication (Egawa et al., 2012; Kim and Lambert, 2002). Thus, it is possible that the viral DNA

replication during stable maintenance phase could be performed solely by host cell replication proteins and viral functions are only required for retention and partitioning of the viral genomes. During the maintenance phase, the BPV1 DNA has been shown to replicate on average only once per cell cycle by a random-choice mechanism thereby keeping the viral copy number constant (Gilbert and Cohen, 1987; Piirsoo et al., 1996; Ravnan et al., 1992). For stable replication of the HPV genome, both ordered once-per-S-phase and random-choice statistical initiation mechanisms have been described (Hoffmann et al., 2006). HPV16 DNA replicated in an ordered once-per-S-phase manner in W12 cells, epithelial cells derived from a cervical lesion of an HPV16-infected patient, while in keratinocyte cell line NIKS, HPV16 replicated randomly. The HPV31 DNA replicated randomly both in HPV31-infected patient derived CIN612 cells and in NIKS cells. In addition to being dependent on the cells that harbour the viral DNA, the mode of replication also seems to be dependent on the E1 protein expression level, as high expression of this protein in W12 cells converted HPV16 DNA replication to random-choice replication. During stable maintenance phase, papillomavirus DNA is replicating by bi-directional (theta-type) replication mode (Auborn et al., 1994; Flores and Lambert, 1997; Gilbert and Cohen, 1987; Yang and Botchan, 1990). The expression of major oncoproteins of HPV, E6 and E7, has also been shown to be necessary for the maintenance of the extrachromosomal forms of HPV DNA likely by facilitating a cellular environment that is conducive to episomal maintenance and by abrogating the check-points that would block the long-term retention of extrachromosomal DNA (Oh et al., 2004; Park and Androphy, 2002; Thomas et al., 1999).

2.4.3. Vegetative replication

The productive stage of the viral life cycle occurs in the terminally differentiating layers of the epithelium where the virus amplifies its genome to thousands of copies per cell for virion assembly. Similarly to initial amplification of viral DNA in establishment phase, the second round of amplification requires viral replication proteins E1 and E2 which increase in abundance following the up-regulation of the differentiation-dependent late promoter (Hummel et al., 1992; Ozbun and Meyers, 1998b). The HPV E6 and E7 proteins are absolutely required to induce a pseudo S phase conducive to viral genome amplification in differentiated epithelial cells as these cells have withdrawn from the cell cycle. Following S phase re-entry, HPV E7 induces prolonged G2 phase in the differentiated cells (Banerjee et al., 2011), during which vegetative amplification of HPV DNA has been shown to take place (Wang et al., 2009). In addition, the E4 protein expression in proliferating epithelial cells has been shown to cause cell cycle arrest in G2 (Davy et al., 2005; Davy et al., 2002; Nakahara et al., 2002). A virus-induced G2 arrest would allow for a highly efficient and rapid viral genomic amplification without hijacking host replication machinery while it is engaged in replicating host DNA.

Both bi-directional replication via theta structures (Auborn et al., 1994) and rolling-circle replication (Burnett et al., 1989; Dasgupta et al., 1992; Flores and Lambert, 1997) have been suggested as the mechanisms for vegetative replication of viral genomes. The differentiated epithelial cells are no longer cycling or producing factors necessary for DNA replication. Since the environment of the differentiated epithelial cells is not favourable for DNA replication and the virus is dependent on these factors for its DNA replication, the virus must overcome these restrictions. Rolling-circle DNA replication is unidirectional, and one initiation event generates multiple copies of the genome thereby facilitating the production of large DNA amounts. As the theta mode of replication requires initiation with every round of replication, the virus may shift from theta replication mode to the rolling-circle replication mode in order to escape the unfavourable conditions of the differentiated cell (Flores and Lambert, 1997).

2.5. E2 as the master regulator of papillomavirus life cycle

2.5.1. Structure and properties of E2 proteins

The papillomavirus E2 gene products are important regulators of viral DNA replication, viral transcription and episomal maintenance. The E2 proteins are relatively well conserved among the papillomaviruses in two functional and structural domains: a transactivation domain (TAD) of about 200 amino acids located within the N-terminal half of the protein, and a dimerization and sequence-specific DNA binding domain (DBD) of about 100 amino acids that is located within the C-terminal region of the protein. These domains are separated by a flexible hinge region, which varies in length (40–200 aa) and sequence composition among different genera of papillomaviruses (Fig. 5) (Giri and Yaniv, 1988; McBride et al., 1989; McBride et al., 1988). The E2 protein binds as a dimer to consensus sequence, $ACCN_6GGT$, present in multiple copies in the URR of all papillomaviruses, through its C-terminal DBD (Androphy et al., 1987). The N-terminal TAD of E2 is responsible for the stimulation of viral DNA replication, transcription, and segregation of viral genomes (Abroi et al., 2004; Bastien and McBride, 2000; McBride et al., 1989; Ustav and Stenlund, 1991). The three-dimensional structures of both domains have been solved for several E2 proteins. The DBD forms a dimeric antiparallel β barrel with surface α-helices serving as recognition helices inserted into the successive major grooves of the DNA binding site (Hegde et al., 1992). The TAD forms a cashew shaped (L-shaped) structure and, as shown for HPV16 E2, consists of two domains, a helical domain containing three anti-parallel α-helices and a curved anti-parallel β-sheet domain (Antson et al., 2000; Harris and Botchan, 1999). The HPV16 E2 TAD forms a dimer both in the crystal structure and in solution (Antson et al., 2000), and this ability to self-interact enables E2 proteins to loop DNA containing widely spaced E2 binding sites (Hernandez-Ramon et al., 2008; Knight et al., 1991; Sim et al., 2008), thereby bringing tissue-specific enhancers and distally bound transcription factors into close proximity to the core transcription complex at the site of transcription initiation.

In addition to the full-length E2 protein, the papillomaviruses encode shorter forms of E2. For BPV1, three species of E2 have been identified. The full-length E2 (48 kDa) is coded by the entire E2 ORF; the E2C or E2-TR (31 kDa) is initiated from an internal ATG in the E2 ORF; and E8^E2 (28 kDa) is translated from alternatively spliced mRNAs by fusing 11 aa from E8 ORF to aa 205 of E2 ORF (Fig. 5) (Choe et al., 1989; Hubbert et al., 1988; Lambert et al., 1989). Similarly to BPV1, HPV11, HPV16, HPV31 and HPV5 have been shown to encode mRNAs coding for short forms of the E2 protein (Chiang et al., 1991; Lace et al., 2008; Sankovski et al., 2014; Stubenrauch et al., 2000). The truncated forms are similar to the BPV1 E8^E2 protein, since they contain a 10–13 residue peptide from an upstream ORF fused to the DBD of E2. The E8^E2 protein has been most frequently described and it has been suggested that all papillomaviruses have the potential to encode its equivalent (McBride, 2013). The shorter forms of E2 act as transcriptional and replicational repressors (Stubenrauch et al., 2007; Stubenrauch et al., 2001; Zobel et al., 2003). In the alpha-papillomaviruses, the residues K5, W6 and K7 of the E8 domain are important for the repression function (Powell et al., 2010; Straub et al., 2014; Stubenrauch et al., 2001; Zobel et al., 2003).

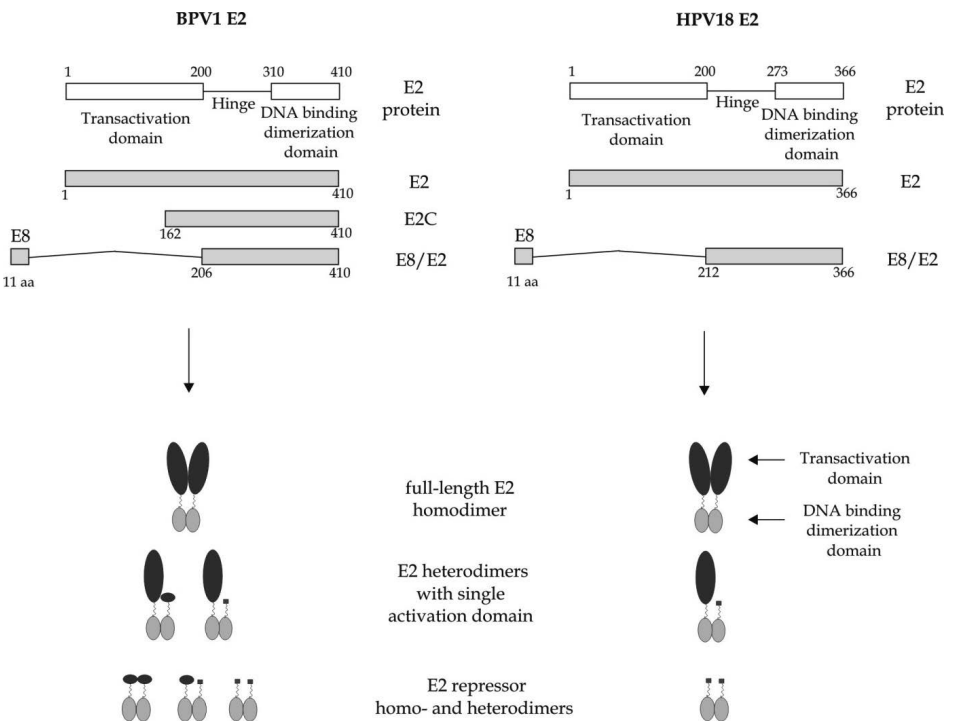

Figure 5. Papillomaviruses encode multiple E2 proteins. In addition to the full-length E2 protein, BPV1 and HPV18 genomes encode N-terminally truncated E2 proteins that serve as transcriptional and replicational repressors. The full-length and shorter forms of E2 are able to form homo- and heterodimer complexes through their common C-terminal DNA binding and dimerization domain. (Kurg, 2011)

The truncated E2 proteins are able to form heterodimer complexes with the full-length E2 and other truncated E2 proteins through their common C-terminal DBD (Fig. 5) (McBride et al., 1989). Although initially it was thought that truncated E2 proteins sequester full-length E2 in inactive heterodimers, the E2 heterodimers with single transactivation domain can interact with viral helicase, E1, and activate papillomavirus DNA replication in a cell-free system (Lim et al., 1998), and serve as activators of transcription and replication in cell culture model systems (Kurg et al., 2006). The relative ratio of E2 proteins in the BPV1-infected cells is 1:10:3 for E2-E2C-E8^E2, however, it has been shown to change during the cell cycle (Hubbert et al., 1988; Yang et al., 1991). The amounts of E2-E2C and E2-E8^E2 heterodimers within the cells are always larger or at least equal to the amount of full-length E2 homodimers, suggesting therefore that E2 heterodimers are the preferential form for E2 protein in infected cells (Kurg et al., 2006).

2.5.2. E2 role in papillomavirus transcription

The E2 protein of BPV1 was first described as a transcriptional activator that activates viral gene expression through E2-responsive elements located within the viral URR (Spalholz et al., 1985). The BPV1 genome contains 17 E2 binding sites, 12 of them are located in the URR (Li et al., 1989). In contrast, the URR in HPVs most often contains only four E2 binding sites whose locations in the URR are highly conserved (Fig. 3). The E2 proteins act as either activators or repressors depending on the context of E2 binding sites and the nature of the interacting cellular proteins. In general, E2 is a transcriptional activator of early genes in BPV1 and a repressor in the mucosal HPVs (Soeda et al., 2006; Spalholz et al., 1985; Thierry and Yaniv, 1987). It has been demonstrated that binding of E2 to its binding sites located upstream from the promoter, such as P_{7940} and P_{89} in BPV1 and the early promoter of cutaneous beta-HPVs, activates transcription (Guido et al., 1992; Haugen et al., 1987; Spalholz et al., 1987). Similarly, transcription is stimulated from heterologous promoters which contain multiple E2 binding sites at some distance from the promoter (Thierry et al., 1990). However, E2 has been shown to repress transcription when E2 binding sites are overlapping the binding motifs for cellular transcription factors. In the mucosal HPV genomes, the repression is mediated through two E2 binding sites immediately adjacent to the TATA box of the major early promoter that regulates expression of the viral oncogenes E6 and E7, as well as E1 and E2. E2 binding to promoter-proximal E2 binding sites sterically hinders the binding of Sp1 and TATA binding protein (TBP) due to partially overlapping binding sequences and prevents the formation of the transcriptional initiation complex resulting in transcriptional repression of the early promoter (Thierry, 2009). E2 binding to the most distal E2 binding site (E2BS-1) can upregulate viral early gene expression (Dong et al., 1994; Rapp et al., 1997; Steger and Corbach, 1997). HPV18 E2 protein binds with the strongest affinity to E2BS-1 and with reduced affinity to sites E2BS-3 and E2BS-4 (Demeret et al., 1997; Steger and Corbach, 1997), which correlates perfectly with the dose-dependent regulation of P_{105} activity by

E2. At low concentrations of E2, E2BS-1 is occupied and the early promoter is activated. As E2 concentrations rise, E2 occupies E2BS-3 and E2BS-4 that are involved in repression. This dose-dependent sequence of DNA binding events correlates with an initial upregulation in early gene expression, increasing the concentration of E2 as well as E6, E7 and E1. Subsequent binding of E2 to sites E2BS-2, E2BS-3 and E2BS-4 leads to repression of transcription of the early promoter and, in parallel to initiation of viral DNA replication, since these E2 binding sites, which are involved in transcriptional silencing, are required for viral DNA replication (Demeret et al., 1995; Sverdrup and Khan, 1995). Binding of E2 to E2BS-1 also counteracts total repression at higher E2 concentrations, ensuring that the E6 and E7 proteins are expressed at a level necessary to maintain a viral replication permissive cellular environment. Integration of viral DNA into the host genome usually disrupts the E2 gene leading to unregulated transcription from the early promoter and accumulation of excessive amounts of E6 and E7, which contributes to malignant progression in high-risk papillomavirus infection. Reintroduction of E2 into cervical cancer cell lines leads to repression of early promoter, thereby reducing E6 and E7 transcription and leading to cellular senescence or apoptosis (Desaintes et al., 1997; Dowhanick et al., 1995; Goodwin et al., 1998; Goodwin and DiMaio, 2000; Nishimura et al., 2000).

E2 regulates viral gene expression by interacting with components of the basic transcriptional machinery and chromatin remodelling complexes. The N-terminal TAD of E2 mediates interactions with several cellular transcription factors such as Sp1, TBP, TFIIB, and chromatin remodelling components such as histone acetylase coactivator CBP/p300, histone acetylase p/CAF, nucleosome assembly protein hNAP1 and chromatin remodelling protein Brm (Kumar et al., 2007; Lee et al., 2002; Lee et al., 2000; Li et al., 1991; Müller et al., 2002; Rehtanz et al., 2004; Steger et al., 1995; Yao et al., 1998). The cellular bromodomain containing protein Brd4 has been described as the major interactor of E2 that plays a central role in both transactivation and repression of the early promoter (Ilves et al., 2006; McBride and Jang, 2013; Wu and Chiang, 2007; You et al., 2004). Brd4 interacts with the N-terminal TAD of E2 protein through its extreme C-terminus (You et al., 2004), directs E2 to transcriptionally active regions of cellular chromatin (Jang et al., 2009) and is required for the transactivation activity of most, if not all, E2 proteins (McPhillips et al., 2006). Brd4 is also a component of the HPV11 E2 transcriptional silencing complex that represses E6/E7 promoter activity (Wu et al., 2006). E2 binding to Brd4 inhibits the association between the positive transcription elongation factor (P-TEFb) and Brd4, leading to active repression of the viral oncogenes (Yan et al., 2010). In addition to Brd4, EP400, a component of the NuA4/TIP60 histone acetyltransferase complex, and SMCX, also known as histone demethylase JARID1C, contribute to E2-mediated transcriptional repression of viral oncogenes (Smith et al., 2010).

The truncated forms of E2 that are lacking the N-terminal TAD also repress transcription. The repressors antagonize the functions of full-length E2 by competitive binding to E2 binding sites (Lim et al., 1998). In addition, the E8 part of E8^E2 significantly contributes to the inhibition of the major early promoter (Lace et al., 2008; Stubenrauch et al., 2000; Stubenrauch et al., 2007; Stubenrauch

et al., 2001) by interacting with cellular co-repressor molecules such the HDAC3/NCoR1 complex (Ammermann et al., 2008; Powell et al., 2010). The E2-E2C heterodimers efficiently activate the E2-dependent promoters (Kurg et al., 2006).

2.5.3. E2 role in papillomavirus genome replication

The role of E2 protein in initiation of viral DNA replication is well understood, E2 participates as a loading factor by recruiting the viral helicase E1 to the origin of replication through protein-protein and protein-DNA interactions (Fig. 4) (Mohr et al., 1990; Sanders and Stenlund, 2000; Sanders and Stenlund, 2001; Sedman and Stenlund, 1995; Sedman et al., 1997; Ustav and Stenlund, 1991). Additional E2 functions include alleviating repression by nucleosomes in the origin (Li and Botchan, 1994) and interacting with RPA (Li and Botchan, 1993) to enhance replication. The role of E2 in the initiation step of viral DNA replication was already described in chapter 2.4 of the thesis.

The truncated E2 proteins act as repressors of viral DNA replication. The HPV E8^E2 protein is a strong negative regulator of viral DNA replication (Lace et al., 2008; Stubenrauch et al., 2000; Zobel et al., 2003) and this involves the recruitment of cellular co-repressor molecules (Ammermann et al., 2008). Elimination of E8^E2 expression results in over-replication of HPV genomes (Lace et al., 2008; Sankovski et al., 2014; Straub et al., 2014; Stubenrauch et al., 2000; Zobel et al., 2003). Despite being a negative regulator, the E8^E2 is essential for long-term episomal maintenance of HPV31 genomes in normal human keratinocytes (Stubenrauch et al., 2000). However, HPV16 E8^E2 represses HPV16 plasmid amplification, but is not required for plasmid persistence and maintenance (Lace et al., 2008; Straub et al., 2014). In BPV1, elimination of E8^E2 expression has little effect on viral processes, whereas disruption of E2C expression increases the level of replication 10–20 fold. Removal of both repressors results in a lower stable copy number (Lambert et al., 1990; Riese et al., 1990), suggesting that at least one of BPV1 repressors is needed for stable replication. Another way of the E2 repressors to regulate papillomavirus replication would be through modulation of the activity of the E2 protein by formation of heterodimer complexes. The E2 heterodimers with single transactivation domain can interact with E1 protein and support *in vitro* replication of BPV1 DNA (Lim et al., 1998). The E2 heterodimers bind DNA sequence-specifically and support replication of BPV1 origin-containing plasmids in cell culture model systems (Kurg et al., 2006).

2.5.4. E2 role in stable maintenance of papillomavirus genome

During latent infection period, the viral genomes are maintained as extrachromosomally replicating elements in the nuclei of proliferating basal keratinocytes. To ensure persistence in infected cells, the viral genomes need to faithfully partition to daughter cells during mitosis and ensure the localization of viral genomes within the nuclear envelope following nuclear reassembly.

Initiation of viral DNA replication requires E1 and E2 proteins and the origin of replication. However, plasmids containing the minimal origin are quickly lost. Long-term replication and maintenance of these plasmids requires additional E2 binding sites in *cis* to the origin (Piirsoo et al., 1996). For BPV1, a sequence in the URR, the minichromosome maintenance element (MME), consisting of at least six E2 binding sites, and the minimal origin as *cis*-elements are required for long-term replication and maintenance of viral genomes (Fig. 2, 3). The MME can be replaced with a sequence of 10 tandem E2 sites suggesting that no particular E2 binding site is essential for stable replication, rather a certain number but not arrangement is required in this process (Piirsoo et al., 1996). Alpha-papillomaviruses contain only four E2 binding sites, which locations in the URR are structurally conserved (Fig. 3). Analysis of episomal maintenance of HPV31 genomes determined that three of the four sites (E2BS-1, E2BS-3 and E2BS-4) are essential for stable maintenance of viral episomes as mutations of these individual E2 binding sites resulted in integration of viral genomes into host chromosomes (Stubenrauch et al., 1998b). However, a recent report found E2BS-3 and E2BS-4 to be minimal *cis*-elements for the segregation of HPV18 genomes during cell division (Ustav et al., 2015). The cooperative binding of the E2 protein to these two binding sites is a major determinant of viral genome segregation efficiency.

The E2 protein is responsible for viral genome maintenance by anchoring viral genomes as well as URR reporter plasmids to host chromosomes in dividing cells (Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). The DBD of E2 protein binds the viral DNA through cognate E2 binding sites and other regions of E2, such as the TAD, simultaneously associate with mitotic chromosomes through protein-protein interactions to facilitate retention, maintenance and partitioning of viral genomes (Fig. 6). The E2 proteins from different genera of papillomaviruses use different cellular targets to achieve accurate segregation of viral genomes during mitosis. In BPV1, E2 TAD is necessary for E2 association with mitotic chromosomes as N-terminally truncated E2C and E8^E2 proteins do not bind mitotic chromatin (Skiadopoulos and McBride, 1998). E2 has been observed associated with host chromosomes at all stages of mitosis (Bastien and McBride, 2000) and also in interphase (Kurg et al., 2005). The E2 protein tethers viral genomes to cellular chromosomes through chromosome adapter protein Brd4 (You et al., 2004) which binds acetylated histones and remains associated with chromatin throughout mitosis (Dey et al., 2003; Dey et al., 2000). Brd4 binds the TAD of E2 and mutations in the TAD compromise both the interaction of E2 with Brd4 and the association of E2 with chromosomes (Baxter et al., 2005; Senechal et al., 2007). The E2 protein colocalizes with Brd4 in punctate speckles on mitotic chromosomes (McPhillips et al., 2006; Oliveira et al., 2006) and stabilizes the association of Brd4 with chromatin both in interphase and mitosis (McPhillips et al., 2006; McPhillips et al., 2005). Brd4 interacts efficiently with the full-length E2 protein homodimer. E2 heterodimer with single TAD binds Brd4 with low affinity and is also defective in the segregation function (Kurg et al., 2006).

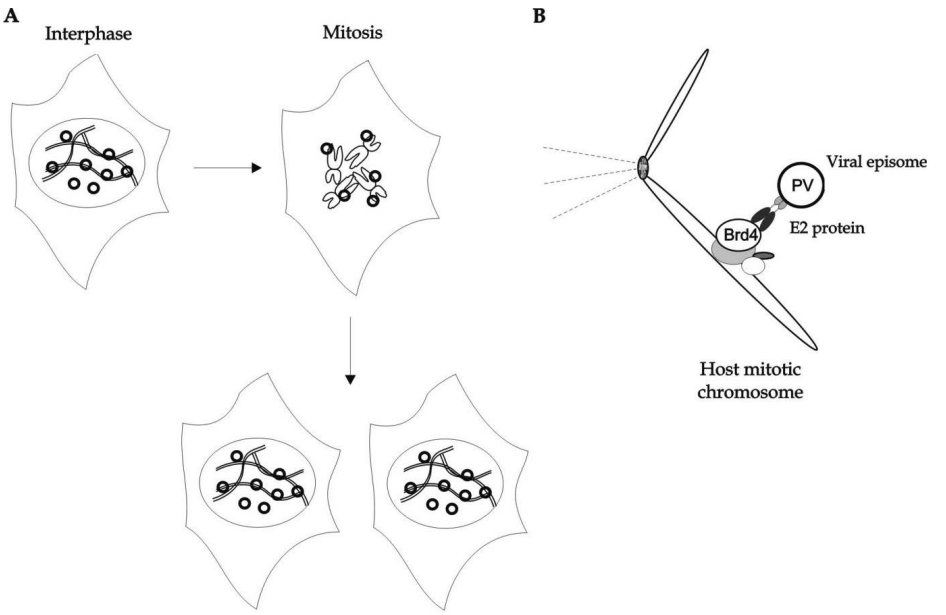

Figure 6. Model of papillomavirus genome partitioning. (A) During mitosis, papillomavirus genomes are partitioned to daughter cells through attachment to cellular mitotic chromosomes. (B) The viral E2 protein binds to the papillomavirus genome and tethers it to mitotic chromosomes by interacting with cellular chromatin-associated proteins, such as Brd4. Adapted from (Kurg, 2011)

E2 proteins from a wide range of papillomaviruses interact with Brd4 to regulate transcription but not all depend on this interaction to efficiently associate with mitotic chromosomes to ensure equal distribution and retention of viral DNA (McPhillips et al., 2006). The E2 proteins of beta-papillomaviruses bind most prominently to pericentromeric regions of mitotic chromosomes and they do not colocalize with Brd4 at this location (Oliveira et al., 2006). The E2 protein of beta-papillomavirus HPV8 binds to ribosomal DNA loci on the short arms of the acrocentric chromosomes and colocalizes with UBF, the RNA polymerase I transcription factor (Poddar et al., 2009). In contrast to BPV1 E2, which binds chromatin through its TAD, the hinge region and the DBD of HPV8 E2 protein are necessary and sufficient for chromosome targeting (Poddar et al., 2009). The E2 proteins of alpha-papillomaviruses bind to Brd4 relatively weakly and do not colocalize with Brd4 on mitotic chromosomes (McPhillips et al., 2005; Oliveira et al., 2006). Instead, several other cellular targets have been proposed to be important for tethering these E2 proteins. E2 and ChlR1, an ATP-dependent DNA helicase important for sister chromatid cohesion, colocalize at early stages of mitosis, suggesting that ChlR1 is required for initial loading E2 onto mitotic chromosomes (Parish et al., 2006). E2 protein of HPV16 localizes with TopBP1, which is involved in transcription, replication, and DNA damage and repair processes, on the chromatin and centrosomes during late stages of mitosis, suggesting that TopBP1 could be the mitotic chromatin receptor for

HPV16 E2 (Donaldson et al., 2007). E2 proteins of HPV11, HPV16 and HPV18 have been found to localize to mitotic spindles and HPV11 E2 also associates with centrosomes during cell division (Van Tine et al., 2004). The TAD and DBD of HPV11 E2 independently associate with the spindles (Van Tine et al., 2004). In addition, E2 protein has been found to colocalize with mitotic kinesin-like protein, MKlp2, in the central mitotic spindle during late mitosis (Yu et al., 2007). Thus, the human papillomaviruses may interact with diverse binding partners for segregation of viral genomes.

2.6. The role of ND10 in papillomavirus life cycle

ND10, also known as PML nuclear bodies (PML-NBs) or PML oncogenic domains (PODs), are small nuclear substructures present in almost all mammalian cells (Ishov et al., 1999). The ND10 ranges in size between 0.2 and 1 μm and in number between 1 and 30 bodies per cell, depending on the cell type and status. The ND10 are dynamic macromolecular structures associated to the nuclear matrix which represent accumulations of multiple cellular proteins that assemble in distinct foci within the nucleus in intimate contact with the surrounding chromatin. The promyelocytic leukemia (PML) protein is the organizer of ND10 which harbours other permanent (DAXX, SP100, SUMO-1) and numerous transient proteins (p53, CBP, HIPK2, components of the DNA repair machinery) recruited in these structures in response to different stimuli (Dellaire and Bazett-Jones, 2004; Negorev and Maul, 2001). The ND10 has been associated with the regulation of several cellular functions, including but not limited to, oncogenesis, DNA damage repair, stress response, senescence, apoptosis, protein degradation, viral infection, and the interferon (IFN) response (Bernardi et al., 2008; Everett and Chelbi-Alix, 2007; Regad and Chelbi-Alix, 2001; Tavalai and Stamminger, 2008). However, despite all this gathered knowledge, the functions of ND10 are still not fully understood.

The ND10 have been implicated to play an important role during the course of infection of a variety of different viruses. These subnuclear structures are preferentially targeted by nuclear-replicating DNA viruses whose initial sites of transcription and development of DNA replication centres are frequently juxtaposed to these domains or their remnants suggesting that the environment at ND10 is particularly advantageous for these viruses. These structures have been linked to IFN system and innate immune signalling since many ND10 proteins, including PML and SP100, are induced after IFN treatment (Regad and Chelbi-Alix, 2001). In addition, several viruses, such as herpesviruses and adenoviruses, encode proteins that colocalize with and sometimes cause catastrophic changes to ND10 by a variety of mechanisms, suggesting that ND10 are involved in intrinsic immunity, which represents the first line of intracellular defense against invading pathogens (Everett, 2001; Everett and Chelbi-Alix, 2007; Regad and Chelbi-Alix, 2001; Rivera-Molina et al., 2013; Tavalai and Stamminger, 2008).

The ND10 have been proposed to play a role in multiple steps in the papillomavirus life cycle. It was first reported that the minor capsid protein L2 from BPV1 associates with these subnuclear structures when exogenously

expressed (Day et al., 1998). This colocalization was subsequently confirmed using the L2 of HPV16 (Heino et al., 2000). In addition, L2 of BPV1 is responsible for the redistribution of the major capsid protein L1 and regulatory protein E2 to ND10 when co-expressed (Day et al., 1998). The L2 of HPV33 has been shown to reorganize the ND10 environment by releasing SP100 and recruiting DAXX into the ND10 structure (Florin et al., 2002b); both activities involve an NDLD peptide motif in L2 (Becker et al., 2003). Reorganization of ND10 by L2 has been observed in cultured cells as well as in productive lesions of the cervix caused by HPV infections (Florin et al., 2002b), suggesting that the interaction of L2 with these subnuclear domains does not appear to be an *in vitro* artefact. DAXX and L2 have been shown to interact with each other (Florin et al., 2002b) and also accumulate in nuclear dots in the absence of PML (Becker et al., 2004). HPV33 L2 localization at ND10 precedes L1 by several hours and L1 accumulates at these subnuclear structures only after L2-induced release of SP100 (Florin et al., 2002a). Other early and late papillomavirus proteins have been found associated with ND10 and its components. Both E1 and E2 of HPV11 colocalize with PML at ND10 if expressed together in the presence of HPV origin containing plasmid in transiently transfected cells (Swindle et al., 1999). The E6 and E7 oncoproteins interact and colocalize with endogenous PML at ND10 (Bischof et al., 2005; Guccione et al., 2004; Guccione et al., 2002). While only HPV18 E6 induced proteosomal degradation of insoluble form of PML isoform IV, both HPV11 and HPV18 E6 could overcome PML IV-induced cellular senescence in primary epithelial cells (Guccione et al., 2004). In addition to targeting the pRb tumor suppressor pathway, E7 protein inhibits PML IV-induced senescence by simultaneously interfering with PML-mediated acetylation and transcriptional activation of p53 by CBP and consequent transcriptional upregulation of p53 response genes that mediate cell cycle arrest (Bischof et al., 2005). The late protein E4 of HPV1 has been shown to induce relocation of PML from ND10 to the periphery of E4-induced nuclear inclusions both *in vitro* and *in vivo* (Roberts et al., 2003). Since E4 expression correlates with the onset of vegetative genome replication, reorganization of the ND10 may be necessary for efficient replication of the virus during the virus-producing phase.

As described above, L2 has been implicated in the delivery of the viral genome into the nucleus to ND10 and establishment of viral infection (Day et al., 2004). The localization of the viral genome to ND10 is comparable to other nuclear DNA viruses. However, in contrast to most DNA viruses, including herpesviruses and adenoviruses, which have developed mechanisms to antagonize the inhibitory function of ND10 early in their replicative phase, localization at ND10 enhances BPV1 early gene expression (Day et al., 2004). L2 of BPV1 also recruits the regulatory protein E2 to ND10 (Day et al., 1998) and can inhibit the transactivation function of E2, but not the capacity of E2 to support viral DNA replication (Heino et al., 2000; Okoye et al., 2005). More recent data show that ND10 proteins have opposing roles in viral transcription, SP100 knock-down increases HPV18 early transcription in primary human foreskin keratinocytes, while PML and DAXX down-regulation reduces it

(Stepp et al., 2013). In addition, SP100 has been identified as an interferon stimulated gene with anti-HPV activity (Habiger et al., 2015) which is consistent with data showing that SP100 acts as a restriction factor for HPV18 during the early stages of infection (Stepp et al., 2013). ND10 structures have also been found in close proximity to HPV DNA replication centres. Using indirect immunofluorescence in combination with fluorescence *in situ* hybridization (FISH), Swindle and others found that HPV11 DNA replication compartments containing the E1 and E2 proteins and replicating DNA partially overlap with PML in a fraction of transfected C33A cells (Swindle et al., 1999). The observed colocalization was reduced when the origin containing plasmid was omitted from the transfection, suggesting that the degree of ND10 localization was influenced by active DNA synthesis. Rivera-Molina and others have also demonstrated that HPV11 DNA/E2 protein complex recruits ND10-associated proteins; HPV DNA/protein complex colocalized with DAXX independently of PML, and with PML independently of the DAXX protein (Rivera-Molina et al., 2012). In addition, the ND10 might be involved in progeny virus assembly and viral DNA encapsidation (Becker et al., 2003; Day et al., 1998; Florin et al., 2002a; Heino et al., 2000). It has been proposed that L2 is recruited to regions of replication at ND10 via E2. Subsequently, L1 could be recruited to replication centres for virus genome encapsidation (Day et al., 1998; Florin et al., 2002a). However, the ND10 structures were found to be absent, both in the presence and in the absence of HPV genomes, in the terminally differentiated layers of the stratified epithelium which serve as sites of progeny virus assembly for papillomaviruses (Nakahara and Lambert, 2007). Nevertheless, L2 of HPV33 still assembles in distinct foci and also recruits L1 to these sites even in the absence of intact ND10 in PML-deficient cells. In addition, L2 aggregates in $PML^{-/-}$ cells also attract DAXX, which would have diffuse nuclear distribution in the absence of PML (Becker et al., 2004). Thus, although ND10 has been connected with all stages of the papillomavirus life cycle, the functional role of these subnuclear structures in papillomavirus infection is still controversial. In the case of BPV1, ND10 seem to positively affect papillomavirus infection as the presence of PML and intact ND10 enhances BPV1 early gene expression in murine fibroblasts (Day et al., 2004). The positive role of PML protein for initial viral transcription and replication has also been confirmed for HPV18 in human keratinocytes (Stepp et al., 2013). Yet others have shown that although the presence of the HPV genomes within the poorly differentiated basal and parabasal layers of the stratified epithelia leads to an increase in the number of ND10 as well as an increase in abundance of post-translationally modified PML protein, PML and ND10 are not required for E2-dependent transcription and viral DNA replication in transfected cells (Nakahara and Lambert, 2007). In summary, these data imply that papilloma-viruses initiate viral transcription and replication, similarly to other DNA viruses, in close proximity to ND10, however, without completely destroying these sub-nuclear structures but rather modestly modify the viral genome's initial environ-ment at ND10 by rearranging or degrading repressive proteins such as SP100 to foster the initial amplification of their own genetic programs.

2.7. The functions of the DAXX protein

2.7.1. The role of DAXX in cellular apoptosis and transcriptional control

The 740-amino-acid human protein DAXX is ubiquitously expressed and highly conserved nuclear protein. It contains two N-terminal paired amphipathic helices (PAHs), a coiled-coil domain, an acidic region and a C-terminal serine/proline/threonine rich region (Hollenbach et al., 1999; Salomoni and Khelifi, 2006). In the cell nucleus, DAXX is predominantly associated with two intranuclear domains: the ND10 and condensed heterochromatin (Ishov et al., 1999).

DAXX was originally described as a protein associated with the death domain of the cell surface receptor Fas in yeast two hybrid assay and thought to be involved in enhanced Fas-mediated apoptosis when over-expressed (Yang et al., 1997). In contrast, various studies have contradicted the original findings and have identified that DAXX is a nuclear protein (Hollenbach et al., 1999; Ishov et al., 1999; Li et al., 2000). Furthermore, depletion of endogenous DAXX protein by RNAi has reported DAXX to function as an anti-apoptotic protein (Chen and Chen, 2003; Michaelson and Leder, 2003). In addition, targeted deletion of DAXX in the mouse results in extensive apoptosis in early mouse development and embryonic lethality by day 9.5 of gestation (Michaelson et al., 1999). Thus, DAXX may have a dual function with respect to apoptosis, depending on the stimulus and the cell type. DAXX may protect from apoptosis at early stages of development, yet be pro-apoptotic in other situations (Michaelson, 2000; Salomoni and Khelifi, 2006).

DAXX also plays an active role in regulation of gene expression. A role for DAXX as a transcriptional repressor was first suggested by Hollenbach et al. who identified that DAXX interacts with Pax3, a member of the paired class homeodomain family of transcription factors, and represses its transcriptional activity (Hollenbach et al., 1999). Since then DAXX has been shown to suppress the activity of a growing number of transcription factors, including ETS1, NF-κB, Smad4, p53 family proteins and nuclear hormone receptors (androgen receptor (AR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR)), through direct protein-protein interactions (Salomoni and Khelifi, 2006; Shih et al., 2007). DAXX represses transcription by interacting with modulators of chromatin structure, namely HDAC1 and HDAC2 (Hollenbach et al., 2002; Li et al., 2000), DNA methyltransferase 1 (DNMT1) and DNMT3A (Muromoto et al., 2004; Puto and Reed, 2008), core histones H2A, H2B, H3 and H4 (Hollenbach et al., 2002), the chromatin-associated protein Dek (Hollenbach et al., 2002) and the X-linked mental retardation and α-thalassaemia syndrome protein (ATRX) (Ishov et al., 2004; Tang et al., 2004; Xue et al., 2003). DAXX forms an ATP-dependent chromatin remodelling complex with ATRX, a member of the SWI2/SNF2 family of ATP-dependent chromatin remodelling proteins, with ATRX being the core ATPase subunit and DAXX being the targeting subunit (Tang et al., 2004; Xue et al., 2003). DAXX and ATRX colocalize in heterochromatic regions in the late S phase of the cell cycle (Ishov et al., 2004). Because the latter part of S phase is used to replicate the repressed genome which requires the reestablishment of epigenetic markers of transcription repression,

DAXX was hypothesized to participate in the post-replication modification of heterochromatin. DAXX acts as a chaperone for histone variant H3.3, and, in complex with ATRX, has been found to be essential for replication-independent deposition of H3.3 at selected heterochromatic regions, including telomeres and pericentric heterochromatin, and to promote H3K9 trimethylation to facilitate the maintenance of heterochromatin through recruitment of methyltransferases (Voon and Wong, 2016). In addition, DAXX has been shown to interact with CENP-C, an intrinsic component of the centromere, and colocalize with interphase centromeres (Pluta et al., 1998).

For its recruitment to ND10, DAXX interacts through its C-terminus with SUMO-modified PML. This interaction is dynamic, cell-cycle-regulated and depends on the SUMOylation of PML (Ishov et al., 1999; Li et al., 2000). The C-terminus in DAXX also associates with other proteins, including transcription factors and chromatin remodelling proteins (Hollenbach et al., 1999; Li et al., 2000; Lin et al., 2006), and in those cases, DAXX cannot be accumulated at ND10. The ATRX protein interacts with the N-terminus of DAXX protein to target DAXX to heterochromatin at the end of S phase. DAXX, on the other hand, recruits ATRX to ND10 during G2 phase (Ishov et al., 2004), suggesting that accumulation of ATRX at ND10 might balance the availability of active ATRX at heterochromatin. Thus, the subnuclear compartmentalization determines the transrepressive effect of DAXX. It seems that DAXX exerts its repressive effect outside of the ND10 compartment as PML inhibits DAXX-mediated transcriptional repression by sequestration of nucleoplasmic DAXX to the ND10 (Li et al., 2000; Lin et al., 2006). In agreement with this notion, DAXX has been found to accumulate at condensed heterochromatin in $PML^{-/-}$ cells (Ishov et al., 1999). Taken together, the ability of DAXX to repress transcription is mediated through protein-protein interactions, subnuclear compartmentalization, protein modification and/or chromatin remodelling.

2.7.2. The role of DAXX in cellular intrinsic immune response against incoming viruses

Intrinsic defence against virus infection is actively studied phenomenon that, unlike the subsequent and complex innate and adaptive immunity pathways, is mediated directly by constitutively expressed and permanently active cellular restriction factors. Studies on the role of ND10 proteins in intrinsic immunity have found DAXX to be involved in this process as various viruses target DAXX in order to favour viral gene expression and replication. The interactions between herperviruses, specifically herpes simplex virus type 1 (HSV-1) and human cytomegalovirus (HCMV), and ND10 have been most extensively studied. HCMV is a member of the beta-subgroup of herpesviruses. Upon entry into the cell, HCMV can either initiate a productive lytic replication cycle or establish a latent infection in which the viral genome is maintained and no progeny virions are produced. Incoming HCMV genomes become associated with ND10 early after infection, resulting in repression of the major immediate early promoter (MIEP) by DAXX and ATRX. DAXX-mediated repression of

HMCV correlates with a transcriptionally repressive chromatin structure around the MIEP and seems to involve the recruitment of HDACs. In order to counteract the repressive effect of ND10, the tegument protein and transactivator pp71 interacts with DAXX which facilitates the displacement of ATRX from ND10 and degrades DAXX in a ubiquitin-independent but proteasome-dependent manner (Hwang and Kalejta, 2007; Lukashchuk et al., 2008; Preston and Nicholl, 2006; Saffert and Kalejta, 2006; Tavalai et al., 2008; Woodhall et al., 2006). This process is vital for the initiation of efficient HCMV gene expression and start of lytic infection. The failure of pp71 to overcome DAXX repression blocks immediate early gene expression and may promote the establishment of latent HCMV infections (Saffert and Kalejta, 2007). Activation of MIEP results in expression of the immediate early protein IE1, one of the regulatory proteins essential for initiating the lytic replication program of HMCV, which causes deSUMOylation of PML and SP100 leading to dispersion of ND10 and inactivation of ND10 accumulations (Tavalai and Stamminger, 2008). Consistent with these effects on ND10, down-regulation of DAXX, ATRX and/or PML has been found to enhance viral replication (Lukashchuk et al., 2008; Preston and Nicholl, 2006; Tavalai et al., 2008), suggesting that these proteins contribute to ND10-mediated viral repression.

HSV-1, a member of the neurotropic alpha subfamily of herpesviruses, establishes a lifelong infection in neural ganglia from which it reactivates periodically to replicate and spread. After entry into the nucleus, parental HSV-1 genomes associate with the periphery of ND10. The interaction between viral E3 ubiquitin ligase ICP0, the immediate early protein that promotes downstream viral gene expression, and ND10 induces the degradation of PML and selected forms of SP100 leading to subsequent dispersal of ND10. Disruption of ND10 by ICP0 is a key event in HSV-1 replication and correlates with the ability of ICP0 to stimulate HSV-1 lytic infection and to induce reactivation from quiescence or latency (Saffert and Kalejta, 2008; Tavalai and Stamminger, 2008). It has been reported that the ICP0-deleted HSV-1 genomes replicate at a greater level in ATRX- and DAXX-depleted cells compared to normal cells (Lukashchuk and Everett, 2010), suggesting that both DAXX and ATRX contribute to the repression of HSV-1 gene expression and infection that occurs in the absence of ICP0.

Similarly to other herpesviruses, the gamma-herpesvirus Epstein Barr virus (EBV) possesses a biphasic life cycle consisting of a productive and a latent phase. However, in contrast to HSV-1 and HCMV, there seems to be no association between the viral episomes and ND10 during latency (Bell et al., 2000), instead EBV genomes can be found in close association with interphase chromosomes. In addition, EBV expresses a set of proteins that are necessary for establishment and maintenance of latency. The major tegument protein BNRF1 is important for supporting EBV primary infection. BNRF1 interacts with DAXX, localizes to ND10 and disrupts the formation of the DAXX-ATRX chromatin remodelling complex. Similarly to HCMV tegument protein pp71, BNRF1 promotes the expression of viral immediate early genes. Knock-down of either DAXX or ATRX results in disruption of viral latency in infected lympho-

blastoid cell lines, suggesting that these proteins play a role in the restriction of viral gene expression and viral replication (Tsai et al., 2011). A more recent study has determined that BNRF1 dissociates ATRX from DAXX to inhibit the DAXX/ATRX-mediated deposition of histone variant H3.3 on the viral genome in order to promote viral gene expression required for latency establishment during early infection (Tsai et al., 2014). Similarly to HSV-1 and HCMV, lytic replication of EBV triggers the dispersion of ND10 proteins and occurs in close association with ND10 remnants (Bell et al., 2000).

DAXX acts also as a negative regulator of human adenovirus type 5 (hAd5) replication during productive infection (Schreiner et al., 2010; Ullman and Hearing, 2008). DAXX/ATRX chromatin remodelling complexes have been shown to play essential role in hAd5 gene expression, depletion of these cellular factors results in a less condensed chromatin state and enhanced viral gene expression (Schreiner et al., 2013). Similarly to HCMV tegument protein pp71, the adenovirus capsid protein IV mediates the initiation of the hAd5 immediate early E1A expression by counteracting DAXX-mediated transcriptional re-pression. The capsid protein IV displaces DAXX from ND10 (Schreiner et al., 2012). The viral early E1B-55K protein subsequently targets DAXX and ATRX for proteasomal degradation during hAd5 productive infection (Schreiner et al., 2013; Schreiner et al., 2010). In contrast to HSV-1, PML is not degraded by adenoviruses but relocalized into track-like structures through the viral E4orf3 protein (Tavalai and Stamminger, 2008).

DAXX is also involved in initiation and maintenance of retroviral epigenetic transcriptional silencing (Greger et al., 2005; Poleshko et al., 2008; Shalginskikh et al., 2012). First, DAXX was reported to interact with avian sarcoma virus (ASV) integrase in complex with viral DNA early after infection and mediate the repression of viral gene expression via the recruitment of HDACs to viral DNA (Greger et al., 2005). In addition, DAXX has been found associated with DNMTs on the viral DNA suggesting that integrated ASV provirus is rapidly chromatinized and repressed by histone deacetylation and DNA methylation as part of DAXX-mediated antiviral response (Shalginskikh et al., 2013). DAXX has also been demonstrated to associate with human immunodeficiency virus type 1 (HIV-1) derived lentiviral DNA through interaction with HIV-1 integrase and recruit HDACs to viral DNA to repress lentiviral gene expression (Huang et al., 2008). Interestingly, a recent report suggested that DAXX may interfere with an even earlier step of retrovirus infection since it was shown to inhibit reverse transciption (Dutrieux et al., 2015).

Taken together, these data support the concept of DAXX as being part of intrincic immune response against incoming viruses. Herpesviruses and adeno-viruses encode proteins that efficiently counteract DAXX-mediated intrinsic defences. However, it should be noted that, in the case of herpesviruses, silencing of lytic phase genes is actually required for establishment of lifelong latent infections.

3. AIMS OF THE STUDY

The studies that formed the basis of this thesis were focused on the life cycle of papillomaviruses, a family of medically important viruses due to established involvement in carcinogenesis. Specifically, I concentrated on two different aspects.

First, I investigated the functional activities of viral E2 protein heterodimers that are formed between the full-length and truncated E2 proteins. This information is of great interest because E2 serves as the master regulator protein that regulates transcription of viral early promoters, and participates in the initiation of viral DNA replication, and in viral genome episomal maintenance. In addition to the full-length E2 protein, all papillomaviruses encode shorter forms of E2 which are able to form heterodimers with the full-length E2 through their common C-terminal DBD. The functions of E2 heterodimers containing only one TAD were poorly characterized until our group used single-chain E2 heterodimers as a model to study the biological activities of E2 heterodimers of BPV1 (Kurg et al., 2006). This work showed that similarly to the full-length E2, the single-chain E2 heterodimer of BPV1 functioned as an effective transcriptional activator of E2-dependent promoters and was able to support replication of BPV1 origin-containing plasmid *in vivo* (Kurg et al., 2006).

The specific objectives of my studies regarding the E2 heterodimers were:

- To analyze further in detail the localization and replication function of BPV1 E2 protein heterodimer with single TAD by using the single-chain E2 heterodimer as a model.
- To construct HPV11 and HPV18 E2 heterodimers with a single TAD and analyze their functional activities.

The second aspect of this thesis was to investigate the possible connection of HPV replication with ND10 nuclear structures and their component, the DAXX protein. The E2 and E1 proteins of HPV have been shown to replicate viral genomes in replication centers that are formed in close association to ND10. This feature is characteristic to many DNA viruses, which also begin their replicative program at these sites. The functional significance of this interaction in virus replication, however, is still unclear.

The specific aim in connection with this aspect was:

- To examine the HPV replication compartments in relation to ND10, and the effect of the DAXX protein on HPV genome replication and transcription.

4. MATERIALS AND METHODS

The detailed descriptions of the materials and methods used in this study can be found in the appropriate sections of each publication. In general, wild-type or mutant viral genomes, plasmids containing viral regulatory *cis*-sequences or expression constructs encoding viral or cellular proteins were transfected by electroporation or lipofection into the cells. For knock-down of DAXX protein, DAXX siRNA was transfected into cells by lipofection prior transfection of viral genomes. The transfected cells were subjected to various analytical methods which are mentioned and, in some cases, briefly described below.

In order to study the role of different E2 proteins on the replication of papillomavirus origin and genomes, and the effect of DAXX down-regulation on replication of HPV18 and HPV11 genomes, low-molecular weight DNA was isolated from transfected cells at given time-points, digested with appropriate linearizing restriction enzymes and/or *Dpn*I to distinguish replicated from nonreplicated DNA, and analyzed either by Southern blotting or by quantitative real-time PCR analysis. The expression level of proteins, and the efficacy of DAXX expression knock-down was evaluated by immunoblotting analysis.

To study biochemical properties of E2 proteins, different methods were used. Biochemical fractionation followed by immunoblotting analysis of fractionated proteins was used to study the localization of E2 proteins in BPV1-transformed cells and in cells transiently transfected with native and single-chain heterodimer BPV1 E2 proteins. This method is described in detail in Ref. I.

To determine the ability of single-chain BPV1 E2 heterodimer to form a complex with E1, we used the co-immunoprecipitation assay followed by immunoblotting analysis of precipitated complexes as described in Ref. I.

Gel shift assay was used to determine the ability of single-chain HPV18 E2 heterodimer protein to bind DNA containing double-stranded E2 binding site (Ref. II).

Dual-luciferase reporter assay, involving the E2-responsive reporter plasmid containing the native HPV URR and a non-specific reporter, was used to compare the transcription repression function of native and codon-optimized HPV E2 and to determine the ability of single-chain HPV E2 heterodimer to repress the HPV early promoter (Ref. II).

To analyze the presence of the putative HPV18 E8^E2 transcript, and the effect of DAXX down-regulation on HPV18 and HPV11 early gene expression, total RNA was isolated from transfected cells and reverse transcribed. In order to analyze the HPV18 E8^E2 and E1 transcripts, the cDNA was amplified with PCR and analyzed by gel-electrophoresis. The purified amplification product in HPV18 E8^E2 transcript analysis was subsequently subjected to sequence analysis. In order to determine the effect of DAXX down-regulation on HPV early gene expression, the cDNAs were analyzed by quantitative real-time PCR (Ref. II, III).

Immunofluorescence analysis either alone or coupled with FISH analysis was used for visualization and localization studies of viral proteins, E1 and E2, or viral DNA, respectively, and ND10 proteins, PML and DAXX, in HPV replication compartments (Ref. III).

5. RESULTS AND DISCUSSION

5.1. Bovine papillomavirus type 1 E2 protein heterodimer with single transactivation domain is functional in papillomavirus DNA replication *in vivo* (Ref. I)

5.1.1. Localization of BPV1 E2 heterodimers is determined by the transactivation domain

Most of our knowledge about the role of E2 in papillomavirus DNA replication is gathered from studies with the full-length E2 homodimer. In order to investigate in detail the localization and replication function of E2 heterodimers with single TAD, we used the same single-chain E2 heterodimer (scE2) as previously described in (Kurg et al., 2006). By using a tethering strategy, the full-length E2 and E2 repressor were joined by a flexible polypeptide to create a single-chain protein capable of forming an intramolecular dimer. The constructed scE2 was fully functional in binding to specific E2 protein binding site. In several biochemical assays used, the scE2 was seen to fold correctly as only intramolecular dimers and not any kind of intermolecular complexes were formed. Thus, monomeric E2 heterodimers with only one TAD could be used to eliminate the competitive binding of other E2 dimers to DNA in order to study directly the biological functions of E2 dimers composed of full-length E2 and E2 repressors. The E2 heterodimer consists of full-length and N-terminally truncated E2 proteins, the cellular compartmentalization of which is different. The full-length E2 protein is mostly associated with cellular chromatin and the N-terminal TAD of the protein is responsible for this association. The truncated E2C protein, on the other hand, is found in soluble nucleoplasm fraction (Kurg et al., 2005). We used biochemical fractionation of U2OS cells, transfected with expression plasmids for E2 and scE2, as depicted in (Fig. 1B in Ref. I) (Kurg et al., 2005), to analyze subnuclear localization of scE2. We found that scE2 fractionated similarly to full-length E2 protein into non-ionic detergent resistant chromatin-nuclear matrix fraction and was released from this fraction by 0.4 M salt, under conditions where chromatin components are extractable but without disruption of the higher structure of the chromatin (Fig, 1D in Ref. I, lanes 4 and 9). We received similar results with BPV1-transformed C127 cells, which maintain and replicate the BPV1 genome as an episome and express all three E2 proteins (full-length E2, E2C and E8^E2) at low, physiological levels (Hubbert et al., 1988). As shown in Fig. 1C in Ref. I, the native E2 heterodimers with single TAD (E2:E2C and E2:E8^E2) fractionated similar to the full-length E2 protein homodimer into high salt-sensitive chromatin fraction (lanes 4 and 5), while E2C and E8^E2 homodimers were found in non-ionic detergent soluble fraction that contains soluble cytoplasmic and nucleoplasmic proteins (lane 2). Taken together, these data suggest that one TAD is sufficient to localize the E2 protein into cellular chromatin which is in agreement with the results that E2

heterodimer with single TAD is able to support papillomavirus DNA replication and transcription, since both activities are conducted through interactions with cellular proteins (Kurg et al., 2006).

5.1.2. The BPV1 E2 heterodimer with single transactivation domain interacts with E1 and initiates replication from different origins in *trans*

The role of E2 protein in viral DNA replication is to load the viral helicase E1 to its cognate binding site in the origin of replication by cooperative binding with E1. Both proteins bind as dimers and the cooperative binding is mediated by an interaction between the TAD of E2 and the C-terminal helicase domain of E1 (Berg and Stenlund, 1997; Mohr et al., 1990; Sanders and Stenlund, 1998; Sanders and Stenlund, 2000; Sanders and Stenlund, 2001; Sedman and Stenlund, 1995; Sedman et al., 1997; Ustav and Stenlund, 1991). By using the co-immunoprecipitation assay in COS-7 cells, transfected with expression plasmids for E1 and full-length E2 or scE2 proteins, we determined that E1 is able to form a complex with E2 heterodimer with single TAD similar to full-length E2 homodimer (Fig. 4 in Ref. I, lanes 3 and 4), and furthermore, under competitive conditions interacts with monomeric E2 TAD better than with dimeric E2 (Fig. 4 in Ref. I, lane 5).

The scE2 is also able to load E1 to the origin of replication as it supports replication of BPV1 origin-containing plasmid (Kurg et al., 2006) and BPV1 URR-containing plasmid (Fig. 2A in Ref. I). The BPV1 origin of replication consists of two E2 binding sites, E2BS-11 and E2BS-12, the E1 binding site and the A/T rich region (Fig. 3) (Ustav et al., 1993; Ustav and Stenlund, 1991; Ustav et al., 1991). Either E2 site alone supports replication, but the presence of both E2 binding sites within the origin is necessary for the full replication activity *in vivo*. Replication pre-initiation complex forms preferentially on the origin sequence by cooperative binding of E1 and E2 to E1 and E2 binding sites separated by 3 base pairs (E2BS-12) (Sanders and Stenlund, 1998; Sedman and Stenlund, 1995; Sedman and Stenlund, 1996; Sedman et al., 1997). E2 bound to the distal binding site (E2BS-11), located 33 base pairs upstream of the E1 binding site, assists in formation of the higher order form of E1 with origin melting activity, which forms by stepwise binding of E1 to the origin. Thus, E2BS-11 and E2BS-12 play separate but synergistic roles in initiation of viral DNA replication that are dependent on their location within the origin (Gillette and Borowiec, 1998; Sanders and Stenlund, 2000; Sanders and Stenlund, 2001). As shown in Fig. 2B in Ref. I, the scE2 is able to support DNA replication from both E2 binding sites in BPV1 origin of replication similar to the full-length E2 protein.

In addition to the TAD of E2 and helicase domain of E1, the DBDs of E1 and E2 also interact and this interaction between the two DBDs only plays a role when the binding sites for the two proteins are proximal, as the E1 binding

site and E2BS-12 in the BPV1 origin (Berg and Stenlund, 1997; Chen and Stenlund, 1998). The interaction between the E2 TAD and E1 helicase domain, on the other hand, shows little dependence on the relative position of the E2 binding sites as this interaction can occur over distances of several kilobase pairs (Ustav et al., 1993). The role played by the interaction between the E1 and E2 DBDs appears to be architectural by generating a sharp bend in the DNA that places the E2 TAD and the E1 helicase domain physically closer to each other, allowing for productive interaction between E1 helicase domain and E2 TAD to take place (Gillitzer et al., 2000). In order to study the effect of E2 heterodimers on papillomavirus DNA replication, we used the E2 double mutant 390/388 (E2(390/388)). Alanine substitutions at positions 390/388 of E2 severely reduce the interaction of DBDs of E2 and E1 and disrupt the productive interaction between the E2 TAD and E1 helicase domain, and therefore are incapable of functioning efficiently from the proximal E2BS-12. At the same time, these mutations have no effect on replication of distal E2BS-11 containing origin (Gillitzer et al., 2000) (Fig. 3C in Ref. I). We co-transfected expression plasmids for E2(390/388) and E2C together with E2BS-11 or E2BS-12 containing origin plasmid (OriA or OriB, respectively) and with E1 expression vector into the CHO cells. Co-expression of E2(390/388) and E2C results in the formation of E2(390/388) homodimers, E2(390/388):E2C heterodimers and E2C homodimers. From these, the latter is unable to support papillomavirus DNA replication (Fig. 3A in Ref. I, lane 4), and E2(390/388) homodimer is able to support the replication of OriA only (lane 3, upper band). The efficient replication of OriB should be achieved only by E2(390/388):E2C heterodimers. Indeed, as shown in Fig. 3A in Ref. I, the heterodimers were able to support replication of both, OriA and OriB (lane 7), suggesting that E2 heterodimer containing one TAD and only one functional DBD is active for replication of OriB. When both proteins, E2 and E2C, carried the mutation, the replication was initiated much more efficiently from OriA compared to OriB (Fig. 3A in Ref. I, lane 8), like in the case of E2(390/388) alone (lane 3). The ratio of quantified replication signals of OriB and OriA is shown in the diagram in Fig. 3B in Ref. I. These data show that the native E2 heterodimers are able to support DNA replication from both E2 binding sites in the BPV-1 origin within the cells. Our data are supported by earlier studies. First, Lim et al. showed that heterodimers composed of BPV1 E2 and E2C enhance the binding of E1 to the origin of replication *in vitro* as efficiently as E2 homodimers and can activate DNA replication in a cell-free system (Lim et al., 1998). Second, disruption of the N-terminal dimerization of BPV1 and HPV16 E2 proteins does not affect their ability to initiate replication suggesting that monomeric TAD is functional in replication (Hernandez-Ramon et al., 2008; Sanders et al., 2007). We suggest that E2 homo- and heterodimers work in concert in viral DNA replication.

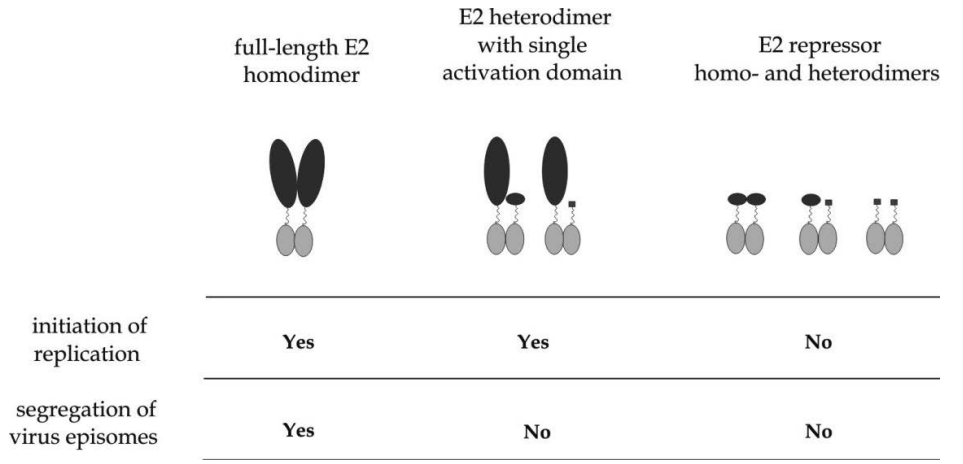

Figure 7. Activities of different E2 homo- and heterodimer complexes in initiation of viral DNA replication and segregation of viral episomes.

5.1.3. The BPV1 E2 heterodimer with single transactivation domain is able to initiate the viral genome replication in *cis*

In the context of full virus genome, the E2 protein has several additional auxiliary roles in papillomavirus DNA replication besides E1 loading. These include its ability to relieve nucleosome-mediated repression by altering chromatin structure within the origin to facilitate the initiation of DNA replication (Li and Botchan, 1994), interaction with and possible recruitment of general replication factors (Li and Botchan, 1993), and tethering of viral episomes to host mitotic chromosomes for their long-term persistence in infected cells (Ilves et al., 1999; Lehman and Botchan, 1998; Skiadopoulos and McBride, 1998). The binding of transcriptionally competent E2 to at least two tandem binding sites has been shown to be required for altering the chromatin structure around the promoter in a yeast model, suggesting that the full-length E2 homodimers are probably needed for this activity (Lefebvre et al., 1997). The chromosomal tethering function of E2 also requires full-length E2 homodimers as the E2 heterodimers with single TAD are crippled in the partitioning and maintenance of viral genomes (Kurg et al., 2006). Therefore, we decided to verify the ability of BPV1 E2 heterodimer with single TAD to initate virus DNA replication in the context of the whole BPV1 genome.

In order to follow the ability of scE2 to support papillomavirus DNA replication in the context of the BPV1 genome, we replaced the E2 ORF with scE2 sequence in BPV1 mutant genomes where initial ATG codons for E2C (BPV E2C$^-$) or E2C and E8^E2 (BPV E2C$^-$E8^E2$^-$) have been mutated. We used BPV1 E2C$^-$ genome because the replication signal of wild-type BPV1 genome in C127 cells was very low and remained sometimes under detection limits. As it has been previously reported, disruption of E2C results in ten-fold increase in

E2 transcriptional activity and ten-to-twenty-fold increase in viral copy number (Lambert et al., 1990; Riese et al., 1990). Transient replication assay of these genomes in C127 cells showed that scE2 initiates BPV1 genome replication in the absence of other E2 proteins (Fig. 5 in Ref. I). However, the replication signals of both BPV1 scE2 genomes decreased in course of time and were undetectable on day eleven after transfection.

The reduced replication signals of BPV scE2 genomes after several cell divisions are, in part, due to the defective partitioning function of the scE2 protein (Kurg et al., 2006). The cellular protein Brd4 is the interaction partner of BPV1 E2 for association of BPV1 genomes to host mitotic chromosomes (You et al., 2004) and the ability of E2 to bind mitotic chromosomes correlates with its interaction with Brd4 (Baxter et al., 2005). The E2 heterodimer with one TAD binds Brd4 with low affinity (Kurg et al., 2006), suggesting that the inability of scE2 to support the partitioning of viral genomes in dividing cells is partially caused by the weakening of the E2-Brd4 interaction.

On the other hand, at least one of two E2 repressors of BPV1 is required for the long-term maintenance of viral genomes and for the virus transforming activity (Lambert et al., 1990; Lehman et al., 1997). Disruption of both E2 repressor proteins, E2C and E8^E2, has been shown to severely reduce the copy number of BPV1 genomes (Lambert et al., 1990). Our results presented in this study are consistent with these reports and support the concept that truncated forms of E2 have a critical regulatory role on E2 function, and suggest that a very well orchestrated control of papillomavirus maintenance and copy number is achieved by finely tuned balance of different E2 proteins. We suggest that E2 heterodimers are able to support quick amplification of virus genomes taking place in limited number of cell cycles similarly to the full-length E2 homodimers. The maintenance of viral genomes in dividing cells, however, is supported solely by the full-length E2 homodimers. This is supported by data from Szymanski and Stenlund who analyzed promoter activities of BPV1 in their natural genomic context in replication-permissive C127 cells. The E2C promoter (P_{3080}) is the most productive promoter upon initial viral infection when the full-length E2 promoters (P_{89} and P_{2443}) have low basal activity. The E2 protein stimulates all the early promoters through common sequences in the URR, however, the various promoters display differential sensitivities to E2, the P_{3080} being the least responsive (Szymanski and Stenlund, 1991). So, the level of E2C is high and full-length E2 low upon initial viral infection, but after some time the level of full-length E2 is increasing due to positive auto-regulation of E2 expression. Presumably, the level of E2 heterodimer with single TAD is also initially high, and the ratio of E2 homo- and heterodimers is therefore also changing during the course of infection. In addition, a dynamic change exists in the ratio of E2 repressors to activators within the cell cycle. In asynchronous and G1 cells, the E2 repressors dominate, but in late S phase and G2/M, the activator is present in about equal levels to that of the repressors (Yang et al., 1991). These findings, together with our results that E2 heterodimers with single TAD are able to initiate BPV1 genome replication suggest that both E2

dimers, and the dynamic balance between these two forms of E2, are important for regulation of virus replication and stable plasmid copy number in papillomavirus-transformed cells.

In conclusion, in Ref. I we investigated in detail the localization and replication function of E2 heterodimers with single transactivation domain and found that single transactivation domain of E2 is sufficient to localize the E2 protein into cellular chromatin, for interaction with viral helicase E1, and for initiation of DNA replication from different papillomavirus origins. E2 heterodimer in the context of the entire BPV1 genome is able to initiate papillomavirus DNA replication, but not to maintain it for a long time (Fig. 7).

5.2. Human papillomavirus E2 protein heterodimer with single transactivation domain initiates HPV18 replication but is not sufficient for long-term maintenance of the virus genome (Ref. II)

5.2.1. HPV18 genome encodes E8^E2 repressor

The activity and amount of functional full-length E2 protein must be very tightly regulated in order to carry out the initiation of viral DNA replication, and control of viral transcription, segregation, and extrachromosomal maintenance of viral genomes in dividing cells. Similarly to BPV1, human papillomaviruses (e.g. HPV11, HPV16, HPV31, HPV5) have been shown to encode mRNAs encoding for short forms of the E2 protein (Chiang et al., 1991; Hubbert et al., 1988; Lace et al., 2008; Sankovski et al., 2014; Stubenrauch et al., 2000). The shorter forms of E2 protein are similar to BPV1 E8^E2 protein, since they contain a small conserved E8 ORF (HPV31, HPV16, HPV11, HPV5), or a fragment of E1 ORF (HPV11) fused to the C-terminus of E2. The shorter forms of E2 protein exert their negative effect on virus replication through modulating the ability of full-length E2 to enhance E1-dependent DNA replication as well as by regulating viral gene expression by displacement of E2 molecules from their binding sites (Chiang et al., 1991; Lim et al., 1998; Stubenrauch et al., 2000). In addition, the E8 domain of the E8^E2 protein itself is an active repressor molecule that functions independently of binding site competition and heterodimer formation with full-length E2 protein (Zobel et al., 2003) inhibiting transcription and DNA replication by interaction with co-repressor molecules (Ammermann et al., 2008; Powell et al., 2010). We investigated whether HPV18, which is the second most prevalent HPV in cervical cancer, expresses an E8^E2 transcript in our model system, U2OS cells transiently transfected with intact HPV18 genomic plasmid. We used the human osteosarcoma cell line U2OS for this and following replication assays for HPV genomes since the U2OS cells have been found to support replication of HPV subtypes belonging to different phylogenetic genera (Geimanen et al., 2011) and gene expression profiles of HPV18, HPV11 and HPV5 have been found to be very similar in U2OS cells

and in keratinocytes, the natural host cells of HPV (Isok-Paas et al., 2015; Sankovski et al., 2014; Toots et al., 2014). Thus, U2OS cells are suitable for studying the replication and transcription properties of HPVs. In order to detect the presence of HPV18 E8^E2 transcript, the total mRNA isolated from U2OS cells transiently transfected with HPV18 DNA was reverse transcribed, amplified by reverse transcription-PCR with primers located within the E2 ORF and upstream of the E8 coding region, and analyzed by gel-electrophoresis. As shown in Fig. 5A, B in Ref. II, the HPV18 genome transfected into U2OS cells encodes the E8^E2 transcript similar to other alpha-papillomaviruses (lane 2). The putative HPV18 E8^E2 protein fuses 11 aa from E8 ORF to aa 212 of E2 ORF by using the splice donor (SD) at nt 1357 and the splice acceptor (SA) at nt 3434 and contains the conserved K5, W6 and K7 residues required for repression activities as confirmed by sequence analysis of the purified amplification product (Fig. 5D in Ref. II) (Powell et al., 2010; Straub et al., 2014; Stubenrauch et al., 2001; Zobel et al., 2003). The replication analysis of HPV18 $E8^{-}$ genome carrying an ATG-to-ACG mutation in the initiator codon of the E8 ORF, that is silent in the overlapping E1 gene, determined that elimination of E8^E2 expression leads to a dramatic increase in transient DNA replication levels in transfected U2OS cells (Fig. 6B in Ref. II), confirming that HPV18 E8^E2 acts as a regulatory repressor protein similarly to other studied E8^E2 proteins (Lace et al., 2008; Sankovski et al., 2014; Straub et al., 2014; Stubenrauch et al., 2000; Zobel et al., 2003). These conclusions are supported by more recent studies of our group and others (Fertey et al., 2011; Reinson et al., 2015; Reinson et al., 2013; Toots et al., 2014; Wang et al., 2011).

5.2.2. Codon-optimization and construction of HPV E2 heterodimers with single transactivation domain

In order to analyze the functional activities of HPV11 and HPV18 E2 heterodimers with single TAD, formed by the full-length E2 and E2 repressor, we decided to use a similar scE2 model as for BPV1 in (Kurg et al., 2006) and Ref. I. The HPV E2 proteins are expressed at very low levels and sometimes remain below the detection level of immunoblotting analysis. Therefore, first, we resynthesized the 5' ends of HPV11 and HPV18 ORFs to replace the infrequently used codons with those most commonly found in mammalian genes (Fig. 1A in Ref. II) (Oliveira et al., 2006) in order to increase their expression level. As shown in immunoblotting analysis in Fig. 1B in Ref. II, codon-optimization (co) increased drastically the expression level of HPV11 and HPV18 E2 proteins in COS-7 cells. Similar results were obtained in U2OS cells (data not shown). To establish that the codon-optimized HPV11 E2 (HPV11E2co) and HPV18 E2 (HPV18 E2co) proteins were functional, we tested their transcription repression function. Using the dual-luciferase reporter assay system, we found that codon-optimized E2 proteins repressed their native promoter in a concentration-dependent manner in U2OS cells (Fig. 2 in Ref. II) and in HaCaT cells (data not

shown). The codon-optimized E2 proteins were stronger repressors than their native counterparts at similar concentrations, which is consistent with the increased expression level of E2co proteins. Thus, the codon-optimized E2 proteins retained the biological activity of the native proteins.

Papillomaviruses use several mechanisms in control of the activity and level of full-length E2 protein within the cell. In addition to direct control of the levels of E2 expression and encoding truncated E2 repressor proteins that counteract the activity of full-length E2, the E2 is also regulated in part at the posttranscriptional level as the sequence of HPV E2 gene contains codons infrequently used in mammalian genes. Similar results, suggesting that different codon usage between the viral and the human genomes accounts for the poor expression of HPV E2, have been published earlier for HPV11 and HPV31 E2 (Oliveira et al., 2006). Enhanced expression by codon-modification has also been shown for HPV16 E7 and E5 (Cid-Arregui et al., 2003; Disbrow et al., 2003). Thus, the nonhuman codon bias of certain HPVs seems to be another mechanism to control the activity of early viral genes and to establish the extrachromosomal maintenance of HPV genomes in dividing keratinocytes. Prevention of excessive viral protein expression by suboptimal codon usage and maintenance of viral genomes in undifferentiated cells at low but constant levels minimizes deleterious effects to the host and may have resulted from selective pressure to evade the immune system in the basal layers of the infected epithelium.

As a next step, the HPV11 and HPV18 E2 single-chain heterodimers were constructed by fusing the coding region of C-terminal DBD in frame to the full-length partially codon-optimized E2 as depicted for BPV1 scE2 protein (Fig. 1A in Ref. II) (Kurg et al., 2006). We constructed two scE2 variants with either a 5- or 21-residue linker (scE2co-5 and scE2co-21, respectively) between the two DBDs. The 21-residue linker was created by adding a synthetic oligonucleotide encoding a glycine-rich (GGSGGGGGSGGGGS) polypeptide tether of 14 amino acids to achieve maximum flexibility for correct folding of the protein (Kurg et al., 2006). On the basis of previous studies, flexible linker of appropriate length and composition, used to monomerize dimeric transcription factors, has little, if any, influence on the native state structure of the protein or domain (Bakiri et al., 2002; Dellarole et al., 2007; Kurg et al., 2006; Sieber and Allemann, 2000). As shown in the immunoblotting analysis in Fig. 1C in Ref. II, all proteins, used in this study, expressed at similar levels and migrated at expected sizes. We also tested the ability of the constructed synthetic scE2 to bind DNA in a gel shift assay (electrophoretic mobility shift assay) with the lysates of transfected COS-7 cells. HPV18E2co and HPV18scE2co-21 were both able to bind to radiolabeled double-stranded E2 binding site (Fig. 1D in Ref. II, lanes 4 and 5) and gave a mobility shift similar to that of BPV1 E2 proteins, demonstrating that both, the full-length HPV18E2co and HPV18scE2co, can bind DNA sequence-specifically. The maintenance of specific DNA recognition after intramolecular dimerization suggests that the single-chain E2 heterodimers folded correctly to form genuine pseudodimers and that such

tethered proteins behave like natural dimers in sequence-specific DNA binding. Our results are in agreement with previous studies that have detected only intra-molecular dimers when using tethered proteins (Bakiri et al., 2002; Dellarole et al., 2007; Kurg et al., 2006; Sieber and Allemann, 2000). Thus, we can rule out the possibility that created single-chain E2 heterodimers might associate with each other to generate an intermolecular dimer with two dimeric DBDs or that linker proteolysis produces a full-length E2 homodimer from the tethered construct.

5.2.3. HPV E2 heterodimer with single transactivation domain initiates replication of URR-containing plasmid and viral genome, but is insufficient for long-term episomal maintenance

Earlier studies have shown that single TAD of E2 is sufficient for initiation of DNA replication in concert with viral helicase E1 (Fig. 7) (Hernandez-Ramon et al., 2008; Kurg et al., 2006; Lim et al., 1998). In our study, we also found that scE2 is able to initiate the replication of HPV11 and HPV18 URR-containing plasmid (Fig. 4A, B in Ref. II) and HPV18 genome (Fig. 6B in Ref. II) in transfected U2OS cells. This confirms that the replication initiation function of E2 proteins is conserved between different types of papillomaviruses. As shown in the Southern blot analysis in Fig. 4 in Ref. II, the ability to initiate replication is comparable between full-length and single-chain E2 proteins using URR-containing plasmids transfected into U2OS cells. Similar results were obtained in human C33A and hamster CHO cells (data not shown). However, in the context of viral genome, HPV18scE2 genome expressing the single-chain E2 heterodimer replicated weaker than the wild-type HPV18 genome (Fig. 6B in Ref. II). It should be noted that we used the sequence of native E2 gene to replace the E2 ORF with scE2 sequence in wild-type HPV18 and HPV18/E8$^-$ genomes to ensure the balance of expression level of viral E1 and E2 proteins from the HPV18 genome. As determined by mRNA analysis of transfected U2OS cells at the earliest time point of replication assay, the amount of E1 transcript was comparable in cells containing either the wild-type HPV18 genome or HPV18scE2 genome (Fig. 5C in Ref. II) suggesting the mutation of HPV18 genome did not change the transcription of HPV18 E1 mRNA. The E8^E2 protein of human papillomaviruses is a strong negative regulator of replication which acts early in the viral replication cycle to negatively regulate the copy number (Lace et al., 2008; Straub et al., 2014; Stubenrauch et al., 2000; Zobel et al., 2003). The E8^E2 protein limits genome replication by repressing both viral transcription and the E1/E2-dependent DNA replication (Fertey et al., 2011; Lace et al., 2008; Straub et al., 2014; Stubenrauch et al., 2001; Zobel et al., 2003; Toots et al., 2014). Both inhibitory activities require the conserved E8 part, which functionally interacts with cellular co-repressors (Ammermann et al., 2008; Powell et al., 2010). It is possible that E8^E2 encoded by the HPV18 genome acts as a dominant negative regulator inhibiting the

action of single-chain E2 heterodimer better than that of the full-length E2 homodimer. However, we cannot rule out the possibility that HPV E2 may directly or indirectly influence the cellular environment and make it favourable for the extrachromosomal replication of the virus, and this function is characteristic for full-length E2 homodimer only. For instance, the pro-apoptotic function of high-risk HPV E2 proteins requires two functional TADs in order to induce cell death as the heterodimers formed between wild-type E2 and N-terminally deleted E2 proteins fail to induce apoptosis in both HPV-transformed and non-HPV-transformed cell lines (Webster et al., 2000). Moreover, the pro-apoptotic activity of the TAD does not involve its transcriptional function, rather it mediates interactions with factors involved in the apoptotic pathway (Demeret et al., 2003).

The full-length E2 protein with two TADs is required for persistent replication of BPV1, the single-chain E2 heterodimer is not sufficient for this activity (Fig. 7) (Ref. I) (Kurg et al., 2006). Consistent with our previous results, we found that wild-type HPV18 genome is able to replicate and maintain in dividing U2OS cells for up to two weeks, while HPV18 genomes expressing the single-chain E2 heterodimer disappeared (Fig. 6 in Ref. II). However, in addition to the full-length E2, HPV18 also needs the E8^E2 protein for long-term maintenance of viral genomes as the replication signal of HPV18 genomes unable to express E8^E2 started to drop after day six despite high transient replication levels, while the wild-type HPV18 genome replicated at a constant copy number within the two weeks of the experiment (Fig. 6 in Ref. II). It could be argued that the high-level replication of E8^E2C mutant genomes is detrimental for cells and by exceeding a critical threshold, triggers potential host defences to elevated viral DNA or increased expression of viral proteins. However, it does not seem to be the case, as clonal populations of human cervical epithelial cells have been derived that harbour and stably maintain high-risk HPV16 genomes extrachromosomally at approximately 1000 viral copies per cell for at least 15 passages (Jeon et al., 1995). In addition, BPV1-transformed cells do not undergo apoptosis or growth arrest under high genome copy number conditions (Lambert et al., 1990; Riese et al., 1990). Interestingly, while the E8^E2 proteins of the high-risk types 16, 18 and 31 display similar inhibitory effects on genome over-replication and E6/E7 promoter activity (Fertey et al., 2011; Lace et al., 2008; Straub et al., 2014; Stubenrauch et al., 2000; Stubenrauch et al., 2007; Stubenrauch et al., 2001; Zobel et al., 2003), the requirement for E8^E2 for plasmid persistence and maintenance differs between different human papillomavirus types as HPV31 E8^E2 and, according to our results, HPV18 E8^E2, but not HPV16 E8^E2, is needed for this activity (Lace et al., 2008; Stubenrauch et al., 2000). Notably, HPV16 is the most potent cancer-inducing HPV that accounts for approximately 50% of cervical cancers and is also highly represented in other cancers related to high-risk HPVs (Gillison and Lowy, 2004; Walboomers et al., 1999). The varying requirements of E8^E2 among different high-risk human papillomaviruses for stable maintenance of viral episomes could also play a role in the greater prevalence of HPV-16 in HPV-associated cancers.

Overall, the episomal maintenance of alpha-papillomaviruses is a complex process which is not yet fully understood. Our data suggest that this is regulated at least on two different levels: first, through the concentration of functional E2 homodimer, and second, through interactions with cellular factors. For BPV1, the E2 protein acts as a tether linking the viral genome to host mitotic chromosomes through interactions with cellular binding partners, one of which is Brd4 (You et al., 2004). The alpha-papillomaviruses target different cellular binding partners, including ChlR1, MKlp2 and TopBP1, for maintenance of viral genomes in dividing keratinocytes (Donaldson et al., 2007; McPhillips et al., 2006; Parish et al., 2006; Yu et al., 2007). Based on our results, we assume that the intact E2 homodimer with two functional TADs is needed for these interactions.

5.2.4. HPV heterodimer with single transactivation domain represses viral early promoter

In case of papillomaviruses, the transcriptional control of viral genes and activation of viral DNA replication are both mediated by the E2 protein. The role of E2 in initiation of virus DNA replication is conserved between different papillomaviruses, but its activity in transcription varies to a large extent. The binding of E2 to its binding sites within the viral URR can either activate or repress transcription of the E6 and E7 oncogenes, depending on the particular virus and the particular E2 protein being studied. The context of E2 binding sites in the URR and the number, arrangement and type of recruited cellular proteins determine the transcriptional activation or repression of the E6/E7 promoter. Binding of BPV1 E2 to its binding sites located upstream of the E6/E7 promoter activates transcription (Haugen et al., 1987; Spalholz et al., 1987; Spalholz et al., 1985). In contrast, the HPV E2 protein is primarily a transcriptional repressor of viral early promoter when expressed from the background of the viral genome in human keratinocytes. The URR of mucosal HPVs contains four E2 binding sites and E2 binding to the two promoter-proximal binding sites has been suggested to compete with the binding of cellular transcription factors due to steric hindrance. Yet, in addition to an intact DBD, which specifically binds the protein to promoter-proximal E2 binding sites in the URR, the transcriptional repression function of E2 also requires a competent TAD suggesting that E2-mediated silencing involves the recruitment of specific factors to the URR (Goodwin et al., 1998; Soeda et al., 2006; Thierry, 2009; Thierry and Yaniv, 1987).

In our study, we did not see any differences between the repression activities of full-length and single-chain E2 proteins in transient reporter assay in transfected U2OS cells as the HPV11 and HPV18 E2 heterodimers with single TAD were able to repress the HPV natural early promoter in a concentration-dependent manner similar to full-length E2 proteins (Fig. 3 in Ref. II). In the case of BPV1, E2 heterodimers with single TAD are able to activate tran-

scription from authentic BPV1 promoter but only to two-thirds of the wild-type protein activity level and are therefore weaker transactivators than E2 dimer with two TADs (Kurg et al., 2006). Brd4 is the major cellular partner required for E2 transcriptional activation in a number of papillomaviruses (Ilves et al., 2006; McPhillips et al., 2006; Schweiger et al., 2006; Senechal et al., 2007). The E2-Brd4 complex is also involved in repression of viral transcription (Wu et al., 2006; Yan et al., 2010). The TAD of E2 is crucial for interaction with the Brd4 protein and E2 binds to Brd4 most efficiently as a dimer (Cardenas-Mora et al., 2008; Kurg et al., 2006). Point-mutations in E2 disrupting the N-terminal dimerization have a major effect on transactivation but do not affect replication (Baxter et al., 2005; Hernandez-Ramon et al., 2008; McPhillips et al., 2006; Senechal et al., 2007), suggesting that N-terminal dimerization may modulate Brd4 binding. The E2 heterodimer with single TAD can bind Brd4 but has a much weaker affinity for Brd4 than the E2 homodimer (Kurg et al., 2006). It has been proposed that two TADs in E2 dimer interact with two copies of the Brd4 protein forming an E2-Brd4 heterotetramer (Cardenas-Mora et al., 2008); dimerization of the E2 proteins through both the C-terminal and N-terminal domains would greatly stabilize such a complex. In the case of E2 heterodimer with single TAD, two E2 heterodimers would be needed to interact with two Brd4 proteins and such a complex is probably less stable. The N-terminal dimerization also enables E2 proteins to loop DNA containing widely spaced E2 binding sites (Antson et al., 2000; Hernandez-Ramon et al., 2008; Knight et al., 1991; Sim et al., 2008), however, the local concentration of E2 interaction surfaces in formed complexes would be lower for E2 heterodimers with single TAD than for E2 homodimers. Alternatively, two functional TADs per dimer might be required for cooperative interactions between E2 dimers. In addition to Brd4, a genome-wide siRNA screen determined that E2 recruits several different cellular factors that contribute to repression of the HPV URR including the histone demethylase JARID1C/SMCX and EP400, a component of the NuA4/TIP60 histone acetyltransferase complex (Smith et al., 2010). This screen revealed that a single cellular protein or pathway is not sufficient for full transcriptional repression function of HPV E2 rather the identified proteins have additive effects on E2-dependent transcriptional repression of HPV URR. Nevertheless, the mechanisms by which E2 represses oncogene expression from the early promoter are still largely unknown. The differences or similarities between full-length E2 homodimer and E2 heterodimer with single TAD in transcription function in BPV1 and mucosal HPVs, respectively, could also be contributed to the importance of E2 transcription function in the life cycle of these viruses. BPV1 is highly dependent on the transactivation function of E2 protein as BPV1 mutant genomes which express replication-competent but transactivation-defective E2 proteins did not transform mouse C127 cells (Brokaw et al., 1996). In contrast, HPV31 genomes containing the transactivation-defective but replication-competent E2 mutant gene were readily able to immortalize keratinocytes as they could be established as stable episomes in transfected cells and expressed early and late viral transcripts at levels similar to

those of wild-type HPV31 (Stubenrauch et al., 1998a). Therefore, any effect of the E2 protein on the expression of the E6 and E7 oncogenes during the normal viral life cycle of mucosal high-risk HPVs has been suggested to be of secondary importance compared to the role of E2 in viral DNA replication (Bechtold et al., 2003; Stubenrauch et al., 1998a).

Taken together, in Ref. II we showed that HPV E2 heterodimer with single transactivation domain represses the HPV early promoter. Similarly to our previous results with BPV1 (Fig. 7) (Ref. I) (Kurg et al., 2006), HPV E2 heterodimer with single transactivation domain is able to initiate replication of URR-containing plasmid and in the context of HPV18 genome, but is not sufficient for long-term replication of HPV18 genomes. We also showed that HPV18 genome has a capacity to encode truncated E2 repressor protein E8^E2 which serves as a negative regulator of HPV18 genome replication.

5.3. DAXX modulates human papillomavirus early gene expression and genome replication in U2OS cells (Ref. III)

5.3.1. The localization of HPV replication foci in relation to ND10 in U2OS cells

After import into the nucleus, the parental genomes of many DNA viruses are targeted to, or nearby, specific nuclear sites, namely ND10 (Everett, 2001; Rivera-Molina et al., 2013; Tavalai and Stamminger, 2008). These nuclear substructures have been implicated in several cellular processes, such as DNA damage repair, transcriptional regulation, cell senescence, apoptosis, and are the primary sites of the interferon-dependent as well as the intrinsic response to infection (Bernardi et al., 2008; Dellaire and Bazett-Jones, 2004; Everett and Chelbi-Alix, 2007; Negorev and Maul, 2001; Regad and Chelbi-Alix, 2001; Tavalai and Stamminger, 2008). Although this cellular response is dedicated to counter invasion of foreign proteins and DNA, these viruses make use of this aggresome response by assembling replication centers and initiating the replication of their DNA in close proximity to ND10 or their remnants. The replication compartments anchor viral genomes, concentrate and compartmentalize viral and cellular factors that are required for viral DNA synthesis, and, as a consequence of their assembly and organization, function as scaffolds that maximize the efficiency of viral replication (Schmid et al., 2014). Compared to other nuclear DNA viruses, such as herpesviruses and adenoviruses, the papillomaviruses also locate to ND10 upon their delivery to the nucleus and replicate their genomes in replication compartments formed adjacent to ND10 (Day et al., 2004; Nakahara and Lambert, 2007; Rivera-Molina et al., 2012; Stepp et al., 2013; Swindle et al., 1999). Although the assembly of replication compartments and their components varies between different virus families, several fundamental similarities still exist. These similarities might originate from the requirement to control common cellular factors that are relocalized to

these sites to be utilized by the virus for its replication and transcription. On the other hand, the common cellular factors may be inhibited by the virus to prevent or control the cellular antiviral defense.

Most established human cell-lines fail to support HPV genome replication. However, both alpha-papillomaviruses, including HPV11 and HPV18, and beta-papillomaviruses are able to replicate, and establish a stable replication as autonomously replicating extrachromosomal plasmids in U2OS cells (Geimanen et al., 2011). Although the U2OS cells are not the natural host cell types of papillomaviruses, HPV genomes are transcriptionally active in these cells and can express the viral early proteins required for the initiation and the establishment of HPV replication (Ref. II) (Geimanen et al., 2011; Isok-Paas et al., 2015; Sankovski et al., 2014; Toots et al., 2014). Thus, these cells appear to contain all the necessary cellular factors required for the replication of papillomavirus genomes and therefore provide a cost-effective system for studying the fundamental processes of papillomavirus replication. Our group has previously shown that replication of HPV genomes takes place in distinct replication compartments in U2OS cells (Geimanen et al., 2011) . The viral proteins E1 and E2, required for the initiation of HPV replication within the cells, have been shown to localize with viral genomes and cellular components in the HPV replication centers ensuring the efficient propagation of the virus (Fradet-Turcotte et al., 2011; Reinson et al., 2013; Sakakibara et al., 2011; Swindle et al., 1999). In our study, we examined the HPV replication foci in U2OS cells in relation to the components of ND10. We first analyzed the localization of HPV E2 protein and cellular DAXX protein, a constitutive component of ND10 (Ishov et al., 1999). By using indirect immunofluorescence, we found that in cells transfected with HPV11 genomes, 34% of the E2 foci overlapped partially with DAXX-containing foci (Fig. 2A, B in Ref. III). In cells transfected with HPV18 genomes, we observed at least partial colocalization of DAXX and HPV DNA in 22% of the HPV18 DNA foci by immunofluorescence analysis coupled with FISH detection of HPV18 DNA (Fig. 2C in Ref. III). In order to confirm the partial colocalization of HPV replication centers and ND10, immunofluorescence analysis was performed with another component of ND10, the PML protein, which functions as the essential organizer of the ND10 (Ishov et al., 1999). As shown in Fig. 3A, B in Ref. III, in cells transfected with HPV11 genomes, 44% of the E2 foci overlapped partially with PML-containing foci, whereas in many cases, HPV replication foci and ND10 localized side by side. In cells transfected with HPV18E8^{-} genomes, 42% of the E1 foci overlapped, at least partially, with PML-specific signal (Fig. 3C, D, E, F in Ref. III). The HPV18E8- genome was used since it replicated at a higher level than the wild-type HPV18 genome (Ref. II) (Reinson et al., 2013), resulting in a higher and more easily detectable HPV18 E1 protein level in the transfected cells. Taken together, we found that a portion of HPV replication centers either colocalize partially or are located adjacent to components of the ND10, the DAXX and PML proteins, in U2OS cells. Our data are consistent with the work of others. Swindle et al. have demonstrated that in C33A cells, transfected with HPV11 origin containing

plasmid and E1 and E2 expression vectors, the viral proteins colocalize, at least partially, with PML in HPV11 replication compartments in a portion of the cells (Swindle et al., 1999). In addition, HPV11 origin DNA/E2 protein complex transfected into cells has been shown to recruit ND10 proteins, whereas HPV DNA/protein complex colocalized with DAXX independently of PML, and with PML independently of the DAXX protein (Rivera-Molina et al., 2012). Thus, the papillomaviruses replicate their genomes in close proximity to ND10 in various cell lines. The partial colocalization or adjacent positioning of replication compartments to ND10 and expansion of larger replication centers away from ND10 (Fig. 2 and 3 in Ref. III) suggests that viral replication does not depend on these subnuclear sites, but only starts there.

The ND10 can act as dynamic sensors of cellular stress that go through structural changes, increasing in their numbers in response to DNA damage. Following DNA damage, several DNA repair and recombination proteins relocate to ND10 in a temporally regulated manner, implicating these nuclear sites in DNA repair (Dellaire and Bazett-Jones, 2004). Interestingly, there are several similarities in the recruitment of ND10 and DNA repair proteins to sites of DNA repair in uninfected cells and to viral genomes in viral replication compartments. HPV DNA replication is also tightly linked to the cellular DNA damage response. Different groups have demonstrated that HPV infection triggers the host cell DDR by activating the ATM-Chk2-dependent pathway (Gillespie et al., 2012; Kadaja et al., 2009a; Moody and Laimins, 2009; Reinson et al., 2013; Sakakibara et al., 2011). The activation of ATM is necessary for efficient, productive amplification of HPV (Anacker et al., 2014). Thus, the cellular DDR is activated to facilitate viral DNA amplification, thereby stimulating papillomavirus replication. Consistent with the role of the host cell DDR in HPV replication, the number of ND10 is increased in the presence of HPV genomes within the poorly differentiated basal and parabasal layers of the stratified epithelia, where the early stage of the viral life cycle takes place (Nakahara and Lambert, 2007). The active recruitment of the cellular DNA repair and recombination proteins to sites of DNA damage and their utilization in HPV replication compartments could explain why the environment around ND10 seems to be particularly advantageous for HPV during several stages of the viral life cycle.

5.3.2. DAXX modulates the transient replication of HPV genomes in U2OS cells

Many DNA viruses that replicate in the nucleus encode proteins that modulate, relocate, or induce the degradation of the cellular factors associated with ND10, suggesting that alteration of ND10 may be a viral strategy to evade a cellular defense mechanism. One of the restricting factors targeted by different viruses is the DAXX protein (Everett, 2001; Rivera-Molina et al., 2013; Schmid et al., 2014; Tavalai and Stamminger, 2008). Therefore, we examined the effect of

DAXX on HPV genome replication. For this, we knocked down the expression of DAXX with siRNA and then transfected the cells with HPV11 and HPV18 genomic DNA. Analysis of newly replicated HPV11 and HPV18 DNA by Southern blotting determined that down-regulation of DAXX repressed the initial replication of both HPV11 and HPV18 genomes (Fig. 4A, B in Ref. III). We also analyzed the HPV DNA levels by quantitative real-time PCR, using mitochondrial DNA as an internal control. Consistent with Southern blot analysis, the quantification of replication products by real-time PCR indicated that knock-down of the DAXX protein reduced HPV DNA replication 2–3 fold (Fig. 4C in Ref. III). In order to determine whether the DAXX protein is involved in papillomavirus early promoter regulation, we studied the expression of viral early genes that were transcribed from transfected HPV genomes in U2OS cells. Analysis of HPV transcript levels revealed that DAXX knock-down prior to transfection of cells with HPV11 and HPV18 genomes decreases viral early gene expression by approximately 2–3 fold (Fig. 5A, C in Ref. III). At the same time, knock-down of DAXX protein did not influence the expression level of cellular housekeeping gene beta-actin (Fig. 5B in Ref. III). In summary, our results show that knock-down of one component of ND10, the cellular DAXX protein, leads to reduced HPV11 and HPV18 early gene expression and viral replication in U2OS cells. Thus, surprisingly, DAXX appears to have a positive role in HPV transcription and replication regulation. This is consistent with a more detailed study by Stepp and others of the roles of three components of ND10 in the early stages of HPV18 infection in primary human keratinocytes – the PML, SP100 and DAXX proteins. They revealed that PML and DAXX knock-down leads to a reduction in HPV18 transcription, while SP100 behaves as a repressor of viral infection (Stepp et al., 2013). The presence of PML and intact ND10 is also associated with enhanced BPV1 early gene expression (Day et al., 2004).

Although DNA viruses from several families are deposited at ND10 and start their transcription and replication at these nuclear sites, initially, the parental viral genomes are most often subjected to repression of viral transcription (Everett, 2001; Rivera-Molina et al., 2013; Tavalai and Stamminger, 2008). The ND10 harbor many trancriptional repressors, some of which are interferon-upregulated (Everett and Chelbi-Alix, 2007; Regad and Chelbi-Alix, 2001), and, generally, several cellular restriction factors contribute to this repression process in a cooperative manner. In order to favour viral gene expression and replication of incoming viral genomes, several viruses target these cellular restricting factors. In case of herpesviruses (HSV-1, HCMV, EBV) and adenoviruses (hAd5), the DAXX protein in co-operation with the ATRX protein contribute to the repression of viral early genes. Disruption of the DAXX-ATRX chromatin remodelling complex by virus encoded proteins leads to initiation of viral gene expression and start of productive infection (Hwang and Kalejta, 2007; Lukashchuk and Everett, 2010; Lukashchuk et al., 2008; Preston and Nicholl, 2006; Saffert and Kalejta, 2006; Schreiner et al., 2013; Schreiner et al., 2012; Schreiner et al., 2010; Tavalai et al., 2008; Tsai et al., 2014; Tsai et al.,

2011; Woodhall et al., 2006). Failure to target DAXX correlates with increased repression of herpesvirus and adenovirus genomes. In contrast, the DAXX protein has not been found to negatively impact the transcription and replication of HPV (Fig. 4 and 5 in Ref. III) (Stepp et al., 2013). Although papillomaviruses, adenoviruses and herpesviruses all begin their replication cycle adjacent to ND10 after entering the nucleus, only adenoviruses and herpesviruses disperse or structurally rearrange ND10 by different mechanisms before replication, whereas papillomaviruses initiate a transcriptional cascade which is followed by replication without causing major disruptive changes in ND10 (Everett, 2001; Ishov and Maul, 1996; Rivera-Molina et al., 2013; Schmid et al., 2014; Tavalai and Stamminger, 2008). In fact, rather than disrupting the actions of DAXX, the minor capsid protein L2 of HPV33 has been shown to recruit DAXX into the ND10 structure (Florin et al., 2002b). This phenomenon has been detected in both cultured cells and in productive lesions of the cervix caused by HPV infections. In addition to the positive role of DAXX and PML in viral transcription and replication early in the HPV life cycle (Fig. 4 and 5 in Ref. III) (Stepp et al., 2013), the presence of PML and intact ND10 has also been shown to enhance BPV1 early gene expression (Day et al., 2004). In that sense, papillomaviruses resemble the Simian virus 40 (SV40) of polyomaviruses, as SV40 gene expression also takes place in replication compartments formed adjacent to ND10, but SV40 does not take part in disrupting these structures (Ishov and Maul, 1996). Also similarly to papillomaviruses, polyomaviruses actively recruit cellular DDR proteins, such as ATM kinase and the MNR complex, to their replication compartments and exploit their functions (Schmid et al., 2014). Thus, ND10 are part of an intrinsic intracellular defence against viral infection and, in general, deposition of ND10 proteins at the sites of viral genomes is aimed at repressing viral gene expression and preventing replication of invading viruses. However, it may be argued that these structures restrict the replication of some viruses more than others, since not all viruses, which replication has been linked to ND10, disturb the integrity of the whole subnuclear structure. Although it has also been suggested that PML and ND10 are not necessarily required for papillomavirus transcription and viral DNA replication in transfected cells (Nakahara and Lambert, 2007), our results and reports of others indicate that these processes are enhanced by DAXX, PML, and the presence of intact ND10 (Fig. 4 and 5 in Ref. III) (Day et al., 2004; Stepp et al., 2013). Alternatively, as the vegetative viral genome amplification, late gene expression and virion production of HPV do not take place in the infected undifferentiated basal cells, but, instead, are restricted to differentiated layers of the epithelium (Doorbar et al., 2012), the repressive actions of the DAXX and PML proteins may not be as needed as they are required to repress the transcription of lytic phase genes of herpesviruses and adenoviruses in cells where their productive replication occurs. In addition, the different basal expression levels of viral promoters could also play a role in the impact of ND10 on the viral life cycle. Nevertheless, papillomaviruses could target other ND10-associated proteins to initiate their replicative program and that actually seems

to be the case. A recent study by Stepp and others determined that unlike DAXX and PML, the knock-down of SP100 enhances HPV18 early transcription in primary human keratinocytes (Stepp et al., 2013). In addition, SP100 is released from ND10 by L2 and possibly degraded (Florin et al., 2002b). Thus, it seems that SP100 is the major cellular restriction factor in case of papillomaviruses and L2-induced ND10 rearrangements mitigate transcriptional barriers that prevent efficient expression of viral early genes important for establishing a latent infection. The DAXX protein most likely functions indirectly to support early transcription and replication of HPVs, for example, by recruiting cellular or viral proteins to viral replication compartments.

To summarize, in Ref. III we found that HPVs replicate their genomes in close proximity to components of the ND10, the DAXX and PML proteins, in U2OS cells. The DAXX protein modulates the early gene expression and the transient replication of HPV genomes. Since HPV replication is reduced in the absence of DAXX, this protein appears to have a positive role in HPV transcription and replication regulation.

6. CONCLUSIONS

1. By using the single-chain E2 heterodimer as a model, I showed that the single transactivation domain is sufficient to localize the BPV1 E2 protein into cellular chromatin. This result is in agreement with the ability of the BPV1 E2 heterodimer with single transactivation domain to function similarly to the full-length E2 as an effective transcriptional activator of E2-dependent promoters and as a competent replication initiator protein.
2. The single transactivation domain of E2 heterodimer is sufficient for interaction with viral helicase E1, and for initiation of DNA replication from different papillomavirus origins, also in the context of the entire viral genome. These results, derived from studies with BPV1, and HPV18 and 11 E2 proteins, confirm that the replication initiation function of E2 proteins is conserved between different types of papillomaviruses. In contrast, the E2 heterodimer with single transactivation domain is not sufficient for long-term replication of papillomavirus genomes. Thus, the dynamic balance between full-length E2 homodimers and E2 heterodimers with single transactivation domain is important for regulation of viral DNA replication and genome copy number in infected cells.
3. HPV18 genome has a capacity to encode truncated E2 repressor protein E8^E2 which, similarly to other E8^E2 proteins studied so far, serves as a negative regulator of HPV18 genome replication. Similarly to the full-length E2 homodimer, the HPV E2 heterodimer with single transactivation domain represses the early promoter of HPV18 and 11.
4. HPV replication compartments are located in close proximity to components of the nuclear substructure ND10, the DAXX and PML proteins, in human osteosarcoma U2OS cells. In that sense, HPV resembles other DNA viruses, such as herpesviruses and adenoviruses, which initiate their replicative program adjacent to ND10.
5. The DAXX protein modulates the early gene expression and the transient replication of HPV genomes. Unlike the restrictive role that DAXX plays in the early life cycle of herpesviruses and adenoviruses, this protein appears to have a positive role in the regulation of HPV transcription and replication in U2OS cells.

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SUMMARY IN ESTONIAN

Papilloomiviiruse E2 valkude rolli uurimine viiruse DNA replikatsioonil

Papilloomiviirused on selgroogsete seas laialt levinud väikesed, koespetsiifilised DNA viirused, mis nakatavad naha või limaskesta epiteelkoes asuvaid keratinotsüüte. Inimese papilloomiviiruse (HPV) suurim meditsiiniline tähtsus seisneb tema võimes põhjustada mitmeid pahaloomulisi kasvajaid, sealhulgas emakakaelavähki, mis on naiste seas esinemissageduselt teisel kohal rinnavähi järel ning kõrge suremusega. HPV-d jagunevad kõrge ja madala vähitekke riskiga viirusteks. Madala riskiga viirused (HPV11, HPV6 jt) põhjustavad healoomulisi vohandeid, näiteks soolatüükaid ja kondüloome, mis tervetel inimestel kaovad immuunsüsteemi toimel aasta või paari jooksul. Pahaloomulised kasvajad võivad aja jooksul areneda rakkudest, mis on nakatunud kõrge riski HPV-dega (HPV16, HPV18 jt). Praeguseks saadaolevad HPV-vastased vaktsiinid on küll efektiivsed uue nakkuse ärahoidmisel, kuid ei paku paraku kaitset kõigi kõrge riskiga HPV-de osas, samuti on nad kulukad ning halvasti kättesaadavad arengumaades, kus leiab aset 80% emakakaelavähist tingitud surmadest. Kuigi maailmas on miljoneid HPV-ga nakatunud inimesi, ei ole tänaseks veel välja töötatud spetsiifilist viirusvastast ravi. Seetõttu on HPV-alane uurimistöö endiselt päevakorral, et paremini mõista viiruse onkogeensust ja leida uusi viise nakkuse ärahoidmiseks ning olemasolevate ravivõtete täiendamiseks.

Papilloomiviiruse E2 valk osaleb viiruse DNA replikatsiooni algatamisel koos viiruse peamise replikatsioonivalguga E1. Lisaks reguleerib E2 valk viiruse varajaste geenide avaldumist ning mängib olulist rolli viiruse genoomi episomaalsel säilimisel kinnitades rakkude jagunemise käigus viiruse genoomid raku kromosoomide külge. Samas ekspresseerib papilloomiviirus lisaks täispikale E2 valgule ka sama valgu lühikesi vorme, mille ülesandeks on reguleerida täispika E2 valgu funktsioone. Eelnevalt on meie grupp näidanud, et täispikast ja transaktivatsiooni domeeni mitteomavast lühikesest E2 valgust moodustunud E2 heterodimeerid on võimelised toetama viiruse varajaste geenide transkriptsiooni ja DNA replikatsiooni. Antud töö esimeses publikatsioonis uurisin detailsemalt E2 heterodimeeri paiknemist rakus ning selle rolli viiruse DNA replikatsioonil veise papilloomiviiruse tüüp 1 (BPV1) E2 näitel. Leidsin, et BPV1 E2 heterodimeer paikneb sarnaselt täispika E2 homodimeerile rakulise kromatiini fraktsioonis, mis on kooskõlas E2 heterodimeeri võimega toetada viiruse geenide transkriptsiooni ja DNA replikatsiooni. Lisaks näitasin, et BPV1 E2 heterodimeer on võimeline siduma viiruse E1 valgu replikatsiooni alguspunktile viiruse genoomis ning toetama viiruse DNA replikatsiooni initsiatsiooni. Samas ei suutnud E2 heterodimeer säilitada viiruse genoomi jagunevates rakkudes pikema aja jooksul.

Käesoleva tööga seotud järgnevas publikatsioonis uurisin HPV18 ja HPV11 E2 heterodimeeride osalust viiruse DNA replikatsioonil ning leidsin, et sarnaselt BPV1 E2 heterodimeerile toetab ka HPV E2 heterodimeer viiruse DNA replikatsiooni initsiatsiooni, kuid pole võimeline tagama viiruse genoomi pika-

ajalist säilimist rakus. Seega on E2 heterodimeeri võime toetada viiruse genoomi replikatsiooni initsiatsiooni konserveerunud erinevate papilloomiviiruste seas ning täispika E2 homodimeeri ja E2 heterodimeeri vaheline dünaamika on oluline reguleerimaks viiruse DNA paljundamist ja säilumist viirusega nakatunud rakkudes. HPV E2 heterodimeer oli võimeline ka maha suruma viiruse varajaste geenide avaldumist samas ulatuses kui täispika E2 homodimeer. Lisaks leidsin, et HPV18 on võimeline kodeerima transaktivatsiooni domeeni mitteomavat E8^E2 valku, mille üheks rolliks on inhibeerida HPV18 genoomi replikatsiooni, käitudes seega sarnaselt teiste papilloomiviiruste seas tuvastatud E8^E2 valkudega.

Käesoleva töö kolmandaks tahuks on papilloomiviiruse DNA replikatsiooni toimumiskoha uurimine rakus. Leidsin, et HPV replikatsioonikeskuste paiknemine raku tuumas kattub osaliselt tuumsete struktuuriüksuste ND10 asetusega. Nimelt paiknesid HPV18 ja HPV11 replikatsioonivalgud E1 ja E2 kas osaliselt koos või kõrvuti ND10 struktuursete valkudega PML ja DAXX, viidates sellele, et sarnaselt teistele raku tuumas replitseeruvatele DNA viirustele (herpesviirused, adenoviirused jt) algatab ka HPV oma replikatsioonitsükli ND10 läheduses. Kuigi ND10 mängib olulist rolli rakku sisenevate viiruste replikatsiooni inhibeerimisel, on mitmed viirused leidnud viise ND10 inhibeerivast toimest vabanemiseks, sealhulgas ND10 struktuuri lõhkumise või ND10 struktuursete valkude lagundamise läbi. Üks ND10 valkudest, mis inhibeerib mitmete ND10 läheduses paljunevate viiruste replikatsiooni, on DAXX valk. Oma töös leidsin, et DAXX valk moduleerib ka HPV18 ja HPV11 varajaste geenide avaldumist ja DNA replikatsiooni. Üllatuslikult ei omanud aga DAXX valk negatiivset toimet, vaid mõjutas positiivselt HPV geenieksressiooni ja genoomi lühiajalist replikatsiooni uuritavates rakkudes.

Kokkuvõtvalt uurisin töö käigus papilloomiviiruse peamise regulaatorvalgu E2 rolli viiruse DNA replikatsioonil ning viiruse replikatsiooni toimumiskohta rakus ja sellega seotud ühe rakulise faktori, DAXX valgu, mõju viiruse replikatsioonile.

ACKNOWLEDGEMENTS

First of all, I would like to thank my parents and sister for their love and support during the length of my studies.

I am very grateful to my supervisor Reet Kurg for her insight, positivity, support and encouragement.

I also would like to express my gratitude to Mart Ustav, Aare Abroi, Toomas Silla, Ivar Ilves and Ene Ustav for their advice and critical discussions.

I thank all the current and former members of our little research group, especially Liisi Võsa, Kadri Õunap and Janika Põder for the moments we spent inside and outside of the lab, for their friendship and support through all the ups and downs.

I would also like to thank other members of the Mart Ustav group and people from the 4th floor, especially Andrei, Mihkel, Kiira, Age and Margit. I also appreciate all the help Merike and Inge provided me.

I am grateful to Anna-Elina Lehesjoki and Kaia Palm for giving me the opportunity to work and learn in their groups during my studies, this gave me new and exciting knowledge of the field.

And last but not least I would like to thank my husband Tanel for his continued love and support and belief in me and my daughter Elis for bringing joy into my life and being the inspiration behind my thesis.

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