

ELINA LOTOTSKAJA-PEREPELENKO

Protein kinases as regulators of
human papillomavirus replication



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LIST OF ORIGINAL PUBLICATIONS

- I. Alla Piirsoo, Marko Piirsoo, Martin Kala, Eve Sankovski, **Elina Lototskaja**, Viktor Levin, Mauro Salvi, Mart Ustav (2019) Activity of CK2 α protein kinase is required for efficient replication of some HPV types. *PLOS Pathogens*, **15**(5), p.e 1007788.
- II. **Elina Lototskaja***, Olga Sahharov*, Marko Piirsoo, Martin Kala, Mart Ustav, Alla Piirsoo (2021) Cyclic AMP-dependent protein kinase exhibits antagonistic effects on the replication efficiency of different human papillomavirus types. *Journal of Virology*, **95**(13): e0025121
*Authors contributed equally to this work
- III. **Elina Lototskaja**, Lisett Liblekas, Marko Piirsoo, Airiin Laaneväli, Ruslan Ibragimov, Alla Piirsoo (2023) Phosphorylation of E2 serine residue 402 is required for the transcription and replication of the HPV5 genome. *Journal of Virology*, **97**(6):e0064323.

My contributions to the listed publications are as follows:

- Ref. I – Performed cloning of HPV-NLuc genomes, analysed their replication efficiency and interpreted the results. Contributed to the preparation of the manuscript.
- Ref. II – Analysed endogenous PKA activity effect on the initial amplification and maintenance of HPV genomes. Performed experiments determining PKA's effect on the HPV replication proteins E1 and E2. Interpreted the results and contributed to the preparation of the manuscript.
- Ref. III – Performed most of the experiments, interpreted the results and contributed to the preparation of the manuscript.

The article not included in this dissertation is as follows:

- I. Ruslan Ibragimov, Sofiya Babok, **Elina Lototskaja-Perepelenko**, Larisa Ivanova, Nika Mikhailava, Lidiia Zaikina, Marko Piirsoo, Alla Piirsoo (2025) Targeting papillomavirus infections: high-throughput screening reveals an effective inhibitor of cutaneous β -HPV types. *Journal of Virology*, **99**: e00918-25.

LIST OF ABBREVIATIONS

A _E	early polyadenylation site
AKAP	A-kinase-anchoring protein
A _L	late polyadenylation site
ATM	ataxia-telangiectasia mutated
BPV	bovine papillomavirus
Brd4	bromodomain protein 4
C	catalytic
cAMP	cyclic adenosine monophosphate
CBD	cyclin-binding domain
CDK	cyclin-dependent kinase
Cdk2	cyclin-dependent kinase 2
ChlR1	ATP-dependent DNA helicase
CK2	casein kinase 2
CRE	cAMP-response element
CREB	CRE-binding protein
CX4945	ATP-competitive CK2 inhibitor
DBD	DNA-binding domain
DDR	DNA-damage response
E1BS	E1 protein binding site
E2BS	E2 protein binding site
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
EV	<i>Epidermodysplasia verruciformis</i>
FGFR	fibroblast growth factor receptor
FMDV	foot-and-mouth disease virus
GPCR	G-protein coupled receptors
Gα _i	inhibitory G protein
Gα _s	stimulatory G protein
HD	helicase domain
HPV	human papillomavirus
HR	high-risk
HSPG	heparan sulphate proteoglycan
IC ₅₀	half maximal inhibitory concentration
KIP1	cyclin-dependent kinase inhibitor
LCR	long control region
LMW	low-molecular-weight
LR	low-risk

MAPK	mitogen-activated protein kinase
MG132	proteasome inhibitor
MKlp2	mitotic kinesin-like protein
Myc	myelocytomatosis oncogene
NES	nuclear export signal
NLS	nuclear localisation signal
NMSC	non-melanoma skin cancer
OBD	origin-binding domain
ORF	open reading frame
ori	origin of replication
p-TEFb	positive transcription elongation factor
PBM	PDZ-binding motif
PCNA	proliferating cell nuclear antigen
PDE	phosphodiesterase
PKA	protein kinase A
PKI	PKA inhibitor
Pol II	polymerase II
pRb	retinoblastoma protein
PV	papillomavirus
R	regulatory
RCR	rolling -circle replication
rDNA	ribosomal DNA
RDR	recombination-dependent replication
RPA	replication protein A
RPC	replication factor C
SB	Southern Blot
Ser/Thr	serine/threonine
SRPK1	serine-arginine protein kinase 1
SWI/SNF	ATP-dependent chromatin remodelling complex
TAD	transactivation domain
TBP	TATA-binding protein
TFIIB	transcription factor II B
TFIID	transcription factor II D
Tyr	tyrosine
UBF	RNA polymerase I transcription factor
URR	upstream regulatory region
VLP	virus-like particle
WT	wildtype

1. INTRODUCTION

Human papillomaviruses (HPVs) are extremely common viral pathogens. Most people are exposed to at least one HPV type during their lifetime, although the majority of infections remain asymptomatic. However, HPV infection can cause benign lesions of the skin or mucosal epithelium, including warts and papillomas. These lesions may regress spontaneously due to immune-mediated clearance or may require local treatment, such as chemical destruction with acids, liquid nitrogen cryotherapy, or surgical removal.

Although most HPV infections are transient, persistent infection with high-risk HPV types can lead to malignant transformation. The association between HPV infection and cervical cancer was established through the work of Professor Harald zur Hausen, whose discovery fundamentally changed the understanding of virus-associated cancers and was recognised with the Nobel Prize in 2008. This discovery initiated a new era in HPV research, highlighted the importance of HPV infection as a major public health issue, and led to extensive research into HPV prevention, pathogenesis, and treatment. Despite significant progress in prevention driven by prophylactic vaccines against the most clinically important mucosal HPV types, there is currently no curative antiviral therapy capable of eliminating ongoing HPV infections. These vaccines remain unequally accessible worldwide and are ineffective against existing infections.

Most HPV-related research has focused on oncogenic viruses associated with cervical and other anogenital cancers. However, cutaneous HPV types are also clinically relevant. Although skin-infecting HPVs usually cause asymptomatic or subclinical infections, they may contribute to extensive and persistent skin lesions in immunocompromised individuals, including patients with HIV infection, organ transplant recipients, patients receiving immunosuppressive cancer therapy, and individuals with inherited disorders such as *Epidermodysplasia verruciformis*. In these settings, infection with members of the β -HPV genus has been associated with an increased risk of non-melanoma skin cancer, particularly in combination with ultraviolet radiation exposure. As a result, the molecular biology of these viruses has become an increasingly important area of research. Nevertheless, therapeutic options for ongoing cutaneous HPV infections are still lacking.

A key requirement of HPV-driven cancer is long-term maintenance of the viral genome in infected epithelial cells, where it replicates in synchrony with host cell DNA and is transferred to daughter cells. Only two viral proteins are required for viral genome replication, E1 and E2. E1 is the only virus-encoded enzyme that functions as an ATP-dependent helicase that initiates and drives viral DNA replication. E2 is a multifunctional regulatory protein that facilitates E1 recruitment, modulates viral transcription, and contributes to the maintenance and segregation of viral episomes during cell division. The functions of E1 and E2 proteins are strongly influenced by the cellular environment, where they are subject to regulation, including phosphorylation by host cell protein kinases.

This thesis focuses on the role of host protein kinases in the regulation of HPV replication, with particular emphasis on the viral proteins E1 and E2. Specifically, it investigates how casein kinase 2 and cyclic AMP-dependent protein kinase affect the replication of different HPV types, including the cutaneous β -HPV5 and the mucosal α -HPV11 and 18. Additionally, this work examines phosphorylation-dependent regulation of the HPV5 E2 protein and identifies functionally important phosphorylation events required for viral transcription and replication. By comparing kinase-dependent regulation across HPV types from different genera, this thesis aims to clarify how host signalling pathways contribute to HPV genome replication and how these mechanisms may differ between cutaneous and mucosal HPVs.

2. LITERATURE REVIEW

2.1. Introduction to Papillomaviruses

Papillomaviruses (PVs) are widespread small DNA viruses that infect the stratified squamous epithelium of skin and mucosal membranes of vertebrates. PVs infect mammals, fish, birds, and reptiles. Detection of the same PV types from the vertebrates indicates that PVs have been developing along with different species for millions of years (Bernard, 1994a). This explains PV's ability to spread between species, causing subclinical infections (Antonsson *et al.*, 2000). Although PV infections are mostly asymptomatic and can be eliminated by the adaptive immune system, in some cases, they can lead to more clearly expressed manifestations, starting from benign lesions, such as warts and papillomas, to malignant neoplasms (e.g., cervical cancer) (Dürst *et al.*, 1983; Gross *et al.*, 1982).

PVs belong to the *Papillomaviridae* family, which currently includes around 600 types, where about 220 infect humans and are referred to as human papillomaviruses (HPVs) (The PapillomaVirus Episteme [PaVE]). HPVs are subdivided into five genera: Alpha(α)-, Beta(β)-, Gamma(γ)-, Mu(μ)- and Nu(ν), where α -, β - and γ -PV genera include the most common and medically essential types. The nucleotide sequence of the L1 capsid protein is the most conserved among HPV types and is therefore used for HPV classification. A novel PV isolate is deemed distinct if its L1 DNA sequence differs by at least 10% from the nearest known PV type (De Villiers *et al.*, 2004).

HPVs infect basal proliferating keratinocytes of the cutaneous and mucosal epithelia. The majority of the *Alphapapillomavirus* members mainly infect the mucosal epithelium of the mouth and the internal and external genital areas. All the remaining HPV types infect the skin. Additionally, HPVs are subdivided into high- and low-risk (HR and LR, respectively) groups according to their oncogenic potential. LR HPVs cause benign lesions, including genital warts (e.g., HPV6 and HPV11) and skin warts (e.g., HPV1, HPV2, HPV4) (La Rosa *et al.*, 2013). HR α -HPV types can induce oncogenic transformations. Additionally, HPV types from the α -genus (26, 30, 31, 33, 34, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82, 85, and 97) are considered probable HR types (International Agency for Research on Cancer, 2025). Although HPV16 and HPV18 are primarily associated with cervical cancer, they have also been identified as etiological agents in other anogenital cancers, such as anal, vulvar, penile, and vaginal cancers (Muñoz *et al.*, 2006; Palefsky, 2010), and in head and neck cancers, especially oropharyngeal carcinomas (Chaturvedi *et al.*, 2011; Smith *et al.*, 2004).

HPV infections of the cutaneous epithelium are mostly asymptomatic or at least subclinical, rarely causing visible warts or irritation. However, in immunocompromised patients, infections with distinct HPV types (e.g. HPV5 and HPV8) combined with prolonged UV exposure can lead to extensive, persistent, and recurrent warts over large areas of the skin, along with a dramatically increased risk of developing non-melanoma skin cancer (NMSC), especially squamous cell

carcinoma (Borgogna *et al.*, 2014; De Koning *et al.*, 2009). Mostly, they include immunocompromised patients with the rare autosomal recessive inherited skin disease *Epidermodysplasia verruciformis* (EV) and patients endured organ transplants, immunosuppressive cancer treatment or HIV infection (International Agency for Research on Cancer, 2025; Orth, 2006).

2.2. Treatment and prophylactic of HPV-related diseases

Currently, no therapeutic drugs are available for the treatment of ongoing HPV infections. Marketed vaccines against up to nine different α -HPV types are only prophylactic and primarily induce neutralising antibody responses that prevent the virus from entering the cell. All vaccines are generated using recombinant DNA technology, with HPV L1 capsid proteins that self-assemble into non-infectious virus-like particles (VLPs). Gardasil is a quadrivalent vaccine containing VLPs of four HPV types (HPV6, HPV11, HPV16, and HPV18). It was approved by the US Food and Drug Administration and also received EU-wide marketing authorisation in 2006 (European Medicines Agency, 2025b). A bivalent vaccine, Cervarix, targets HPV16 and HPV18 and was licensed in 2007 (European Medicines Agency, 2023; Harper, 2009). The nonavalent vaccine Gardasil 9, containing VLPs of the nine HPV types, was approved by the US Food and Drug Administration in 2014 and received EU-wide marketing authorisation in 2015 (European Medicines Agency, 2025a). In addition to the HPV types covered by Gardasil, it includes HPV31, HPV33, HPV45, HPV52, and HPV58. The viral oncoproteins E6 and E7 are the most targeted antigens for the development of therapeutic vaccines to date. Despite the variable success of preclinical studies and clinical trials of different vaccines (DNA-based VGX-3100, mRNA BNT113, and an LNP-formulated self-amplifying E7 RNA), these studies still suggest that E6 and E7 can be effective targets for virus-induced malignant growth inhibition (ClinicalTrials.gov; Ramos Da Silva *et al.*, 2023; K. Zhou *et al.*, 2023).

Despite preventive efforts, HPV-related diseases continue to spread widely, and treatment remains necessary. Treatment of HPV-induced benign proliferative lesions focuses on their removal or destruction, and on boosting the local immune response, rather than on the elimination of the viral infection, which may lead to recurrence (Leslie *et al.*, 2023). First-line therapies usually comprise the use of topical keratolytic agents (e.g., podophyllotoxin, imiquimod, sinecatechin), cryotherapy, and electrosurgery (Workowski and Bolan, 2015). For larger keratinised warts, surgical excision is considered more effective (Leung *et al.*, 2018). Additionally, different acids (e.g., salicylic and trichloroacetic) cause gradual peeling and elimination of warts (Leslie *et al.*, 2023). When HPV infection results in neoplastic alterations or invasive carcinoma, therapeutic approaches must target the dysplastic or malignant cells that harbour viral oncogenes. Treatments offered include local ablative therapies for precancerous lesions and multimodal cancer therapy for invasive cancers.

2.3. Viral genome organisation and encoded proteins

PVs are small, approximately 55 nm in diameter, non-enveloped viruses with an icosahedral capsid. The capsid encloses the viral genome, which is approximately 8 kb of circular, double-stranded DNA, bound by host histones into chromatin-like structures. The viral genome consists of three regions: the upstream regulatory region (URR), also called the long control region (LCR), which contains binding sites for viral and cellular proteins and is essential for the initiation of viral gene expression and genome replication; the early (E) region encoding genes expressed during the early phases of the infection cycle; and the late (L) region encoding capsid proteins. Two polyadenylation sites (pA) - the early pA_E and the late pA_L - define the termination and proper maturation of the mRNAs transcribed from open reading frames (ORFs) (Figure 1).

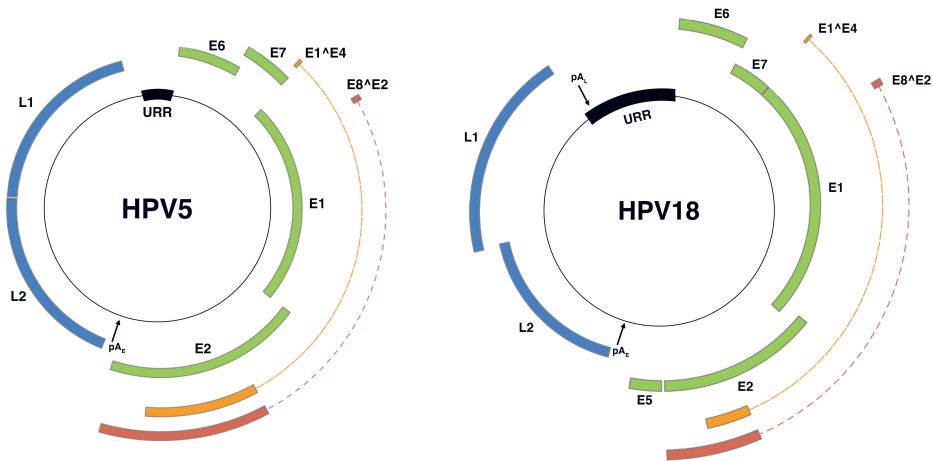


Figure 1. Genomic organisation of β -HPV5 and α -HPV18. The ORFs of the HPV early proteins (E) are shown in green, late proteins (L) in blue and URR in black. E1^{E4} and E8^{E2} spliced transcripts are shown in orange and red, respectively. Late (pA_L) and early (pA_E) polyadenylation sites are indicated with arrows. Genome maps are adapted from the PaVE database (<https://pave.niaid.nih.gov>).

The E1 and E2 proteins of different HPV types are functionally well-conserved and are crucial for viral DNA replication. E1 is an ATP-dependent helicase that initiates viral DNA replication at the origin of replication (ori), while E2 serves as a multifunctional regulator, facilitating E1 recruitment to the ori and modulating viral transcription. The E2 spliced variant, E8^{E2}, acts as a repressor of transcription and replication, hence modulating viral gene expression and genome copy number. E1 and E2 involvement in the viral life cycle is discussed in Chapter 2.4, and structures, molecular mechanisms, and regulation of these proteins are discussed in detail in Chapter 2.5.

The number and identity of other early region genes vary across PV genera, contributing to functions such as cell cycle entry, modulation of the host immune response, and virus release (Doorbar *et al.*, 2012).

Although it is classified as an early protein, E4 is primarily produced as a fusion protein from the alternatively spliced transcript, where the N-terminal short peptide originates from E1 ORF and the C-terminal sequence corresponds to E4. The functions of E4 are linked to the late stages of viral infection, and its expression is, therefore, elevated in the parabasal layer of the epithelium. E4 engagement with the mitotic cyclin B-cyclin-dependent kinase 1 (CDK1) complex causes the G2/M phase cell cycle arrest, delaying mitotic progression and promoting viral genome maintenance in differentiated epithelial cells (Davy and Doorbar, 2007; Knight *et al.*, 2004).

Not all PV genera encode E5 proteins, as it is specifically expressed in α -HPVs and some δ - and κ -PVs of other host species (PaVE database). E5 is a small trans-membrane protein found in the endoplasmic reticulum, Golgi apparatus and nuclear membranes (Conrad *et al.*, 1993). Engagement with various cellular proteins is crucial for E5 biological activity, which subsequently regulates cell transformation, immune response evasion, and viral pathogenesis, thus contributing to the enhanced oncogenic functions of α -HPVs (Cortese *et al.*, 2010).

E6 and E7 are viral oncoproteins that play direct roles in host cell transformation, immortalisation, and carcinogenesis. E6 binds to the tumour suppressor protein p53, a key transcription factor involved in DNA damage response and apoptosis. E6 promotes ubiquitin-mediated degradation of p53, leading to the accumulation of mutations, the prevention of apoptosis, and enhanced proliferation of HPV-infected cells (Scheffner *et al.*, 1990). E7 binds to the retinoblastoma (pRb) tumour suppressor that normally sequesters the E2F transcription factor in a cell cycle-dependent manner. E7-mediated inhibition of pRB promotes uncontrolled entry into S phase, leading to dysregulated DNA synthesis and cellular proliferation (Dyson *et al.*, 1989). The combined action of E6 and E7 facilitates genomic instability, mutation accumulation, and disruption of DNA damage repair mechanisms, thereby significantly increasing the risk of malignant transformation.

Proteins L1 and L2 are crucial for the final stages of the HPV life cycle. L1 is the major capsid protein, forming the icosahedral viral shell, interacting with the cell surface receptors (e.g. heparan sulphate proteoglycan [HSPGs]), and initiating attachment to the host epithelial cells (Knappe *et al.*, 2007). L2 is the minor capsid protein that contains a nuclear localisation signal (NLS) and facilitates viral genome trafficking into the host nucleus after viral entry (Wang and Roden, 2013).

2.4. The life cycle of HPV

HPV infects proliferating keratinocytes within stratified mucosal and cutaneous epithelia. The renewal of these tissues is facilitated by the deep basal layer, which comprises two essential cell populations: a limited number of slowly dividing, long-lived stem cells that are crucial for lifelong tissue maintenance, and a significantly larger population of their progeny, referred to as transiently amplifying (TA) cells. To establish a persistent infection, the virus targets both TA- and stem cells, as stem-like niches appear well suited for long-term persistence, while TA cells provide the rapid proliferation needed for initial genome amplification and subsequent differentiation to facilitate late gene expression and virion assembly (Strati, 2017). Since HPV cannot penetrate intact epithelium, it reaches basal keratinocytes through minor abrasions, wounds or hair follicles. Generally, the virus is transmitted via skin-to-skin or mucosal contact, typically during sexual activity, childbirth, or direct surface contact (Kines *et al.*, 2009; Schmitt *et al.*, 1996). To anchor HPV to the target cell, the carboxyl-terminal domain of L1 initially binds to HSPGs on the extracellular matrix (ECM) or the plasma membrane (Giroglou *et al.*, 2001). Next, host proprotein convertases (e.g., furins) cleave the L1 capsid protein, exposing the L2 minor capsid protein and facilitating dissociation from HSPGs, which triggers internalisation (or virus entry into the host cell) (Richards *et al.*, 2013). HPV internalisation is initiated by the association of the L2 with secondary receptors and happens via clathrin-mediated endocytosis (Evander *et al.*, 1997; Schelhaas *et al.*, 2012). After the viral DNA is uncoated through the endosomal pathway, the L2/viral DNA complex is retrogradely transported to the Golgi apparatus and further directed to the nucleus to initiate transcription. Since L2 facilitates viral DNA attachment to mitotic chromosomes, its movement from the Golgi to the nucleus is closely related to mitosis (Ozbun and Campos, 2021).

After the viral genome is imported into the host cell nucleus, it undergoes initial amplification up to 50-300 copies per cell (Williams *et al.*, 1994). Maintaining a limited number of HPV DNA copies allows the virus to produce necessary and sufficient levels of early gene products while minimising cellular damage and avoiding detection by the host immune system.

After the establishment of a persistent infection, PVs enter a stable maintenance phase (latent phase) in which viral genomes, distributed equally to daughter cells as extrachromosomal elements, replicate in synchrony with host cell DNA.

The third phase of HPV infection is initiated in terminally differentiating cells in the upper epithelium layers, during which vegetative viral DNA amplification, reaching up to 1000 copies per cell and late viral gene expression are activated. This productive phase culminates in the synthesis of capsid proteins, the assembly of viral particles, and the release of new virions. An overview of the HPV infection is depicted in Figure 2. All three stages will be reviewed in detail in the following paragraphs.

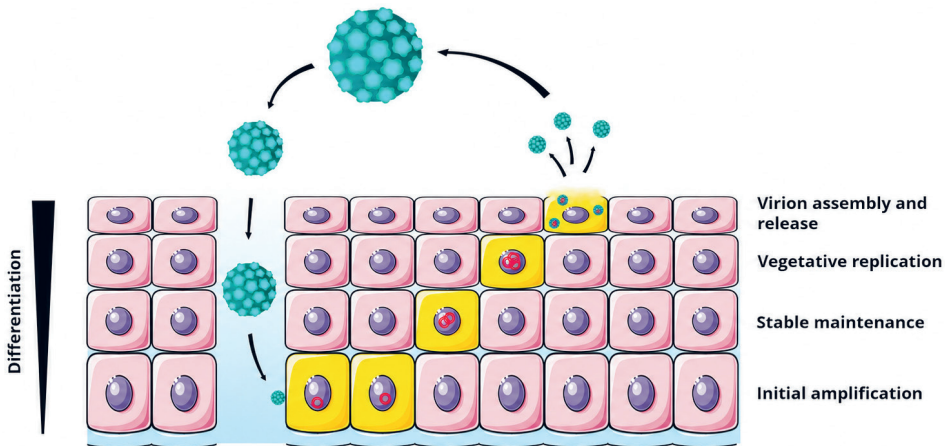


Figure 2. A simplified overview of the productive HPV infection in stratified epithelium. Infected cells are highlighted in yellow, and uninfected (normal) cells are shown in pink. Viral entry at the basal layer and virion release from the upper layers are indicated. The vertical axis on the left represents the stage of epithelial cell differentiation, while a schematic outline of the major phases of the HPV life cycle is provided on the right.

2.4.1. Initial viral genome amplification

After entering the host cell, the viral DNA is transported to the cell nucleus. Since HPV does not carry any pre-synthesised viral replication proteins, such as E1 and E2, the early gene transcription must first be initiated. The early viral promoter (P97 in HPV16 and HPV31, P105 in HPV18) is activated by binding of the host transcription factors (Ap-1, NF-1, Oct-1 and Sp1) (Bernard, 1994b). As a result, the early viral proteins needed for viral genome replication (E1 and E2) and for cell transformation (E6 and E7) are expressed.

For the initiation of the HPV genome replication, several elements are needed: a highly conserved *cis*-acting DNA element *ori*, an adjacent A/T-rich region with an E1 binding site (E1BS) and the viral replication factors E1 and E2 (Remm *et al.*, 1992; Ustav *et al.*, 1991). All remaining proteins required for replication are provided by the host cell (Ustav *et al.*, 1991; Ustav and Stenlund, 1991). These include a replication protein A (RPA), a single-stranded-DNA-binding protein, replication factor C (RPC), proliferating cell nuclear antigen (PCNA), DNA polymerase (α -primase and DNA polymerase γ), several cell cycle-related proteins and topoisomerases (Kurg, 2011).

To launch the initial replication of the HPV DNA, the E2-dimer binds to the palindromic consensus site 5'–ACCG(N)₄CGGT–3' within the *ori*, referred to as the E2 binding site (E2BS). The number of E2 binding motifs can differ among PV genera (Figure 3). For example, α -PVs contain four and β -PVs contain five E2BSs, while seventeen E2BSs are found in δ -PVs (Laaneväli *et al.*, 2019; Li *et al.*, 1989), of which a subset contributes to efficient genome maintenance (M. Piirsoo *et al.*, 1996). In addition to differences in E2BS number, location and distribution of these sites relative to the corresponding promoter also differ between PVs (Rogers *et al.*, 2011).

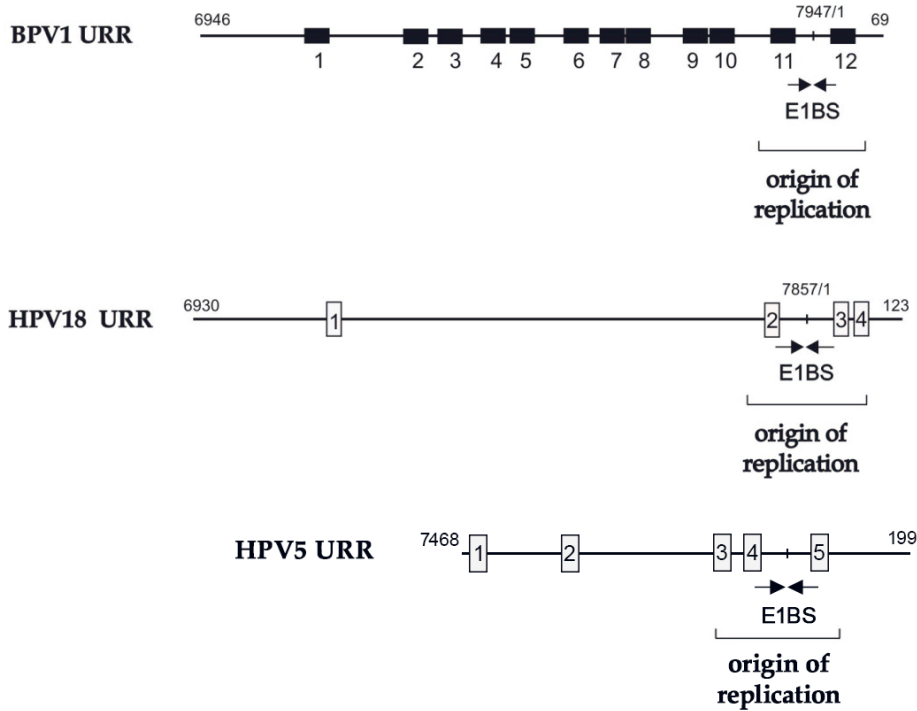


Figure 3. The URR of bovine PV 1 (BPV1), HPV18 and HPV5. The E1BSs are depicted with arrows, and the E2BSs are shown with boxes. The figure is adapted from (Kurg, 2011; Laaneväli *et al.*, 2019).

PV's DNA replication is initiated with the formation of the E1- and E2-complex and E1/E2 cooperative binding at adjacent sites within the ori (Berg and Stenlund, 1997; Mohr *et al.*, 1990). While E1 independently possesses low sequence specificity, E2 promotes ori-specific binding and minimises off-target interactions by recruiting E1 to the ori via direct contact between E2 transactivation domain (TAD) and E1 helicase domain (HD) (Stenlund, 2003) (Masterson *et al.*, 1998; Moscufo *et al.*, 1999; Yasugi *et al.*, 1997). E1-E2-ori complex formation is followed by enrolment of an additional E1 dimer and the displacement of E2 in an ATP-dependent manner (Sanders and Stenlund, 1998). As a result, double E1 trimers are formed on the ori, followed by double E1 hexamer assembly in the presence of ATP. The generated E1 double hexamers as ATP-dependent helicases unwind the DNA double helix in advance of replication fork progression (Schuck and Stenlund, 2005; Sedman and Stenlund, 1998) (Figure 4).

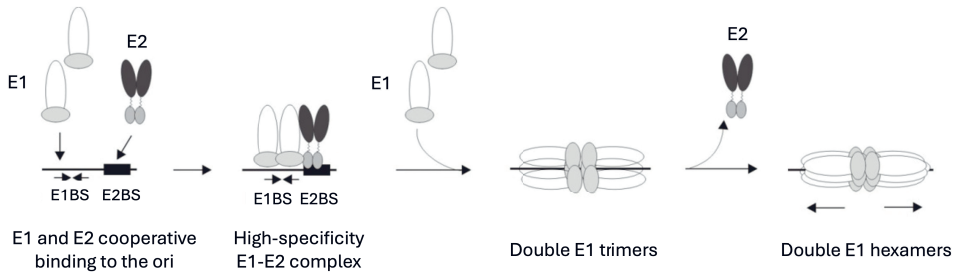


Figure 4. A schematic overview of the viral initiation complex assembly. Replication initiation begins with the cooperative binding of E1 and E2 dimers to their respective binding sites (E1BS and E2BS) within the ori, forming a highly specific E1-E2-DNA complex. Subsequently, additional E1 molecules are recruited to the complex, leading to the displacement of E2. To acquire helicase activity, E1 proteins assemble into a double hexameric complex. The figure is adapted from (Kurg, 2011).

Replication of the HPV genome occurs via bidirectional theta structures (intermediates that resemble the Greek letter θ [theta]) (Flores and Lambert, 1997) with two replication forks, which progress in opposite 5' to 3' directions, resulting in the continuous synthesis of the leading strand and discontinuous synthesis of the lagging strand (Okazaki fragments). Expansion transpires along the circular genome until replication forks converge at the ori. As a result, two daughter molecules are formed, containing one strand from the parental DNA and the other newly synthesised strand (Figure 5).

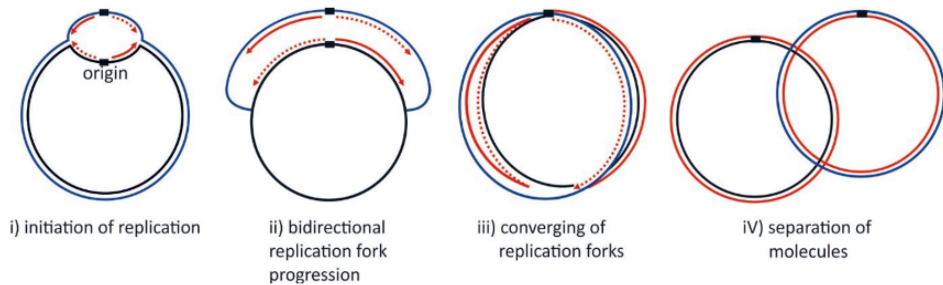


Figure 5. A simplified overview of the bidirectional theta replication. Replication begins at the ori with the formation of two replication forks that advance bidirectionally across the genome. As replication progresses, the forks move in opposite directions and ultimately converge on the opposite side of the genome. Upon fork convergence, the daughter DNA strands are segregated. The figure is adapted from (Liblekas *et al.*, 2021).

2.4.2. Stable maintenance replication

The initial replication phase is crucial for infection establishment and is thought to be rapid. After the infection is established, PVs enter a latent phase in which viral DNA is stably maintained at a nearly constant copy number in proliferating basal cells. This process is believed to occur through maintenance replication. First, replication is tightly regulated to occur approximately once per cell cycle, enabling the viral genome to double each cycle. Second, there is nearly equal segregation of the extrachromosomal genome into the nuclei of the daughter cells. E2 is the key viral protein that mediates the viral genome partitioning and maintenance during the latent phase of infection. Host cellular components involved in the efficient segregation and the tethering mechanism are described in Chapter 2.5.3.3.

For BPV-1 (Bovine papillomavirus 1), the viral genome utilises random-choice replication during the maintenance phase (Gilbert and Cohen, 1987; M. Piirsoo *et al.*, 1996; Ravnan *et al.*, 1992). This process initiates replication, ensuring that, on average, each genome copy is duplicated once per cell cycle, thus maintaining a constant viral copy number. In the case of HPV, two modes of the replication mechanisms used for the maintenance phase have been described (Hoffmann *et al.*, 2006). HPV16 and HPV31 replication during the latent phase was observed in HPV-positive cell lines W12 and CIN612, respectively, which were derived from the naturally infected tissues (Bedell *et al.*, 1991; Stanley *et al.*, 1989). Results were controversial: HPV16 DNA replicated in a once-per-S-phase manner in the W12 cells, whereas in the HPV31-positive CIN612 cells, a random-choice mode was observed. Nonetheless, the individual introduction of HPV16 and HPV31 DNAs into the immortalised keratinocyte cell line NIKS resulted in random-choice replication for both types. This observation indicates that HPV DNA latent phase replication, whether via a random-choice or once-per-S-phase mechanism, may be affected by the cell type harbouring the viral episome. An alternative explanation posits that in W12 cells, the viral genome is integrated, rendering E1-independent replication and synchronising it with the host's DNA replication, which occurs once per cell cycle. In contrast, in CIN612 cells, HPV31 persists in an episomal form. Additionally, the replication mode appears to depend not only on the host cell type but also on E1 protein expression levels, as high E1 expression in W12 cells shifts HPV16 DNA replication to a random-choice mechanism (Hoffmann *et al.*, 2006).

2.4.3. Vegetative viral DNA replication

Following the maintenance phase, the infection enters the vegetative replication phase as infected basal cells leave the basal layer, detach from the basement membrane, and migrate into the suprabasal layers of the stratified epithelium. Typically, in this process, cells complete the proliferative program and enter the differentiation pathway, leading to cell cycle exit and the termination of cell division. Unfortunately for HPV, all cellular DNA replication factors and polymerases are

produced only in the mitotically active cells. However, HPV has managed to overcome this obstacle. The viral E6 and E7 proteins are pivotal in disrupting cellular pathways that trigger growth arrest during differentiation and in establishing and maintaining a cellular environment conducive to viral genome replication (Cheng *et al.*, 1995; Stubenrauch and Laimins, 1999). In the case of α -PVs, E5 also contributes to efficient productive replication by modulating the cell cycle progression and inhibiting apoptosis (Fehrmann *et al.*, 2003; Genther *et al.*, 2003). In addition, several studies have shown that expression of the E4 protein causes cell cycle arrest in G2 (Knight *et al.*, 2006; Nakahara *et al.*, 2002). Consequently, the initial E6/E7-mediated cell cycle progression ensures the availability of the replication machinery, whereas the subsequent E4-induced G2 arrest prevents entry into mitosis. This coordination enables HPV to effectively utilise host replication factors, thereby minimising competition with host DNA synthesis and enhancing viral genome amplification (Moody, 2017).

Activation of the productive phase of the viral life cycle leads to amplification of viral genomes to thousands of copies per cell (Bedell *et al.*, 1991; Ozburn and Meyers, 1998). To enable rapid, high-level viral genome amplification when cellular replication machinery is limited, HPV induces the formation of the viral replication foci. These nuclear replication foci represent “factories” where components required for viral DNA synthesis are concentrated (Swindle *et al.*, 1999). To recruit all the necessary components for viral DNA synthesis, viral proteins induce the DNA-damage response (DDR), leading to activation of the ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) pathway markers (Mehta and Laimins, 2018).

In the vegetative replication phase, the virus switches from bidirectional theta replication, prevalent during the maintenance phase, to a different replication mechanism that supports robust amplification of viral genome copy numbers in differentiated keratinocytes. Early studies indicated a transition to rolling-circle replication (RCR) (Dasgupta *et al.*, 1992; Flores and Lambert, 1997); however, contemporary research has refined this model. Recent findings suggest that what appears to be “rolling-circle” replication in differentiated cells may represent a form of recombination-dependent replication (RDR), often referred to as repair-driven replication (Sakakibara *et al.*, 2013). Consistent with this, replication foci are enriched not only for DDR markers but also for factors involved in homologous recombination (HR) (Gillespie *et al.*, 2012; Maher *et al.*, 2011). These events create a cellular environment that favours HR, which is initiated during the late S and G2 phases without competition from host DNA synthesis. In this context, HPV genomes can undergo RDR accompanied by progressive head-to-tail oligomerisation, generating high-molecular-weight concatemeric episomal DNA. These concatemeric intermediates are later processed into unit-size circular viral genomes, enabling robust vegetative genome amplification (Orav *et al.*, 2013; Sakakibara *et al.*, 2013).

2.5. E1 and E2 as master regulators of the HPV infection cycle

All PVs encode the E1 and E2 proteins, which function together for HPV genome replication and persistence. E1 is the ATP-dependent helicase and the only viral enzyme essential for viral replication. E2 acts as a multifunctional regulator of various aspects of the viral life cycle. Although E1 and E2 perform different molecular functions, they act in concert throughout the HPV life cycle.

2.5.1. Structure of E1

The E1 ORF is considered the largest and most conserved among PVs' early ORFs (Bergvall *et al.*, 2013). With a size ranging from 600 to 650 amino acids, the E1 protein is divided into three functional segments.

The N-terminal domain, 200 amino acids in length, has variable sequences among the PVs but still contains conserved motifs: bipartite nuclear localisation signal (NLS), a Crm1-dependent nuclear export signal (NES), a cyclin-binding domain (CBD) for interaction with cyclins A/E in complex with cyclin-dependent kinase 2 (Cdk2) and phosphorylation sites for different kinases (Lentz *et al.*, 1993; Ma *et al.*, 1999; Xiao and Wilson, 1994). Differences in the N-terminal region of E1 across PV types are thought to arise from adaptation to different hosts and epithelial tissues.

A central part of the E1 is an origin-binding domain (OBD) (also known as DNA-binding domain, DBD) that recognises and binds to the specific E1BS within the ori, which is a crucial step in the initiation of the viral replication (Thorner *et al.*, 1993).

The C-terminal helicase domain (HD) is responsible for assembly into hexamers and unwinding DNA during replication. The stepwise initiation complex assembly is detailed in Chapter 2.4.1. The helicase domain of E1 can also be divided into three functional segments: first, the minimal oligomerisation domain, used for the correct formation of the hexameric complex (Bergvall *et al.*, 2013); second, the ATP-binding module that binds and hydrolyses the ATP (Titolo *et al.*, 2000); and the C-terminal brace necessary for the assembly and stabilisation of the E1 hexamer (Whelan *et al.*, 2011) (Figure 6).

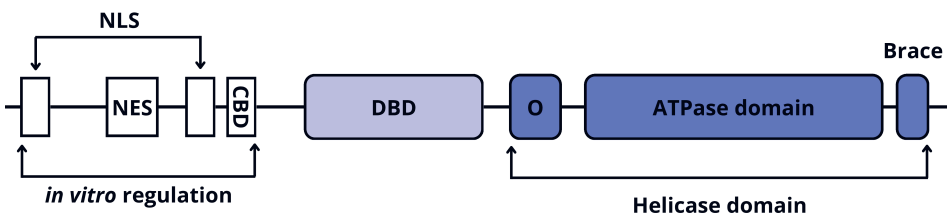


Figure 6. Schematic representation of the E1 structure. The N-terminal regulatory region, with a bipartite nuclear localisation signal (NLS), nuclear export signal (NES), and cyclin-binding domain (CBD), is shown in white boxes. The DNA-binding domain (DBD) is highlighted in light purple, and the C-terminal helicase domain of E1, including the oligomerisation domain (O), ATPase domain, and brace, is shown in deep purple.

2.5.2. Structure of E2 and E8^ΔE2

The full-length E2 is functionally well conserved among all PVs and consists of two functional domains: an N-terminal transactivation domain (TAD) of about 200 amino acids, which is pivotal in regulating viral transcription, replication and episomal maintenance; and the C-terminal DNA-binding/dimerisation domain (DBD) of about 100 amino acids in length that recognises palindromic E2 binding sites in the URR. These domains are connected by the variable-length region of ~40–200 amino acids (depending on the PV type), called the hinge (Figure 7) (Giri and Yaniv, 1988; A. A. McBride, 2013). The hinge is the least conserved and most flexible region of E2 and is largely unstructured, allowing relative mobility between TAD and DBD (Winokur and McBride, 1992).

Apart from the full-length E2, all PVs also encode its N-terminally truncated versions, most frequently described as E8^ΔE2. It is generated via alternative splicing, fusing a 10-13 amino acid segment encoded by the E8 ORF (present in the E1 ORF) through the hinge region with the C-terminal DBD of the E2 (Stubenrauch *et al.*, 2000) (Figure 7). Consequently, E8^ΔE2 lacks the N-terminal transactivation domain of E2, while retaining the ability to dimerise and bind E2-BSs. This enables E8^ΔE2 to compete with full-length E2 for DNA binding and thereby function as a negative regulator (Stubenrauch *et al.*, 2001; Zobel *et al.*, 2003).

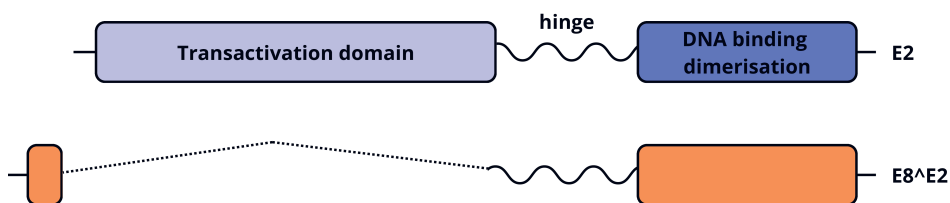


Figure 7. Schematic overview of E2 and E8^ΔE2 protein structures. The N-terminal transactivation domain of E2 is depicted in light purple, and the C-terminal DNA-binding domain is shown in deep purple. These domains are separated by a hinge region, illustrated as a curved line. The fusion protein E8^ΔE2 is shown in orange, with its N-terminal portion derived from E8 ORF and the central and C-terminal parts corresponding to E2.

2.5.3. Functions of E1, E2 and E8^ΔE2

2.5.3.1. E1 role in HPV replication

Helicase E1 is the catalytic driver of HPV genome replication and the only enzyme encoded by HPVs (L. Yang *et al.*, 1993). To promote replication of the viral genome, E1 interacts not only with E2 but also directly binds the host's replication protein A (RPA), which stabilises single strands of the DNA (Loo and Melendy, 2004) and with the DNA polymerase α -primase complex, which provides primers needed for replication initiation and lagging strand DNA synthesis (Park *et al.*, 1994). Interaction of the E1 DBD with topoisomerase I enhances E1 ori-

binding specificity and helps relieve superhelical tension in the viral DNA as the forks progress (Hu *et al.*, 2006).

Because uncontrolled E1 activity is detrimental to host cells, its interactions and localisation are tightly regulated throughout the replication cycle. Besides interaction with the replication factors, E1 has also been shown to associate with other cellular proteins, including p80/Uaf1, hSNF5, histone H1, E1BP, and p56. The N-terminal region of E1 interacts with the cellular WD-repeat-containing protein p80 (USP1-associated factor 1 [Uaf1]), playing a key role in the maintenance phase of the viral life cycle (Côté-Martin *et al.*, 2008). In addition, E1 undergoes regulated nucleocytoplasmic shuttling driven by its NLS and NES motifs. CRM1-dependent nuclear export, modulated by cyclin A/E-Cdk2 phosphorylation, is dispensable for transient replication but is important for long-term episome maintenance, likely by limiting excessive nuclear E1 accumulation (Fradet-Turcotte, Moody, *et al.*, 2010). Association with hSNF5 (a protein from the SWI/SNF complex) has been shown to be required for HPV replication (D. Lee *et al.*, 1999), whereas interaction with histone H1 may relieve chromatin-mediated repression of viral DNA replication (Swindle and Engler, 1998).

Beyond its canonical helicase role, E1 is strongly linked to viral genome amplification in differentiated cells, where E1 levels increase during the productive phase. It was shown that E1 co-localises with DDR factors at viral replication foci (Fradet-Turcotte *et al.*, 2011; Sakakibara *et al.*, 2011). Consistent with a direct role in DDR activation, nuclear accumulation of E1 is associated with activation of ATM/ATR signalling. Both its origin-binding function and ATPase-helicase activities are required for this response and for early S-phase arrest (Fradet-Turcotte *et al.*, 2011; Sakakibara *et al.*, 2011). Taken together, these findings indicate that E1 not only unwinds DNA for replication but also helps to establish replication foci that concentrate DDR components and shape the cellular environment supporting viral genome amplification.

2.5.3.2. E2 role in HPV transcription

Aside from its role in replication initiation, E2 acts as a major regulator of HPV transcription primarily by recruiting cellular factors to viral genomes (Phelps and Howley, 1987; Spalholz *et al.*, 1985). Depending on the context of E2-BSs and the nature of the interacting cellular proteins, E2 proteins can function either as transcriptional activators or repressors.

As an activator, E2 DBD binds to consensus E2 sites located in the 5' region of the viral URR, distal to the early promoter. On the other hand, transcriptional repression occurs when E2-BSs are positioned proximal to the early promoter and overlap cellular transcription factor motifs, thereby preventing formation of the transcriptional initiation complex (Thierry, 2009). The outcome of E2 regulation is also defined by the levels of the E2 protein (Fujii *et al.*, 2001; Steger and Corbach, 1997). It has been demonstrated that the HPV18 E2 protein exhibits the highest binding affinity for E2BS-1, whereas binding to the promoter-proximal low-affinity sites E2BS-3 and E2BS-4 is reduced (Figure 8) (Demeret *et al.*, 1997;

Steger and Corbach, 1997). Consequently, at low E2 concentrations, E2BS-1 is occupied, allowing the early promoter to remain activated. As E2 accumulates, occupancy extends to E2BS-2 and the promoter-proximal E2BS-3 and E2BS-4, which interfere with host transcription factor binding and switch regulation toward repression. This progressive, E2 dose-dependent occupancy provides a gradual mechanism for early gene expression control and links repression of the early promoter to the onset of viral DNA replication, since these specific E2 binding sites are crucial for silencing early gene expression and facilitating replication initiation (Figure 8).

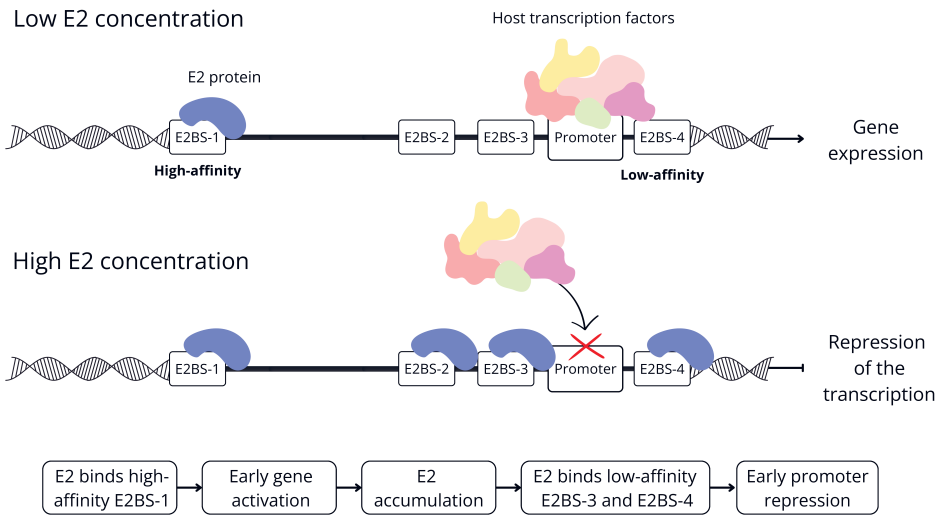


Figure 8. Dose-dependent transcriptional regulation by E2 proteins. At low concentrations, E2 binds to the high-affinity E2BS-1, supporting activation of early transcription. At higher E2 levels, low-affinity sites (E2BS-3/4) in proximity to the promoter become occupied, thereby preventing host transcriptional factors from binding and repressing early gene expression.

Interactions with the basic components of the transcriptional machinery and chromatin remodelling complexes help the E2 protein modify viral gene expression. The TAD of the E2 serves as a platform for assembling various cellular transcription factors, including specificity protein 1 (Sp1), TATA-binding protein (TBP), transcriptional factor II B (TFIIB) (Benson *et al.*, 1997; Breiding *et al.*, 1997; Rank and Lambert, 1995; Yao *et al.*, 1998). It also recruits chromatin-remodelling complexes, such as histone acetyltransferases p300/CBP, the histone acetylase p/CAF, and the SWI/SNF chromatin remodelling complex via Brm (Brahma protein) (Kumar *et al.*, 2007; D. Lee *et al.*, 2002; Li *et al.*, 1991; Müller *et al.*, 2002; Rank and Lambert, 1995). The bromodomain protein (Brd4) is another pivotal E2 co-factor that has been described as having both transcriptional activation and repression functions of the early promoter (Ilves *et al.*, 2006; A. McBride and Jang, 2013). Brd4 acts as a linker between the E2 protein and the

positive transcription elongation factor (P-TEFb) and Mediator complexes, connecting them to the general transcription machinery (Jang *et al.*, 2005; Schweiger *et al.*, 2006). It has been shown that Brd4 serves as a critical element of the HPV11 E2 transcriptional repression complex, which suppresses E6/E7 promoter activity (Wu *et al.*, 2006). Brd4 promotes the recruitment of E2 to the E6 promoter, and upon binding to the promoter, E2 inhibits transcription by preventing transcription factor II D (TFIID) attachment to the TATA box and by restricting RNA polymerase II (Pol II) from reaching the E6 promoter region, thus suppressing viral oncogene expression.

2.5.3.3. E2 role in HPV genome maintenance

After the initial amplification and during latent infection, the HPV genome is sustained as an episomal element in the nucleus of infected proliferative keratinocytes. To ensure persistence in infected cells, the viral episomes must be accurately partitioned to daughter cells during mitosis, preventing loss during basal cell division and facilitating proper localisation within the re-forming nucleus after mitotic exit. This partitioning requires the presence of the E2 protein in *trans* and several E2 binding sites in *cis* relative to the viral replication origin (M. Piirsoo *et al.*, 1996). The C-terminal domain of the E2 binds to the viral genome via E2BSs, whereas the N-terminal domain associates with host mitotic chromatin, forming an analogy of the ‘bridge’ (Ilves *et al.*, 1999; Skiadopoulos and McBride, 1998). However, different PV genera use distinct cellular partners to anchor E2 to mitotic chromatin (Oliveira *et al.*, 2006). Brd4 is the best-characterised of these proteins. It has been demonstrated that in the case of BPV1 and some α -HPVs, E2 interaction with Brd4 through TAD is crucial for the viral plasmid segregation function of E2 (Helfer *et al.*, 2013; A. McBride and Jang, 2013; You *et al.*, 2004). Brd4’s capacity to bind acetylated histones results in its association with chromatin during mitosis (Dey *et al.*, 2000, 2003), aligning with observations that E2 can remain linked to cellular chromosomes throughout mitotic stages (Bastien and McBride, 2000). Nonetheless, this mechanism is not universal. In β -HPVs, E2 proteins predominantly localise to pericentromeric regions of mitotic chromosomes and use a Brd4-independent pathways for genome tethering (Oliveira *et al.*, 2006). The HPV8, for instance, anchors to ribosomal DNA (rDNA) loci via the UBF, a transcription factor for RNA polymerase I (Poddar *et al.*, 2009). Unlike BPV1 E2, which requires its TAD for chromatin binding, HPV8 E2 employs its hinge region and DBD for mitotic chromosome association (Poddar *et al.*, 2009).

More recent work has expanded this classical chromatin-tethering model of E2-mediated genome maintenance by identifying additional host factors that support segregation and episome stability. In addition to Brd4, the DDR scaffold protein TOPBP1 has emerged as an important E2 partner, particularly in HPV16, where the E2-TOPBP1 interaction is required for efficient plasmid segregation or retention during mitosis (Prabhakar *et al.*, 2022). This interaction also promotes increased E2 levels during mitosis, supporting the idea that TOPBP1 contributes to both segregation and E2 stability. In line with this, disruption of the E2-

TOPBP1 complex has been reported to compromise E2 and viral genome stability during the viral life cycle and can promote viral genome integration (Prabhakar *et al.*, 2023). Recent studies further suggest that E2-TOPBP1 does not act solely as a passive tether but can activate DDR, promoting TOPBP1 association with mitotic chromatin and thereby supporting efficient viral genome segregation (Prabhakar and Morgan, 2024). Taken together, these findings collectively demonstrate that E2-mediated maintenance involves both chromatin tethering and coupling to host genome surveillance pathways to ensure stable segregation and preservation of viral episomes during host cell division.

2.5.3.4. E8^ΔE2 functions in the HPV life cycle

The E8^ΔE2, as a truncated form of E2 lacking the N-terminal TAD, exerts its impact at several stages of the HPV life cycle, serving as a principal regulator of both viral DNA transcription and replication. In several HPV types, E8^ΔE2 is transcribed using a separate promoter that is distinct from the major early promoter (Sankovski *et al.*, 2014; Straub *et al.*, 2015), and therefore its expression is not modulated by enhancer elements in the URR. Instead, the constitutive activity of the E8 promoter depends on two conserved elements, including CE2 and CE3, which interact with cellular proteins. Moreover, E8^ΔE2 is capable of inhibiting its own promoter in an E8 domain-dependent manner, indicating negative autoregulation of its expression (Straub *et al.*, 2015). This unique utilisation of the promoter and its autoregulatory capabilities underscores the specific functional identity of E8^ΔE2, resulting in the interplay between E2's structural domains and regulatory elements derived from E8 (Stubenrauch *et al.*, 2001; Zobel *et al.*, 2003).

As a transcriptional repressor, E8^ΔE2 antagonises the functions of E2 by competitive binding to the E2BSs within the regulatory region (Lim *et al.*, 1998). The N-terminal part of E8 recruits the cellular co-repressor complexes NCoR/SMRT-HDAC3. As a result, the engaged complexes modify chromatin structure, leading to transcriptional silencing (Ammermann *et al.*, 2008; Powell *et al.*, 2010) and repressing the expression of the HPV early proteins. E8^ΔE2 also acts as a viral DNA replication repressor. Experimental disruption of E8^ΔE2 results in excessive viral DNA amplification (Kurg *et al.*, 2010; Lace *et al.*, 2008; Sankovski *et al.*, 2014; Stubenrauch *et al.*, 2000). The replication repression is also thought to occur by competing for E2-binding sites. Although functioning as a negative regulator, E8^ΔE2 is crucial for the sustained episomal preservation of HPV31 genomes in keratinocytes (Stubenrauch *et al.*, 2000). Nonetheless, E8^ΔE2 of HPV16 inhibits plasmid amplification, yet is not essential for plasmid persistence and maintenance (Lace *et al.*, 2008; Straub *et al.*, 2014).

2.6. Regulation of the HPV life cycle by host protein kinases

Proteins undergo a range of post-translational modifications that regulate their activity. Among all, phosphorylation, the covalent addition of a phosphate group, is one of the most common. Phosphorylation induces functional alterations in substrate proteins, affecting their stability, enzymatic activity, intracellular localisation, or binding affinity with other proteins. This reaction is catalysed by protein kinases that transfer the phosphate group from ATP to substrate proteins. The process occurs within the catalytic domain of the kinase, which contains an ATP-binding pocket and a specific recognition site (a phosphorylation consensus motif) for the substrate. The hydroxyl (-OH) group of the substrate's target amino acid engages the terminal phosphate of ATP, resulting in the cleavage of the high-energy bond. This leads to the release of ADP and the establishment of a covalent bond between the phosphate group and the substrate. This alteration is not permanent and can be reversed or dephosphorylated (Figure 9).

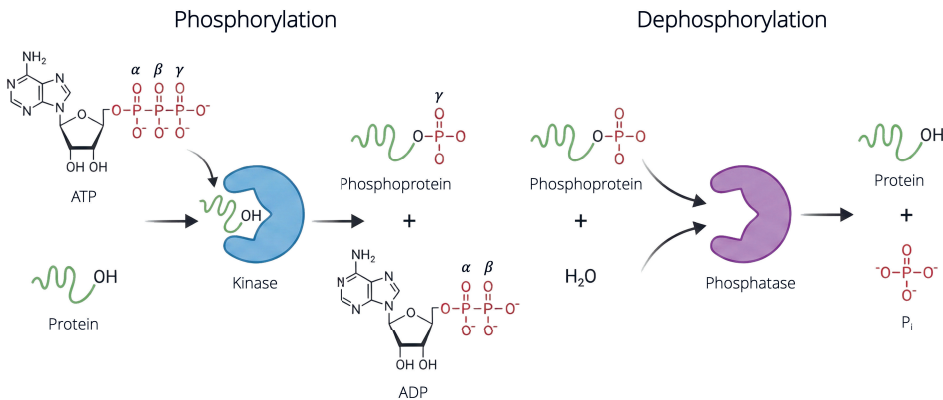


Figure 9. Schematic overview of protein phosphorylation and dephosphorylation. A kinase (blue) conducts transfer of the terminal phosphate (γ) from ATP to the substrate (green), and a phosphatase (purple) removes the phosphate by hydrolysis, releasing P_i (inorganic phosphate).

Protein phosphatases are responsible for the removal of the phosphate groups, making the whole system a dynamic and reversible process. Kinases and phosphatases consistently work together to alter the phosphorylation state of proteins and function as a molecular switch that may be rapidly activated or deactivated. In eukaryotic cells, most protein kinases target either serine, threonine or tyrosine residues, while a minority can phosphorylate all three (Pollard *et al.*, 2017). The human kinome comprises approximately 500 protein kinase genes, representing about 2% of all human protein-coding genes (Manning *et al.*, 2002). This indicates the extensive importance of protein phosphorylation in cellular control.

Both E1 and E2 are phosphoproteins, and their activities are tightly regulated by host cell kinases. Phosphorylation of specific sites can modulate E1/E2

properties, including DNA binding, nuclear localisation, half-life, protein-protein interactions, and replication capacity. Although the host cell encodes hundreds of kinases, the following overview focuses on those for which direct phosphorylation of HPV replication regulators has been experimentally established and linked to specific functional outcomes, making them the most informative examples of kinase regulation over the viral life cycle.

Mitogen-activated protein kinases (MAPKs), including the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), are essential signalling proteins that are involved in different signalling pathways and regulate cell growth, differentiation, stress responses and apoptosis (Cargnello and Roux, 2011; Pearson *et al.*, 2001). In HPV11, ERK and JNK directly phosphorylate E1 at two serine residues (S89 and S93) located adjacent to the NLS. Phosphorylation at these sites is essential for the nuclear import of E1. Alanine substitutions at S89/S93 markedly reduced E1 nuclear accumulation, whereas phosphomimetic substitutions partially restore nuclear localisation and, consequently, replication efficiency (J.-H. Yu *et al.*, 2007). Additionally, E1 contains two conserved MAPK docking motifs near the C-terminus. These motifs are thought to anchor ERK/JNK to E1 for efficient phosphorylation of the NLS-proximal serines. Mutating either docking motif or pharmacologically inhibiting MAPKs impairs E1 nuclear import. Both motifs and their associated MAPK target sites exhibit high conservation across various PVs, including HR α - and cutaneous β -HPVs. This conservation suggests that MAPK-mediated regulation of E1 is a prevalent strategy among PVs. Furthermore, this mechanism is proposed to play a crucial role in the establishment of persistent HPV infection (J.-H. Yu *et al.*, 2007).

Serine-arginine protein kinase 1 (SRPK1) belongs to serine-threonine kinases that phosphorylate serine residues in SR/RS dipeptide motifs. Its function is to phosphorylate SR-family splicing factors, promoting their nuclear import and pre-mRNA splicing. As a result, SRPK1 also influences downstream steps in mRNA metabolism, such as mRNA export and translation. In addition, SRPK1 has been implicated in chromatin reorganisation, metabolic signalling, and regulation of cell cycle and p53 (Giannakouros *et al.*, 2010). The hinge regions of E2 proteins from many HPV types across different genera contain several SR/RS motifs, suggesting they are plausible SRPK1 substrates. It has been demonstrated that, in the case of HPV5, the E2 hinge region contains numerous SR motifs (27 in total), at least one of which has been phosphorylated. This modification resulted in the relocalisation of E2 from the nucleus to the cytoplasm (Lai *et al.*, 2000). SRPK1-dependent regulation of E2 subcellular trafficking was also demonstrated for HPV1. Mutating the putative phosphoacceptor serines or inhibiting SRPK1 increased the fraction of cells showing prominent nucleolar E2 accumulation, suggesting that SRPK1 phosphorylation modulates E2 intranuclear localisation rather than acting as a simple switch of nucleolar targeting signal (Prescott *et al.*, 2014). Moreover, SRPK1 was demonstrated to interact with E1^{E4} proteins from HPV1, HPV16 and HPV18. In differentiated HPV1 wart tissue and keratinocytes, E1^{E4} forms cytoplasmic inclusion bodies and recruits SRPK1 into these aggregations, leading to local accumulation of the kinase. The binding inhibits SRPK1

autophosphorylation, which may stabilise the kinase within the inclusions. This sequestration reduces SRPK1 availability for its cellular substrates and limits its ability to perform its normal cellular functions, thereby creating a more favourable environment for the virus to complete its replication cycle and be released from the cell (Bell *et al.*, 2007).

Cyclin-dependent kinases (CDKs) are crucial regulators of cell cycle progression also capable of modulating transcription in response to various extra- and intracellular signals. Their enzymatic activity depends on the binding of a specific cyclin, which provides structural elements required for kinase activation (Malumbres, 2014). In the context of HPV infection, CDK activity, especially CDK2, helps couple E1/E2-driven replication to the host cell cycle. In HPV11 E1, the CRM-dependent NES promotes cytoplasmic export, whereas phosphorylation of S107 within the NES by CDK2 inhibits its activity and promotes E1 retention in the nucleus during S phase, when viral DNA replication occurs (Deng *et al.*, 2004). Together with the MAPK-mediated stimulation of nuclear import described above, this mechanism regulates the nuclear accumulation of E1. In addition to localisation control, CDK2-dependent phosphorylation has been linked to efficient initiation of viral DNA replication. Besides the N-terminal serine residues, T468 within the C-terminal helicase domain has also been implicated in replication initiation. Disrupting cyclin-CDK2 docking and/or mutating the CDK phosphorylation sites reduces E1/E2-dependent origin replication (Ma *et al.*, 1999). CDK2 also targets the E2 protein, demonstrated *in vitro* by the phosphorylation of HPV16 E2 at T286 within its DBD (Johansson *et al.*, 2009). Increased E2 phosphorylation and stability during S-phase suggest that CDK2 may regulate E2 function in viral DNA replication alongside E1 during this cell cycle stage.

The fibroblast growth factor receptors (FGFRs) are transmembrane receptor tyrosine kinases activated by FGF ligands. FGFRs mediate cell growth, proliferation, differentiation and migration, depending on the cell's nature (Touat *et al.*, 2015). FGFR3 is proposed to phosphorylate the E2 protein during the early stages of infection within proliferating basal keratinocytes, consequently reducing its ability to support viral DNA replication and episome maintenance. Upon keratinocyte differentiation, FGFR3 expression is downregulated, permitting the dephosphorylation of E2. Since E2 localises in the nucleus, this model is consistent with the ability of FGFR family receptors to undergo intracellular trafficking and nuclear translocation (Tuzon *et al.*, 2019; Y. Xie *et al.*, 2020). This regulatory shift facilitates the reactivation of E2 function, enabling robust amplification of the HPV genome in differentiated suprabasal cells (F. Xie *et al.*, 2017).

Focal adhesion kinase (FAK) is a cytoplasmic non-receptor tyrosine kinase that functions as a central mediator of integrin- and growth-factor-dependent signalling, linking extracellular matrix adhesion to cell survival, proliferation, migration, and cytoskeletal organisation (Sulzmaier *et al.*, 2014). In HPV studies, FAK was shown to bind HR HPV E2 and increase its tyrosine phosphorylation during initial amplification. Depletion of FAK increased HPV16 DNA content, suggesting that after viral infection, HPV may utilise host kinase machinery to

restrict excessive E2-dependent viral genome replication (Jose *et al.*, 2023). Protein tyrosine kinase 2 (Pyk2) is a FAK-related non-receptor tyrosine kinase involved in calcium-dependent signalling, stress responses, cell adhesion-associated signalling, and activation of downstream MAPK pathways. Pyk2 is expressed in epithelial cells and has also been implicated in cancer-associated processes, including cell proliferation, migration, invasion, and tumour progression (Shen and Guo, 2018). Similar to FAK, Pyk2 has been linked to the negative regulation of HPV replication, as it phosphorylates HPV E2 at Y131 within the TAD, interferes with viral genome replication, and may help maintain the viral genome at a low copy number during early stages of infection (Jose *et al.*, 2020).

2.6.1. Protein kinase A

The human kinome is divided into nine sub-families, one of which is the AGC group of protein kinases. This group encompasses the serine/threonine kinases of the protein kinase A, G and C families. These enzymes are responsive to changes in local concentrations of cytoplasmic second messengers, including cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), as well as lipids (Leroux *et al.*, 2018). cAMP-dependent protein kinase, also called protein kinase (PKA), is one of the best-described members of the AGC kinases.

2.6.1.1. PKA structure and regulation

PKA exists in its inactive form as a tetrameric holoenzyme, comprised of two regulatory (R) and two catalytic (C) subunits (Krebs and Beavo, 1979). The R subunits of PKA are classified into two distinct types, RI and RII, each with two isoforms: RI α , RI β , RII α , and RII β . PKA holoenzymes containing RI subunits are referred to as type I PKA, while those with RII subunits are termed type II (Corbin *et al.*, 1977). These isoforms differ in their tissue distribution, subcellular localisation, and expression levels and have been suggested to exhibit varying sensitivities to cAMP, contributing to the functional diversity of PKA signalling (Cadd and Stanley McKnight, 1989). The catalytic subunits of PKA, namely C α , C β and C γ , are encoded by the *PRKACA*, *PRKACB* and *PRKACG* genes, respectively. C α and C β subunits exhibit widespread expression in various tissues, with C α is the predominant isoform. The C γ subunit is primarily restricted to germ cells in the human testis (Beebe *et al.*, 1990; S  berg *et al.*, 2013).

As the PKA has a broad spectrum of intracellular targets and lacks the localisation domains, its binding to specific substrates has to be targeted by a scaffold protein. A-kinase-anchoring proteins (AKAPs) are crucial for the subcellular targeting of PKA in proximity to preferred substrates (Scott *et al.*, 1990). The amphipathic helix of AKAP binds to the inactive PKA via the R subunits, guiding the formed PKA-AKAP complex to cellular organelles, membranes or structural proteins (Carr *et al.*, 1991). AKAPs facilitate the localisation of PKA in proximity to target substrates and enzymes that govern its activation and deactivation.

The control of PKA catalytic activity is primarily governed by the second messenger cAMP, which upon binding, triggers a series of conformational and functional alterations. cAMP regulates various biological processes in cells: cellular metabolism, ion channel activation, gene expression, cell growth, differentiation, and apoptosis. The intracellular cAMP concentrations are predominantly regulated by G protein-coupled receptors (GPCRs), which react to external ligands, including hormones and neurotransmitters (Cabrera-Vera *et al.*, 2003). Upon ligand interaction, the GPCR undergoes a conformational alteration that promotes the exchange of GDP for GTP on the associated $G\alpha$ subunit. The GTP-bound form of $G\alpha_s$ (stimulatory G protein) dissociates from the β and γ subunits and activates adenylyl cyclase, a membrane-bound enzyme that catalyses the conversion of ATP to cyclic AMP (cAMP) (Figure 10). In contrast, GPCRs associated with $G\alpha_i$ (inhibitory G protein) limit adenylyl cyclase activity, therefore down-regulating cAMP synthesis. The equilibrium between $G\alpha_s$ - and $G\alpha_i$ -mediated signalling controls the overall concentration of cAMP within the cell. $G\alpha$ subunits hydrolyse GTP to GDP, deactivating themselves, whereas phosphodiesterases (PDEs) convert cAMP to AMP, therefore maintaining stringent temporal regulation of cAMP-dependent pathways (Alberts *et al.*, 2008; Sassone-Corsi, 2012).

The PKA activation occurs upon binding of four cAMP molecules to its R subunits, with two cAMP molecules associating with each subunit. This interaction causes a conformational change that initiates the dissociation of the C subunits, while the R subunits continue to associate with cAMP (Figure 10). The released C subunits acquire catalytic activity following ATP binding (London *et al.*, 2020). The activity of PKA is tightly regulated by cytosolic cAMP concentrations, and even slight variations in cAMP levels can result in substantial alterations in kinase activity. As previously noted, the degradation of cAMP is primarily mediated by PDEs, which, as a result, leads to the downregulation of PKA activity (Plattner and Bibb, 2012). In contrast, intracellular signals such as bicarbonate and calcium ions act to elevate cAMP levels, thereby enhancing PKA activation (Y. Chen *et al.*, 2000). Beyond cAMP-mediated regulation, PKA activity is also modulated through various phosphorylation events, including autophosphorylation and phosphorylation by other regulatory kinases (Byrne *et al.*, 2016).

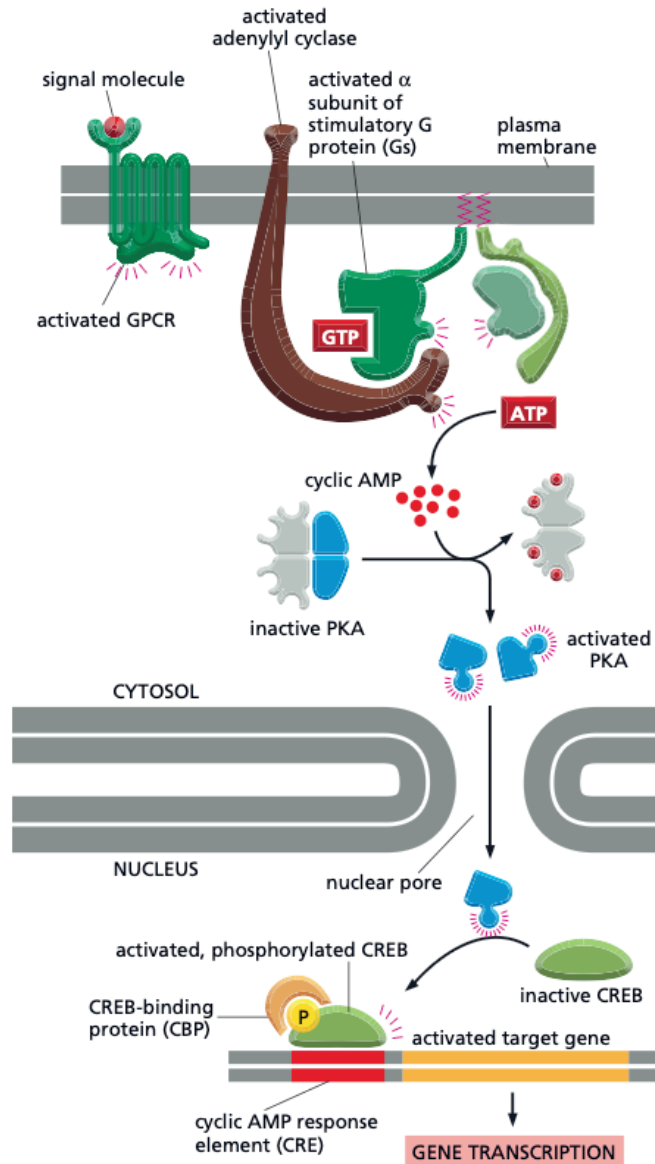


Fig 10. PKA-dependent regulation of gene transcription by cAMP. The pathway starts with the binding of a signal molecule to the GPCR located on the cell membrane. This activates the G_s protein, which then stimulates the enzyme adenylyl cyclase. Consequently, the intracellular concentration of the second messenger cAMP increases rapidly. The elevation of cAMP activates PKA by dissociating its catalytic subunits. The active subunit enters the nucleus and phosphorylates the gene regulatory protein CREB. Upon phosphorylation, CREB recruits the coactivator CBP and binds to CRE, which initiates the transcription of target genes. The figure is adapted from (Alberts *et al.*, 2008).

2.6.1.2. PKA role in cell and HPV viral cycle regulation

PKA have a large spectrum of functions, depending on the cell type and nature, influencing signal mechanisms in different regulatory pathways. The primary physiological function of PKA is in the regulation of glucose homeostasis and the storage of triglycerides (Miyoshi *et al.*, 2006; H. Yang and Yang, 2016). Additionally, PKA phosphorylates many intracellular signalling and effector proteins, for instance, also in the nucleus to alter gene transcription. All genes regulated by PKA contain a *cis*-acting DNA element sequence CRE (cAMP-response element). Phosphorylation of the CRE-binding protein (CREB) leads to its binding to CRE-containing target genes and recruits RNA polymerase II, stimulating gene transcription (K. A. W. Lee and Masson, 1993) (Figure 10). Furthermore, PKA controls the cell cycle regulation (Matsuda *et al.*, 2014) and has been found to act in the cell proliferation and differentiation pathways (F.-X. Yu *et al.*, 2013).

PKA, as a ubiquitous cell kinase, has been demonstrated to be implicated in multiple stages of the HPV life cycle. In β -HPV8, PKA phosphorylates the E2 hinge region at serine-253, promoting binding to host chromatin and mitotic chromosomes (Sekhar and McBride, 2012). Interestingly, the HR α -HPV16 E2 has a functionally analogous site (Ser-243 in the hinge) that is phosphorylated and required for tethering to mitotic chromatin via the Brd4 protein (Chang *et al.*, 2014). In addition to E2, PKA signalling interacts with the HPV life cycle concerning viral and cellular gene expression. PKA is significantly expressed in the basal layers of the epithelium of the skin and cervix, which are vulnerable to HPV infection (Bernard, 1994a). Expressed in the upper levels of epithelial cells, the E6 protein contains a C-terminal PDZ-binding motif (PBM) that allows it to bind and degrade certain host tumour suppressor proteins. Deluri *et al* have demonstrated that PKA negatively regulates HPV18 E6's PDZ interactions, limiting the oncoprotein's ability to drive cell proliferation (Delury *et al.*, 2013).

2.6.2. Casein kinase 2

In 1954, casein kinase 2 (CK2) was demonstrated as the first protein kinase by Burnett and Kennedy (Burnett and Kennedy, 1954). They used casein protein as an *in vitro* kinase assay substrate and showed robust phosphorylation activity towards it, as it possesses accessible serine and threonine residues near acidic amino acids. However, casein is unlikely to be a natural substrate of CK2 and is phosphorylated by another kinase. Nonetheless, the name remained unchanged to prevent confusion with kinases identified later (Venerando *et al.*, 2014). CK2, in contrast, phosphorylates hundreds of other substrates and is involved in virtually all cellular processes.

2.6.2.1. CK2 structure and regulation

CK2 is a ubiquitously expressed heterotetrameric holoenzyme, composed of two catalytic and two regulatory subunits. Catalytic subunits (α and/or α') are encoded by two different genes: *CSNK2A1* and *CSNK2A2*, respectively. Mammalian tetrameric CK2 complexes can enclose identical (two CK α or two CK α') or non-identical (one CK α and one CK α') catalytic subunits (Gietz *et al.*, 1995). In contrast, regulatory subunits (β) are present in only one form and are encoded by a single *CSNK2B* gene. Although CK2 α and CK2 α' subunits share ~90% sequence identity, there are distinctions between their specificity and functionality, for example, β subunit binding capacity, subcellular localisation, specific protein interactions and subunit's regulation during cell cycle progression (Bosc *et al.*, 1995; Gietz *et al.*, 1995; Heriche *et al.*, 1997; St-Denis *et al.*, 2011; I. J. Yu *et al.*, 1991).

In contrast to most protein kinases, CK2 does not require activation by second messengers and is constitutively active. To maintain its high activity, CK2 α acquires an unusually long activation loop, making extensive contact with the N-terminal lobe (Sarno *et al.*, 2002). This interaction relieves CK2 of the need for phosphorylation of the activation lobe or binding of second messengers for activity. Although the free CK2 α is catalytically active on its own, CK2 β still plays an essential role in modulating substrate specificity, stabilising the holoenzyme, and regulating its autophosphorylation (Donella-Deana *et al.*, 2001; Poletto *et al.*, 2008; Zhang *et al.*, 2002). In the absence of the CK2 β , CK2 α can undergo autophosphorylation at Tyr182 (CK2 α) or Tyr183 (CK2 α'), whereas binding of CK2 β counteracts this modification (Niefind *et al.*, 2001). Within the assembled holoenzyme, CK2 β can also be phosphorylated *in trans* by a catalytic CK2 α or CK2 α' subunit. This phosphorylation occurs at conserved N-terminal serine residues and contributes to the regulation of CK2 β stability (Zhang *et al.*, 2002).

2.6.2.2. CK2 role in cell and HPV viral cycle regulation

CK2 localisation has been detected within multiple subcellular compartments, including the nucleus, cytoplasm, organelles, and even the plasma membrane and its outer surface (Faust and Montenarh, 2000). This widespread localisation enables CK2 to interact with a broad range of substrates in diverse cellular environments, thereby contributing to the regulation of numerous biological processes and highlighting its pleiotropic nature. One of the key processes modulated by CK2 is the cell cycle, where it acts at multiple checkpoints to coordinate proper progression and division. For example, CK2 β regulates the stability of the Wee1 protein, thereby promoting the G₂/M transition (Yde *et al.*, 2008). CK2 also impacts the G₁/S transition by phosphorylating the CDK inhibitor KIP1, leading to p27's degradation (Tapia *et al.*, 2004). Additionally, CK2 has been shown to modulate levels of key cell cycle regulators through control of protein synthesis (Homma *et al.*, 2005). Furthermore, CK2 plays a crucial role in transcription and various

signalling pathways by phosphorylating key regulators such as transcription factors (e.g., NF- κ B, p53) and signalling intermediates (e.g., I κ B α , β -catenin), thereby promoting cell survival, proliferation, and stress responses (Jaksch and Thams, 2014; Kato *et al.*, 2003; Meek and Cox, 2011; D. H. Song *et al.*, 2003). CK2 is also known to participate in the regulation of apoptosis (Litchfield, 2003).

The HPV's reliance on host cellular machinery suggests CK2 as a possible regulator of the viral life cycle. The N-terminal domain of the E1 protein and the hinge region of the E2 possess phosphorylation sites that are targeted by CK2. Phosphorylation of the E1 protein in BPV1, HPV11, and HPV31, and of the E2 protein in BPV1, reduces their DNA-binding capacity, thereby hindering the initiation of viral replication. Consequently, CK2 is considered a negative regulator of PV replication (Schuck *et al.*, 2013). CK2 may also act as a positive regulator. It has been shown that CK2 phosphorylates Brd4, resulting in tighter interactions between E2 and Brd4 (Iftner *et al.*, 2017). Additionally, phosphorylation of HPV16 E2 by CK2 promotes its interaction with host protein TopBP1 (Prabhakar *et al.*, 2021). This E2-TopBP1 complex is crucial for tethering viral genomes to mitotic chromosomes and for optimal viral DNA segregation to daughter cells.

3. OBJECTIVES OF STUDY

The biological activity of PV proteins relies on tightly regulated post-translational modifications to coordinate genome maintenance and produce new viral copies. While the life cycle and virus-host interactions of HR mucosal α -HPVs have been extensively studied, significantly less is known about how host cell signalling pathways regulate other HPV types and whether these mechanisms differ between genera. In particular, the roles of protein kinases CK2 and PKA in modulating the functions of the viral proteins E1 and E2 have not been systematically compared across HPV types. This gap is especially relevant for β -HPVs, such as HPV5, a cutaneous type classified as a probable HR HPV, which contribute to carcinogenesis under conditions of immunosuppression and UV exposure. A deeper understanding of the mechanisms by which host-cell signalling controls β -HPV replication, and the ways in which these processes differ from α -HPVs, is essential both for advancing basic knowledge and for identifying new antiviral therapies.

The general objective of this thesis was to identify and characterise functionally important phosphorylation sites in the HPV5 E2 protein, and to determine how two host protein kinases, CK2 and PKA, regulate HPV's replication and transcription, using β -HPV5 in comparison with the α -HPV types 11 and 18.

The specific objectives of this study were as follows:

1. To analyse the phosphorylation pattern of β -HPV5 E2 protein, to identify the highest scoring phosphoresidues of E2 and functional consequences of these phosphorylation events (Ref. **III**);
2. To investigate the role of CK2 in regulating the replication of several α - and β -HPV genomes by combining RNAi-mediated depletion and pharmacological inhibition of CK2 α and CK2 α' in human cells permissive for HPV replication (Ref. **I**);
3. To investigate and compare the role of PKA signalling in regulating the initial amplification and maintenance phases of α - and β -HPV genome replication, with the further goal to identify key viral targets of PKA (Ref. **II**).

4. MATERIALS AND METHODS

The materials and methods employed in this thesis are described in detail within the appropriate sections of each publication. However, several explanations that facilitate the clarity of this work will be provided.

Most of the experiments were carried out using the U2OS cell line, originally established in 1964 from a moderately differentiated osteosarcoma of the tibia of a 15-year-old girl. U2OS cells exhibit an epithelial-like morphology, retain functional tumour suppressor proteins such as p53 and pRb, commonly inactivated in other cancer cells, and display elevated expression of the c-Myc oncogene (Isfort *et al.*, 1995; Wesierska-Gadek and Schmid, 2005). The overexpression of the c-Myc oncogene is thought to impair the normal control of cell division in U2OS cells, allowing them to keep dividing even under high-density conditions, which is likely to result from c-Myc activating genes involved in cell growth and proliferation. As one of the earliest established human cell lines, U2OS is widely used in biomedical research, particularly for studies of DNA replication, repair, and virus-host interactions. Importantly, U2OS cells can be transfected with high efficiency and support HPV genome replication during both transient and stable phases, while also permitting amplification of the viral genome, which characterises the productive phase of the viral life cycle (Geimanen *et al.*, 2011; Orav *et al.*, 2013; Sankovski *et al.*, 2014). Notably, U2OS cells do not support the expression of viral structural (late) proteins or the production of infectious virions. While this feature limits the system's ability to fully recapitulate the complete HPV life cycle, it ensures laboratory safety by eliminating the risk of viral infection during handling.

In the present study, the transient and stable stages of HPV replication were simulated by transfecting U2OS cells with HPV genomes and propagating monoclonal cell lines carrying extrachromosomal viral genomes. Circular HPV genomes were produced using the minicircle protocol described by Kay *et al.* (2010), which relies on an arabinose-inducible bacterial recombination system. In this system, the Φ C31 integrase excises the HPV genome from the plasmid to form a minicircle, while the SclI endonuclease degrades the remaining plasmid backbone, ensuring that only minicircle DNA is produced (Kay *et al.*, 2010).

Although U2OS cells provide a robust and experimentally convenient system for studying HPV genome replication, they are not the natural host cells of HPV. Therefore, selected experiments were also performed in human primary epidermal keratinocytes (HPEKs), which are non-transformed epithelial cells derived from human epidermis. However, compared with immortalised cell lines, HPEKs are more technically demanding, costly, with a limited lifespan and lower transfection efficiency. Thus, U2OS cells were primarily used as a robust and reproducible model for mechanistic analysis, whereas HPEKs were included to evaluate selected observations in a more physiologically relevant keratinocyte-based system.

5. RESULTS AND DISCUSSION

5.1. Generation and validation of the HPV NLuc-marker genomes as a quantitative readout for viral DNA replication (Ref. I)

Since the viral genome replication is tightly linked to HPV pathogenesis and persistence, reliable and sensitive methods for monitoring replication are critical for both basic research and therapeutic development. Traditionally, HPV genome replication is analysed by Southern blotting (SB) or quantitative PCR (qPCR), both of which are time-consuming, expensive, and require prior purification of total or extrachromosomal DNA. Recently, luciferase-based HPV replication assays have been developed (Fradet-Turcotte, Morin, *et al.*, 2010); for instance, Renilla luciferase (RLuc) activity has been shown to correlate with HPV genome copy number and was used for screening small-molecule inhibitors targeting the HPV life cycle (Toots *et al.*, 2017). However, the use of RLuc in studying the HPV life cycle has been limited by several factors. One of the main drawbacks is the length of the RLuc coding sequence, which increases the size of the HPV genome by 936 nucleotides, potentially affecting genome stability and replication efficiency. Additionally, RLuc has relatively low luminescent output that hinders its usage in drug screening assays, especially those designed for high-throughput settings employing minimal cell numbers and medium volumes. To overcome these limitations, we employed a novel luminescent reporter protein, NanoLuc (NLuc), which offers several advantages over RLuc, including a significantly smaller size (172 vs. 312 amino acids), enhanced stability, and approximately 100-fold greater luminescent signal. We generated novel HPV5, HPV11 and HPV18 genomes (HPV5NLuc, HPV11NLuc and HPV18NLuc, respectively) by inserting an NLuc reporter in-frame with E2, immediately after the E1 stop codon. The insertion includes the foot-and-mouth disease virus (FMDV) self-processing 2A protein followed by the full-length E2 ORF, resulting in an NLuc fusion protein containing the first 24 amino acids of E2 at the N-terminus and the FMDV-2A sequence at the C-terminus (Figure 11). The FMDV-2A protein mediates ribosome skipping during translation, allowing two separate protein products to be generated from a single ORF. This 657 bp insertion is significantly smaller than the previously reported HPV18RLuc construct (1077 bp), making it better suited for high-throughput screening of HPV inhibitors (Figure 11).

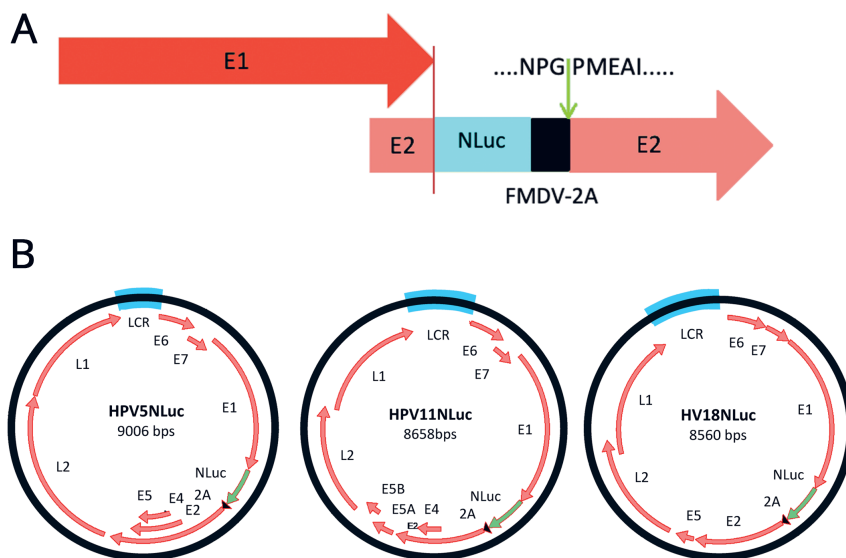


Figure 11. Schematic representation of the NLuc reporter construct and genome maps of HPV5NLuc, HPV11NLuc and HPV18NLuc. A. The NLuc coding sequence (turquoise) and the self-processing FMDV-2A peptide (black) were inserted into the E2 reading frame (light red) immediately downstream of the E1 (red) stop codon. The FMDV-2A protein mediates ribosome skipping at the sequence LLNFDLLKLAGDVESNPG↓P, resulting in separation of the upstream NLuc-containing fusion protein from the downstream full-length E2 protein. The green arrow indicates the ribosome-skipping site between glycine and proline. B. The position of the long control region (LCR) is shown in blue, the inserted NLuc is shown as a green arrow, and the FMDV-2A protein sequence is shown as a black arrow.

The replication efficiency of the generated reporter genomes was assessed in U2OS cells using a luciferase assay. NLuc activity was measured and normalised to alkaline phosphatase activity that represents a number of viable cells (Fig. 3A in Ref. I). Alongside, the same samples were analysed using SB, and the signals were quantified. Notably, normalised NLuc activity strongly correlated with SB-derived viral genome copy numbers. Linear regression analysis confirmed a statistically significant correlation ($R^2 = 0.96$, $p < 0.0005$), supporting the NLuc activity as a reliable tool for quantification of NLuc-positive viral genome replication. To further validate the constructs in a more physiologically relevant setting, replication was also tested in human epithelial keratinocytes (NHEKs) using the luciferase assay (Fig. 3F in Ref. I). All the tested genomes replicated in NHEKs. Collectively, these results demonstrate that the developed HPV genomes expressing NLuc reporter provide a robust and sensitive tool for quantitative analysis of HPV replication, suitable for both basic research and high-throughput applications. Recently, the HPV5NLuc genome was successfully employed in a high-throughput screening of a small-molecule library, leading to the identification of a compound with extensive inhibitory activity against β -HPV replication (Ibragimov *et al.*, 2025).

5.2. Phosphorylation of E2 Serine Residue 402 Is Required for the Transcription and Replication of the HPV5 Genome (Ref. III)

The HPV5 E2 protein is phosphorylated at multiple sites

Although β -HPVs have been studied less extensively than HR mucosal α -HPVs, primarily due to their less established role in carcinogenesis, certain types, such as HPV5 and HPV8, are known to be associated with cutaneous malignancies, particularly in immunosuppressed individuals. To enhance our understanding of the molecular determinants of their pathogenicity, it is crucial to characterise host-dependent changes in viral regulatory proteins. Phosphorylation, a reversible post-translational modification, regulates protein stability, localisation, and activity. While the phosphorylation of the HPV8 E2 protein has been documented (Sekhar and McBride, 2012), the phosphoacceptor sites and phosphorylation-dependent regulation of HPV5 E2 remained uncharacterised.

To address these questions, we first examined and confirmed the phosphorylation status of HPV5 E2, both endogenous and overexpressed in human cells, using an anti-phosphoserine antibody or *in vitro* kinase assay, respectively (Fig. 1B in Ref. III). In both cases, the E2 proteins were initially immunoprecipitated to enhance specificity and enrich the amount of the analysed protein. This result indicates that HPV5 E2 is subjected to phosphorylation by protein kinases expressed in U2OS cells. Interestingly, Coomassie staining revealed additional unknown proteins in the E2 immunocomplexes, suggesting that E2-targeting protein kinases could be coimmunoprecipitated with the E2 protein (Fig. 1C left panel in Ref. III).

To analyse the phosphorylation pattern of HPV5 E2 and identify specific protein kinases present in the immunocomplexes, mass-spectrometry (MS) analysis was conducted. The amount of the immunopurified endogenous E2 protein was too low for MS analysis, and therefore, we proceeded with the codon-optimised overexpressed Flag-tagged E2. MS analysis covered approximately 70% of the E2 sequence and identified serine-402 (S402) as a phosphorylated residue with the highest site-localisation probability in three independent immunocomplexes. The S402 of HPV5 is situated at the C-terminal end of the hinge region, near the DBD and shows 64% conservation among 53 different β -HPV E2 proteins, with adjacent residues being co-conserved. This preservation motif suggests that phosphorylation at S402 may serve as a common regulatory mechanism among β -HPVs, potentially mediated by a shared host kinase(s). A similar motif was also identified in the hinge region of HPV31 E2, where residues S266 and S269 have been previously characterised as phospho-acceptor sites (Van De Poel *et al.*, 2018). In contrast, this motif is absent in other α -HPV types (e.g. HPV11, 16, 18, 45), which lack the extended hinge region, highlighting a genus-specific regulatory divergence in E2 phosphorylation. Also, our MS analysis revealed four protein kinases (PKAC α , CK2 α 1, DNA-PK α , and SRPK1)

present in the E2 immunocomplexes (Table 1 in Ref III), suggesting that these enzymes might specifically interact with and phosphorylate HPV5 E2 protein.

Phosphorylation of E2 S402 is required for HPV5 replication and E2 transcriptional activity

To investigate the functional significance of S402 phosphorylation in HPV5 E2, we first assessed the replication efficiency of the genomes carrying nonphosphorylatable alanine (A) or phosphomimetic aspartic acid (D) mutations at the S402 residue (HPV5-E2-S402A and HPV5-E2-S402D, respectively). These mutations in the E2 ORF also affected the partially overlapping E8^{E2} and E1^{E4} ORFs, as they share the N-terminal part of E2 (Fig. S1 in Ref. III). The obtained modified genomes did not replicate in U2OS cells (Fig. 2A in Ref. III), likely due to the impaired activity of the mutated E2 proteins.

We next generated HPV5 genomes encoding Flag-tagged E2 variants HPV5-E2^{241Flag} and HPV5-E2^{264Flag}, where the numbers indicate the amino acid residue preceding the Flag insertion, which showed enhanced replication efficiency compared to the wildtype (WT) genome (Fig. S3A left panel in Ref. III). These short 10-aa inserts may result in conformation more favourable for some biochemical properties of full-length E2, e.g. DNA binding, affinity to E1, or affinity to cellular interactors, leading to the potentiated replication efficiency of the genomes. In this background, the nonphosphorylatable mutants displayed more than 10-fold reduction in replication, while the phosphomimetic mutations partially rescued this defect by at least 2-fold (Fig. S3C right panel in Ref. III). These results indicated that phosphorylation at the respective S402 site is required for efficient HPV5 genome replication, and that the functional effect of the phosphomimetic substitution is detectable only in the context of the elevated E2 activity.

To validate the gained findings in a more biologically relevant system, we performed similar experiments in the HPEKs. Likewise, the S402A mutant genome showed drastically reduced copy numbers compared to WT, while S402D replicated more efficiently than S402A (Fig. 2C in Ref. III). Interestingly, the magnitude of the difference between WT and mutant genomes was smaller in HPEKs than in U2OS cells. This may reflect differences in replication competence, proliferation rate, and kinase expression profiles between immortalised and primary cells.

Beyond its role in replication, E2 also functions as a transcriptional regulator. To assess the impact of S402 phosphorylation on viral transcription, quantitative reverse-transcription (qRT)-PCR analysis of the transcription pattern of the WT or E2-S402 mutant HPV5 genomes was performed in both U2OS and HPEKs. In U2OS cells, the S402A genome exhibited approximately 10-fold lower viral early gene mRNA expression levels compared to the WT genome, whereas S402D partially restored their expression (Fig. 2D left panel in Ref. III). A similar tendency was observed in HPEKs (Fig. 2D right panel in Ref. III), though the

differences between WT and mutant genomes were less pronounced, possibly due to reduced cell cycle progression and replication efficiency in primary keratinocytes. Collectively, our findings suggest that phosphorylation at S402 is essential for the transcriptional activity of the E2 protein in both U2OS cells and HPEKs.

The overexpressed E2-S402A/D proteins maintain E1-driven replication but fail to induce viral transcription

Viral genome replication and transcription are mutually dependent, as changes in viral genome abundance directly affect transcript levels and vice versa. Therefore, using the replication- and transcription-competent genomes, we could not determine whether reduced replication was a consequence of impaired transcription or whether the causal direction was reversed. To separate the replication and transcriptional roles of E2, we performed complementation assays using the E2- and E1/E2-deficient HPV5 genomes along with the expression vectors encoding the N-terminally Flag-tagged E2, either WT or bearing S402A and S402D mutations.

To investigate the potency of the E2 proteins to trigger the HPV5 replication and transcription, we used an E2-deficient (HPV5-E2fs) genome that is replication-incompetent due to a frameshift mutation in the E2 ORF. Replication was triggered only when WT E2 was supplied in *trans*, whereas no replication was observed in the presence of E2-S402A and E2-S402D proteins (Fig. 3B in Ref. III). In these experiments, the over-expressed WT E2 activated the early promoter and boosted E1 transcription, and *de novo* synthesised E1 in cooperation with WT E2 induced replication. Therefore, the E2's transcriptional and replication roles were indistinguishable in these experimental settings, and the question of whether the replication defect was due to poor transcriptional activation of E1 or a direct loss of E2 replication-related functions remained unsolved. To distinguish E2's replication function from its transcriptional role, an E1/E2-deficient HPV5 genome (HPV5-E1fs-E2fs) along with co-overexpressed E1 was used. This construct contains the frameshift mutation in the E1 ORF from HPV5-E1fs and a frameshift mutation in the E2 ORF. In this case, neither the WT E1 expression nor viral genome replication occurs in the presence of overexpressed E2 until E1 is added in *trans*, which allows for evaluating the activity of E2 solely as a replication factor. As a result, a 4-fold decrease compared to WT was observed in the case of E2-S402A. E2 expression vector bearing phosphomimetic mutation restored replication to some extent, but was still 2-fold lower than that of WT (Fig. 3C in Ref. III), confirming that S402 phosphorylation is critical for E2's direct function as a replication factor.

In order to assess the activity of the overexpressed E2 proteins as transcriptional factors, E2 variants were expressed without E1 to eliminate the influence of the replicated genome. Compared to WT E2, the transcriptional activity of E2-S402A was reduced by approximately 4-fold, although the mRNA expression levels of overexpressed E2 were similar. E2-S402D showed 20-50% stronger

transcriptional activation activity compared to E2-S402A (Fig 3E in Ref. III). Gained results conclusively demonstrate that phosphorylation at S402 affects not only the transcriptional activity of the E2 protein but also its ability to support replication of the viral genome, suggesting that these activities are closely interconnected yet mechanistically different.

Phosphorylation of S402 has no effect on some E2 activities

Despite the overexpression of the E2-S402D phosphomimetic variant alongside the nonreplicating HPV5-E1fs-E2fs genome, leading to a 2-fold decrease in E1 mRNA levels relative to WT E2, no viral genome replication was detected under these conditions. The initial low level of E1 expression, prior to genome replication, was presumed adequate to initiate replication; thus, this outcome was unexpected. A modest reduction in E1 transcript levels alone is unlikely to fully explain the complete loss of replication observed with E2-S402D. Furthermore, in experiments where E1 was provided in *trans*, circumventing the requirement for E2-mediated transcriptional activation, replication levels facilitated by E2-S402A or E2-S402D were notably lower than those attained with WT E2. These findings suggest that, beyond its role in transcriptional activation, additional functional aspects of E2 are likely regulated by phosphorylation at serine 402. To investigate this, we assessed key structural and functional properties of E2, including its nuclear localisation, protein stability, DNA binding, affinity to E1, E2's ability to form homodimers, and interaction with BRD4 protein. The absence of the S402 residue is likely to impede nuclear localisation, DNA binding, and E2 dimerisation, given the close proximity of the putative NLS N-terminus and DBD C-terminus. Based on the results obtained, we were unable to identify the impaired E2 functions, as the WT and S402-mutant E2 proteins exhibited similar functions.

The observed replication deficiency of the S402A mutant, despite relatively modest reductions in E1 transcript levels, raises the possibility that phosphorylation at this site modulates additional, replication-relevant interactions. In addition to its role in E1 recruiting to the viral origin of replication, E2 is known to recruit host replication factors, such as topoisomerase I, replication protein A or DNA polymerase ϵ (Chojnacki and Melendy, 2018; Clower *et al.*, 2006; Muller and Demeret, 2012), to the viral origin of replication. It is therefore plausible that the phosphodeficient S402A variant exhibits reduced affinity for one or more of these host factors, impairing its ability to support viral DNA synthesis.

Another possibility is that E2 phosphorylation at S402 may influence the expression of cellular proteins that facilitate viral replication. While this function has not been fully characterised, E2 has been demonstrated to modulate host gene expression in other contexts (Evans *et al.*, 2019; A. A. McBride, 2013). Whether this regulatory activity is affected by S402 phosphorylation remains to be determined.

5.3. Activity of CK2 α protein kinase is required for efficient replication of some HPV types (Ref. I)

Multiple cellular protein kinases are involved in the regulation of PV E1 and E2 proteins, including mitogen-activated protein (MAP) kinases, casein kinases 1 and 2, cyclin-dependent kinases (CDKs), and fibroblast growth factor receptor 3 (FGFR3). Casein kinase 2 (CK2) is a ubiquitously expressed, dual-specificity serine/threonine kinase that plays a significant role in numerous cellular processes (see Chapter 2.6.2) and is also recognised for its anti-apoptotic functions, leading to frequent upregulation in various cancers (Franchin *et al.*, 2017). In the context of PVs, CK2-dependent phosphorylation of BPV E1 and E2 proteins impairs their DNA-binding capacity and reduces viral genome replication (Schuck *et al.*, 2013). The phosphorylation of potential CK2 target sites within the hinge region of BPV E2 facilitates its proteasomal degradation, thereby reducing replication efficiency (Schuck *et al.*, 2013). However, CK2 activity can exert proviral effects; specifically, CK2-mediated phosphorylation of the host protein BRD4 enhances its interaction with HR HPV E2 proteins, facilitating viral genome tethering during mitosis and promoting genome maintenance (Iftner *et al.*, 2017; Wu *et al.*, 2016). *In vitro* studies indicate that CK2 can reduce E1 DNA-binding activity in both HPV11 and HPV31; nevertheless, the overall effect of the CK2 activity on HPV genome replication within a cellular environment remains insufficiently defined. This disparity underscores the necessity for a methodical examination of CK2's function in regulating HPV replication among different viral types.

The CK2 α catalytic subunit is required for the transient replication of the different HPV genomes

To assess the overall role of CK2 catalytic activity in HPV replication, we conducted transient replication assays in U2OS cells utilising HPV5 (β genus, cutaneous), HPV11 (α genus, low-risk, mucosal), HPV16, HPV18 and HPV31 (all α genus, HR, mucosal) genomes, subjected to varying concentrations of the ATP-competitive CK2 inhibitor CX4945 (Fig. 1A and 1D in Ref. I). CX4945 inhibited the replication of HPV5, HPV11, and HPV18 genomes in a concentration-dependent manner, while HPV16 and HPV31 replication was not suppressed; instead, genome levels increased in response to higher concentrations of CX4945, indicating that CK2 kinase activity may differentially modulate replication among HPV types. The inhibition of HPV replication induced by CX4945 is unlikely to result from indirect cellular effects, as only minor alterations in cell cycle distribution were observed, and cell viability remained largely unaffected at early time points when replication suppression was observed. Furthermore, the replication of certain HPV types (HPV16 and HPV31) increased despite reduced cell viability at later time points, thereby supporting the notion of a virus type-specific effect resulting from CK2 inhibition. Several factors may explain this

effect. Although CX4945 is a potent CK2 inhibitor ($IC_{50} \sim 1$ nM), it also targets other kinases at nanomolar concentrations (Siddiqui-Jain *et al.*, 2010), raising the possibility of off-target effects that may enhance HPV16 and HPV31 replication. Additionally, CX4945 has CK2-independent activity as a splicing inhibitor (Kim *et al.*, 2014), which could differentially affect viral gene expression across HPV types. Alternatively, CK2 may support replication of specific HPV types through kinase-independent functions, as seen in melanoma cells, where CK2 α helps sustain ERK (extracellular signal-regulated kinase) activity (B. Zhou *et al.*, 2016).

The CK2 functions as a heterotetrameric enzyme and consists of two highly homologous (almost 90%) catalytic subunit forms (α and α') that still exhibit differences in specificity and functionality. Their involvement in the regulation of transient and stable HPV replication was evaluated using siRNAs targeted to either of the CK2 catalytic subunits (Fig. 1D and 4C in Ref. I). Silencing of the CK2 α subunit resulted in marked inhibition of replication across all tested HPV types, including HPV16 and HPV31. A comparable effect was observed upon co-transfection with siRNAs targeting both CK2 α and CK2 α' . In contrast, knock-down of CK2 α' had no inhibitory effect on replication of any tested HPV types except HPV16, and even in this case, CK2 α depletion exerted a more pronounced impact. Collectively, these findings indicate that CK2 α , but not CK2 α' , functions as a positive regulator of transient replication of HPV5, HPV11, HPV16, HPV18, and HPV31 in U2OS cells and stable replication of HPV31b in CIN612 cells. The gained results bear two possible explanations. One possibility is that proteins involved in HPV replication contain phosphorylation sites specifically targeted by CK2 α . Alternatively, cellular levels of CK2 subunits may be skewed in favour of CK2 α' , leaving sufficient active CK2 α' even after siRNA-mediated knock-down. However, the former explanation appears more likely, as CK2 α and CK2 α' mRNA levels were similarly reduced (to ~ 3 –5%), and neither protein was detectable by immunoblotting in our RNAi experiments. Additionally, combined knock-down of both subunits did not produce a stronger inhibitory effect on HPV replication. We also observed that inhibition of stable HPV31b replication in CIN612 cells was milder than the inhibition of transient HPV31 replication in U2OS cells. This is consistent with the replication dynamics: during stable replication, the viral genome is duplicated in synchrony with host DNA, whereas in transient replication, the viral genome amplifies with each cell cycle. Consequently, fewer initiation events occur during stable replication, making it less sensitive to perturbation.

The gained results were confirmed using CK2 overexpression constructs with and without point mutations (CK2 α , CK2 α' , CK2 α K68R and CK2 α' K69R, respectively) in the ATP-binding pockets. Only the CK2 α construct was able to enhance the replication of HPV11 and HPV18, indicating that the presence and activity of CK2 α are required to modulate HPV replication (Fig. 2B and 2C in Ref. I).

CX4945 and CK2 α RNAi induce degradation of HPV11 and HPV18 E1 helicase

Multiple investigations have shown that BPV1 E1 and E2 proteins undergo phosphorylation by CK2, which subsequently impairs their DNA-binding capability (Lentz, 2002; McShan and Wilson, 1997; Schuck *et al.*, 2013). A significant number of the identified phosphorylation sites are conserved throughout HPV E1 and E2 proteins. The functional significance of these CK2 sites has been explored using site-directed mutagenesis in BPV genome and replication studies; however, the findings have been inconsistent. Some studies indicate that E1 phosphosite mutations have no effect on transient or stable replication of BPV, while mutations in E2 phosphosites have a positive effect on replication (Schuck *et al.*, 2013). Another study demonstrates that a single glycine substitution in BPV1 E1 serine 48, which is phosphorylated by CK2, is essential for viral genome replication in C127 cells (McShan and Wilson, 2000). Collectively, these discrepancies suggest that the role of CK2 in regulating papillomavirus replication proteins may be context-dependent and highlight the need for further investigation.

To gain insight into the molecular mechanisms of CK2-dependent downregulation of HPV replication, we generated HPV11 and HPV18 genomes containing an HA-epitope in the E1 ORFs. Since the previously described transient replication assays of HPV11 and HPV18 revealed that CX4945 mimics the effect of siCK2 α , the use of CX4945 seemed an advantageous alternative to CK2 α RNAi: pharmacological intervention with CK2 kinase activity influences all cells in the population and is much faster than RNAi-mediated knockdown of protein expression, which effectiveness depends on transfection efficiency. To analyse the HA-tagged E1 protein levels in the presence of CX4945, U2OS cells were transfected with the corresponding genomes and treated with 6 μ M CX4945 on the second day after transfection (Fig. 5B in Ref. I). In contrast to CX4945-treated cells, the levels of HPV18E1HA and HPV11E1HA DNA increased over time in the control cells. The levels of E1 protein decreased markedly upon CX4945 challenge already after 24h of treatment, whereas a decrease in HPV DNA copy number was not detected at this time point. Alongside, HPV11 and HPV18 genomes were readily detectable after 72h of treatment with CX4945, whereas none of the E1 protein was detected at this time point. Additionally, the levels of cytoplasmic and nuclear HPV18 E1 protein in U2OS cells treated with CX4945 or siCK2 α were lower compared to respective controls (Fig. 5C in Ref. I). The possibility of transcriptional downregulation of *E1* expression was excluded by quantifying HPV18-derived transcripts after treatment with CX4945 or CK2 α silencing. RT-PCR and qPCR analysis of *E1*, *E2*, *E1*^{E4} and *E8*^{E2} mRNAs demonstrated that transcript levels remained unchanged under both conditions (Fig. 5D and S5C in Ref. I). These results indicate that the observed reduction in E1 protein levels results from post-transcriptional mechanisms, rather than diminished transcription.

To clarify the mechanism underlying the observed decrease in E1 protein levels and to exclude potential CK2-independent off-target effects of CX4945,

U2OS cells transfected with either HPV11 or HPV18 genomes were treated concurrently with CX4945 and the proteasome inhibitor MG132 (Fig. 6A and S5D in Ref. I). CX4945-induced reduction of E1 protein levels was partially rescued in the presence of MG132, indicating that E1 undergoes proteasomal degradation upon CK2 inhibition. In contrast, treatment with CX4945 for 6h led to approximately 50% decrease in E1 protein levels, which was reversed to a substantial extent when MG132 was co-administered. Furthermore, nuclear and cytoplasmic fractionation assays demonstrated a marked reduction in E1 levels within 4h of CX4945 treatment. Co-treatment with MG132 restored HPV18 E1 protein levels to those observed in control cells. Although we cannot fully exclude the possibility of N-terminal proteolysis of full-length E1, the data strongly suggest that CK2 α catalytic activity is required for post-translational stabilisation and nuclear retention of E1 proteins.

The role of CK2 in regulating E1 protein stability has not been previously described. In contrast, CDK2-mediated phosphorylation has been shown to stabilise BPV1 E1 *in vitro* (Cueille *et al.*, 1998; Maleles *et al.*, 2002), and *in vivo* studies demonstrated that CDK activity is required for nuclear retention of HPV E1 (Deng *et al.*, 2004; Ma *et al.*, 1999). Our findings now suggest that CK2 α catalytic activity similarly contributes to post-translational control of HPV E1, promoting its stabilisation and nuclear localisation. CK2 serves as an additional post-translational checkpoint that PVs utilise to ensure an adequate supply of helicase for genome amplification.

Proteasome rescue experiments suggest that CK2-mediated phosphorylation protects the protein from degradation. This may occur by masking a degradation signal or by helping the protein recruit protective factors, preventing its recognition by the cell's ubiquitin system. Similar CK2-dependent mechanisms have been observed: phospho-deficient mutants of the adenovirus E1B-55K protein lose stability and show enhanced ubiquitination *in vivo* (Ching *et al.*, 2012), Epstein-Barr virus EB2 becomes rapidly degraded when CK2 contacts in its N-terminus are disrupted (Medina-Palazon *et al.*, 2007), and in cytomegalovirus, CK2 phosphorylation of IE2 at residues adjacent to a SUMO-interaction motif enhances SUMOylation that stabilises the protein in a more degradation-resistant conformation (Tripathi *et al.*, 2019). This suggests that various DNA viruses utilise the same host kinase to maintain essential regulatory proteins. Due to the abundance, constitutive activity, and drugability of CK2 (e.g., silmitasertib/CX4945, currently in clinical trials), its inhibition offers a viable antiviral strategy against HPV. However, the kinase may have contrasting effects in different pathogens; for instance, inhibition of CK2 can stabilise the hepatitis C virus NS2 protein (Franck *et al.*, 2005). Therefore, the overall impact of CK2 inhibition should be assessed on a case-by-case basis for each virus.

5.4. Cyclic AMP-Dependent Protein Kinase Exhibits Antagonistic Effects on the Replication Efficiency of Different Human Papillomavirus Types (Ref II)

PKA has kinase activity-dependent antagonistic effects on the initial amplification of the α - and β -HPV genomes

In addition to previously characterised protein kinases involved in the regulation of the HPV life cycle, the cAMP-dependent protein kinase (PKA) has also been implicated as a potential regulator of HPV replication. Although its role in this context remains underexplored, PKA was selected for further investigation based on several observations supporting its biological relevance. It is endogenously expressed in keratinocytes, the natural host cells for HPV, and is activated by cAMP, a key regulator of keratinocyte proliferation and differentiation (Grandjean-Laquerriere *et al.*, 2005; Zippin *et al.*, 2010). Prior studies suggest that PKA influences the HPV life cycle by phosphorylating viral proteins such as the E2 transcription factor in β -HPV8 and the E6 oncoprotein in α -HPV18 (Delury *et al.*, 2013; Kühne *et al.*, 2000; Sekhar and McBride, 2012). Furthermore, bioinformatic analysis revealed multiple PKA consensus motifs (R-R/K-X-S/T) in the E1 and E2 proteins across various HPV types, suggesting potential direct regulatory roles in viral replication.

To investigate of the role of PKA activity in the life cycle of different HPV types, we first analysed the expression of different PKA catalytic subunits (PKA C), as the human genome encodes three distinct genes for PKA C (*PRKACA*, *PRKACB*, *PRKACG*). The expression was analysed in HPEKs, CIN612 and U2OS cells, which are permissive for HPV replication, using RT-PCR. Only *PRKACA* and *PRKACB* mRNAs were expressed in the used cell lines (Fig. 1A in Ref. II). As the PKA α and PKA β proteins encoded by *PRKACA* and *PRKACB*, respectively, share 92% identity, PKA α was chosen for further research because of its higher expression level in the target cells. To investigate the role of PKA catalytic activity in the regulation of HPV replication, we generated constructs encoding N-terminally Flag-tagged WT PKA catalytic subunit α (hereafter referred to as PKA), along with two catalytically inactive mutants: PKA(T197A) and PKA(K72R). The T197A mutation disrupts phosphorylation at threonine 197, a critical activation site within the activation loop. The K72R mutation targets the conserved lysine in the ATP-binding pocket, thereby abolishing ATP binding and catalytic function. The enzymatic activity of the generated constructs was verified by their ability to phosphorylate a known PKA target, the Nth1 protein, *in vitro*, showing kinase activity only for PKA WT (Fig. 1B in Ref. II).

To assess the impact of PKA catalytic activity on the initial amplification of viral genomes, U2OS cells were co-transfected with expression vectors encoding Flag-tagged subunits and either HPV11 (LR α -type), HPV18 (HR α -type), or HPV5 (HR β -type). SB analysis revealed robust inhibition of HPV5 replication

under overexpressed PKA settings. In contrast, both α -HPVs (HPV11 and HPV18) replication was stimulated (Fig. 1C in Ref. II). Quantification showed that catalytically active PKA increased HPV11 and HPV18 genome copy numbers by approximately 2- and 10-fold, respectively (Fig. 1D in Ref. II). Both PKA mutants also enhanced replication, though less effectively, with PKA(T197A) having a greater impact than PKA(K72R). This observation aligns with previous findings showing that the K72R mutation in the ATP-binding domain abolishes catalytic activity, whereas the T197A mutation in the activation loop significantly impairs, but does not eliminate, kinase function (Iyer *et al.*, 2005; Yonemoto *et al.*, 1997). Notably, both mutants retain the ability to form holoenzyme complexes with endogenous PKA subunits, potentially titrating endogenous PKA inhibitors (PKI) or increasing the number of cAMP-responsive holoenzymes containing at least one active catalytic subunit.

To evaluate the role of endogenous PKA activity in early HPV replication, we focused on HPV5 and HPV18 in U2OS cells, excluding HPV11 due to its minimal response to PKA overexpression. Pharmacological activation of PKA using IBMX (competitive non-selective phosphodiesterase inhibitor), forskolin (adenylate cyclase activator), or dbcAMP (cAMP analogue) led to rapid CREB1 phosphorylation, confirming effective stimulation of the pathway and presence of the active endogenous PKA in the cells (Fig. 2A in Ref. II). Consistent with the results obtained using overexpressed PKA, activation of endogenous PKA enhanced HPV18 replication, with forskolin showing the strongest effect (Fig. 2B and 2D in Ref. II). During the early phase of initial replication, HPV18 genome copy numbers increased by approximately 2- and 3-fold on days 2 and 3 post-transfection. However, as the viral genome approached maintenance phase levels, the stimulatory effect of PKA activators diminished. In contrast to HPV18, HPV5 replication was significantly inhibited by IBMX, whereas forskolin demonstrated a less pronounced suppressive effect (Fig. 2C in Ref. II). The findings were additionally corroborated through the application of the PKA ATP-binding site competitive inhibitors KT 5720 and H89. PKA inhibition resulted in a modest increase in HPV5 replication, likely due to the low basal PKA activity in unstimulated U2OS cells, as evidenced by the minimal levels of phospho-CREB1 (Fig. 2A in Ref. II). Variations in the effectiveness of different pharmacological PKA activators, such as IBMX and forskolin, may originate from their off-target activities. IBMX, a nonspecific phosphodiesterase inhibitor, can also stimulate cGMP-dependent kinase pathways, while forskolin selectively activates membrane-bound adenylate cyclase but does not affect the soluble form. The results collectively indicate that PKA activity exerts a negative regulation on HPV5 replication.

Elevated activity of PKA suppresses transient replication of HPV5 in HPEKs

To extend our observations to a more physiologically relevant model, we repeated the experiments in HPEKs. Upon calcium-induced differentiation, HPEKs support an increase in HPV genome copy number, reflecting, to some extent, the environment of a vegetative phase of viral infection. Calcium-mediated differentiation of HPEKs was confirmed by immunoblot analysis of the differentiation markers involucrin and keratin 10, which showed increased protein levels after 2 days of calcium treatment. Additionally, phosphorylated CREB1, a target of both activated PKA and calcium influx, was elevated during differentiation. The replication efficiency of the HPV5 genome in HPEKs was measured using nanoluciferase activity from the modified HPV5-NLuc genome. As previously described in Ref. I (Chapter 5.1), the NLuc activity is directly proportional to the viral genome copy number and therefore can be used to measure the replication efficiency of a transfected genome.

The HPV5-NLuc was co-transfected with PKA or PKA(T197A) constructs into HPEKs, and the luciferase activity was measured on the 3rd day post-transfection. Similarly to results obtained in U2OS cells, PKA repressed HPV5-NLuc genome replication by more than 2-fold (Fig. 3A left panel in Ref. II). A statistically insignificant, mild repressive effect of the mutant PKA was observed, with a decrease in copy number of approximately 20%. Analogously, the influence of the PKA activity on the HPV18 replication efficiency was measured. The PKA was able to induce HPV18 genome copy numbers by about 3.8-fold (Fig. 3A right panel in Ref. II).

To further assess the role of PKA activity, normal and differentiating HPV5-NLuc transfected HPEKs were treated with IBMX or H89. In line with the findings from U2OS cells, elevated endogenous PKA activity repressed HPV5-NLuc replication, while inhibition of PKA by H89 enhanced viral genome amplification. Similar trends were observed in differentiating HPEKs, where calcium treatment increased HPV5-NLuc copy numbers; this increase was reduced by IBMX and further enhanced by H89 (Fig. 3C left panel in Ref. II). In contrast, forskolin and IBMX stimulated HPV18 replication by approximately 2.5- and 1.5-fold, respectively, with calcium-induced differentiation further enhancing this effect (Fig. 3C right panel in Ref. II).

PKA represses the replication of the HPV5 genome but stimulates HPV18 replication during the maintenance phase of viral infection

Our research group has developed and reported HPV5-positive (HPV5⁺) and HPV18-positive (HPV18⁺) stable cell lines generated with U2OS cells. In stable cell lines, HPV genomes replicate synchronously with cellular DNA as multicopy episomes, mostly as viral genome oligomers of various sizes (Geimanen *et al.*,

2011; Sankovski *et al.*, 2014). Introduced cell lines serve as effective models for investigating the maintenance and replication of HPV genomes, akin to persistent infection. In case of HPV18⁺ cells, it has been shown that cells initiate a second round of amplification of the viral genome when cultured at confluence for 3 to 6 days, mimicking to some extent the events taking place during the last stage of productive infection (Geimanen *et al.*, 2011; A. Piirsoo *et al.*, 2020).

In order to determine the influence of modulation of PKA activity on the maintenance replication, HPV5⁺ and HPV18⁺ cells were challenged with IBMX or forskolin for 2, 4 and 6 days (Fig. 4A in Ref II). Extracted low-molecular-weight (LMW) DNA visualised using SB showed an increase in the copy number of the HPV18 genome in response to the PKA activators. In contrast, both PKA activators had a negative effect on HPV5 maintenance replication and was observed in both subconfluent (2 days) and confluent (4 and 6 days) cultures. Of note, the HPV5 genome copy number, unlike HPV18, remained stable in confluent cells, as previously described (Sankovski *et al.*, 2014). To determine if the copy number changes were also evident in the structure of the viral episomes, we subsequently analysed uncut LMW DNA. In untreated HPV5⁺ cells, the blot displayed multiple episomal forms; however, a predominant oligomeric band (indicated by an asterisk in Fig 4C in Ref. II) constituted approximately 30% of the total HPV5 signal. Upon treatment with IBMX or forskolin, the signal from all smaller HPV5 replicons decreased, while the dominant band remained relatively unchanged. A comparable pattern was observed for HPV18 upon depletion of its E1 helicase (A. Piirsoo *et al.*, 2020).

PKA directs HPV5 E2 protein to proteasomal degradation

Since PKA activation had a strong negative effect on HPV5 replication, the next step was to investigate the impact of PKA-activating agents on the viral proteins E1 and E2. To enable detection of the endogenous E1 and E2 proteins, we used a modified HPV5 genome, HPV5-E1HA-NLuc-E2Flag. This construct was generated from the HPV5-NLuc genome (Chapter 5.1) by inserting an HA tag at the beginning of E1 ORF (after the 15th nucleotide) and a Flag-tag after the ATG codon of the full-length E2. The introduced modifications did not impair the replication of the HPV5-E1HA-NLuc-E2Flag genome (Fig. 5A in Ref. II). Overexpression of WT or mutant PKA, as well as modulation of endogenous PKA activity, influenced genome copy number in a manner similar to that observed for HPV5 and HPV5-NLuc (Fig. 5B in Ref. II). To assess the levels of endogenous E1 and E2 proteins, U2OS cells were transfected with HPV5-E1HA-NLuc-E2Flag genome and treated with IBMX, forskolin or dbcAMP. Immunoblot analysis detected that elevated PKA activity reduced E2 protein levels without affecting E1 (Fig. 5C in Ref. II). Among the activators, IBMX caused the most pronounced decrease in E2, whereas forskolin had the mildest effect, correlating with their respective impacts on HPV5 replication.

The reduction in HPV5 E2 protein levels may result from either protein degradation or transcriptional repression. It was previously shown that E2 proteins of

different α -HPV types are subjected to ubiquitin-mediated proteasomal degradation through their amino-terminal activation domain (Bellanger *et al.*, 2001, 2010; Gagnon *et al.*, 2009). To distinguish between these mechanisms, HPV5-E1HA-NLuc-E2Flag-transfected U2OS cells were treated with IBMX for varying durations, with or without the proteasome inhibitor MG132 (Fig. 5D in Ref. II). E2 protein levels decreased after 2 hours of IBMX treatment but were restored by MG132, indicating proteasome-mediated degradation. E1 levels remained unchanged.

To exclude PKA-mediated effects on HPV5 transcription independent of E2, replication-deficient HPV5E1⁻ and HPV5E2⁻ genomes were transfected into U2OS cells and challenged with IBMX or dbcAMP for 8 hours. qPCR analysis showed that in HPV5E1⁻ genomes, PKA activation reduced E8^{E2}, E1 and E2 transcripts but did not affect E1^{E4}, E6 or E7, which are transcribed from distinct promoters (Fig. 5G in Ref. II). In contrast, no significant transcriptional changes were observed in HPV5E2⁻ genomes upon PKA activation (Fig. 5H in Ref. II). These data indicate that E2-independent viral transcription was not perturbed by elevated PKA activity.

The proteasomal degradation of the HPV5 E2 protein driven by PKA-dependent phosphorylation can be explained by two mechanisms. First, phosphorylation could create a recognition site for a specific ubiquitin ligase (Z. Chen *et al.*, 1995; A. Song *et al.*, 1998; Yaron *et al.*, 1997). Alternatively, phosphorylation may induce conformational changes in the E2 protein, allowing ubiquitin to bind more easily and target the protein for proteasomal degradation (Abu Hatoum *et al.*, 1998). Based on the obtained results, it is not possible to determine which mechanism is responsible for the proteasomal degradation of the HPV5 E2 protein. Notably, CK2-mediated phosphorylation induces conformational changes in the BPV1 E2 protein, promoting its degradation in Delta-PVs (Penrose *et al.*, 2004).

The strong negative effect of PKA on HPV5 replication suggests that β -HPVs may prefer to infect skin cells with low PKA activity. In contrast, α -HPVs can infect mucosal epithelial cells, where PKA activity is usually higher. It is still not fully known which exact cells are the main targets of HPV infection in either the skin or mucosa, because the stem cells in these tissues are not well understood (Egawa *et al.*, 2015). However, some evidence points to the idea that β -HPVs might infect cells with low PKA activity. For instance, one group of skin stem cells is found in the bulge region of hair follicles (Blanpain and Fuchs, 2009), which relies on Sonic Hedgehog (Shh) signalling via Gli transcription factors (Brownell *et al.*, 2011). This pathway is functional only in cells with low PKA activity, as PKA-mediated phosphorylation promotes Gli degradation (Sheng *et al.*, 2006). It has been suggested that these hair follicle stem cells could be the natural targets of β -HPVs in healthy humans (Gheit, 2019).

HPV18 E8^ΔE2 transcripts and E1/E2 proteins are targets of PKA activity

Having characterised the mechanism underlying PKA's repressive impact on HPV5 replication, we next sought to elucidate how PKA stimulates HPV18 replication. To address this question, U2OS cells were co-transfected with replication-deficient HPV18E1⁻ genome and either an empty vector or constructs encoding WT or mutant PKA. qPCR analysis was used to assess expression of the viral regulatory genes E1, E2 and E8^ΔE2. While E1 and E2 mRNA levels remained consistent across all conditions, E8^ΔE2 transcript levels were reduced by approximately 95% in cells expressing catalytically active PKA (Fig. 6A in Ref. II).

To determine whether PKA-mediated enhancement of HPV18 replication is solely due to downregulation of the E8^ΔE2 repressor, we employed the E8^ΔE2-deficient HPV18E8⁻ genome. While the loss of E8^ΔE2 alone substantially increased replication, overexpression of PKA further elevated HPV18E8⁻ genome copy numbers, though less dramatically than in the WT context. Quantitative analysis showed a 3.5-fold increase in HPV18E8⁻ copy number upon PKA overexpression and a 2-fold increase with forskolin treatment, indicating that E8^ΔE2 downregulation is not the only mechanism by which PKA enhances HPV18 replication. To investigate additional mechanisms, we examined E1 and E2 protein levels in cells transfected with the HPV18-E1HA-NLuc-E2Flag genome and treated with forskolin. A moderate but statistically significant increase in both E1 (1.8-fold) and E2 (1.5-fold) protein levels was observed, suggesting that PKA also promotes replication by enhancing the expression or stability of these essential viral replication factors.

These findings support a model in which PKA promotes HPV18 replication through a combination of E8^ΔE2 repression with upregulation of E1/E2 expression. The precise mechanism of E8^ΔE2 downregulation, whether via p1193 promoter interference or altered splicing, remains unclear, but PKA clearly acts as a positive regulator in the HPV18 life cycle.

5.5. GENERAL DISCUSSION

Protein phosphorylation is one of the most widespread and dynamic mechanisms by which cells regulate protein functions. By altering protein stability, localisation, conformation and interaction partners, phosphorylation allows cells to rapidly adapt to changes in intra- and extracellular conditions. Since HPV replication is completely dependent on the host cell environment, viral proteins are unavoidably embedded in this regulatory network. E1 and E2, the two central viral regulators of viral genome replication and transcription, do not therefore function as isolated viral factors, but as substrates and sensors of host cell signalling pathways.

Although the host cell encodes several hundred protein kinases, only a limited subset of kinase-dependent regulatory mechanisms has been experimentally characterised for the HPV E1 and E2 proteins. MAPKs such as ERK and JNK phosphorylate HPV11 E1 and promote its nuclear import (J.-H. Yu *et al.*, 2007). CDK2 regulates E1 nuclear retention and replication activity (Fradet-Turcotte, Moody, *et al.*, 2010; Johansson *et al.*, 2009; Ma *et al.*, 1999). SRPK1 phosphorylates the E2 proteins of HPV5 and HPV1, affecting their subcellular localisation (Lai *et al.*, 2000; Prescott *et al.*, 2014). FGFR3 phosphorylates a conserved tyrosine residue in E2 and restricts E2-dependent transcription, replication and episome maintenance (F. Xie *et al.*, 2017). Pyk2 and FAK have also been found implicated in HR-HPV E2 phosphorylation, leading to negative regulation of viral genome replication (Jose *et al.*, 2020, 2023). In some cases, the conservation of kinase target motifs or the analysis of related HPV types suggests that these mechanisms may extend beyond a single viral type. However, for most kinases, the functional consequences have been examined only in selected viral proteins or in a limited number of HPV types. Therefore, it has remained difficult to determine whether a given kinase-dependent mechanism represents a broadly conserved principle of PV biology or a type-specific adaptation.

The results of this thesis demonstrate that kinase-mediated regulation is not a marginal or type-restricted phenomenon. Instead, both mucosal and cutaneous HPV types are controlled through phosphorylation-dependent mechanisms. However, the consequences of this regulation are strongly HPV type-specific, as the same cellular kinase pathway may support replication of one HPV type while suppressing another. Besides type-specificity, kinase activity also depends on the viral protein targeted and, probably, on the cellular context in which the viral genome is maintained.

The identification of HPV5 E2 S402 as a functionally important phosphorylation site illustrates the precision of this regulation. This residue is highly conserved among β -HPV E2 proteins, suggesting that it may represent a shared regulatory element within cutaneous HPV types. Mutation of S402 strongly impaired HPV5 genome replication and reduced E2-dependent transcriptional activation, although several basic properties of E2, including stability, dimerisation, localisation, and interaction with E1 and Brd4, were not detectably altered. This indicates that S402 phosphorylation does not detectably affect the basic

structural and molecular properties of E2. Instead, it may regulate more specific E2-dependent functions, such as the recruitment of additional cellular cofactors, the formation of transcriptionally active E2-containing complexes, or the modulation of host-cell genes required for efficient viral genome replication. This possibility is consistent with previous work showing that HPV16 E2 can act as a regulator of host cell transcription (Evans *et al.*, 2019). The kinase responsible for S402 phosphorylation remains unknown. However, PKA, CK2 and several proline-directed kinases were identified as possible candidates. It is plausible that multiple kinases can phosphorylate S402, ensuring modification of this functionally important residue under different cellular conditions. Such redundancy could prevent dependence on a single kinase and thereby reduce the virus's evolutionary vulnerability.

The CK2 results reveal a broader level of kinase-dependent regulation of HPV replication. RNAi-mediated depletion of CK2 α inhibited replication of several HPV types from different genera, including cutaneous β -HPV5 and mucosal α -HPV11, HPV16, HPV18 and HPV31. This suggests that CK2 α acts as a broadly supportive host factor. We have linked this effect to maintenance of E1 protein levels, since CK2 depletion reduced its stability and thereby limited E1 availability during viral replication. In this context, CK2 α can be considered a host cell factor that creates a permissive environment for viral DNA synthesis by preserving the functional pool of E1. Interestingly, CK2 inhibition with CX4945 increased replication signals for HPV16 and 31, suggesting that pharmacological treatment may introduce compound-specific or off-target effects. This observation is important from a therapeutic perspective as pharmacological kinase inhibition cannot be assumed to produce uniform antiviral effects across HPV types. Depending on the viral and cellular context, inhibition of the same kinase pathway may suppress replication of some HPV types, while producing neutral or even replication-enhancing effects in others.

The most remarkable example of type-specific kinase regulation was observed for PKA. Activation of PKA promoted replication of the α -HPV11 and 18, whereas the same pathway inhibited replication of the β -HPV5 genome. Mechanistically, these opposite outcomes were linked to different viral targets. In HPV5, elevated PKA activity led to proteasome-dependent degradation of E2, thereby limiting the availability of a key transcriptional and replication regulator. In contrast, PKA activity reduced the level of the E8^{E2} repressor in HPV18, shifting the balance toward enhanced replication. Therefore, PKA alters the internal balance between viral activators and repressors, and the outcome depends on how each HPV type has wired its regulatory circuit.

A key question raised by these findings is how HPV replication is regulated in cells where multiple kinase pathways, including CK2 and PKA, are simultaneously active. If CK2 α and PKA are active simultaneously in HPV-infected cells, their effects on viral replication are unlikely to be determined simply by kinase presence or absence. This is relevant because mucosal α -HPVs, such as HPV11, 16, and 18, and cutaneous β -HPV5 infect related but distinct keratinocyte

environments. Although both mucosal and cutaneous HPVs rely on basal keratinocyte infection and differentiation-dependent replication, the tissues they infect differ in keratinisation, differentiation dynamics, environmental exposure, and cellular transcriptional programmes. These differences may influence kinase activity, localisation, and substrate accessibility. CK2 is a broadly expressed, constitutively active kinase involved in cell proliferation, survival, and stress responses, and its nuclear and cytoplasmic localisation can vary with cell-cycle stage and cellular conditions (Borgo *et al.*, 2021; Filhol *et al.*, 2003; Litchfield, 2003). In keratinocytes, CK2 has also been linked to hyperproliferation, abnormal differentiation, and inflammatory signalling (Huang *et al.*, 2023). In contrast, PKA activity is dynamically regulated by cAMP and spatially organised by AKAPs (Kritzer *et al.*, 2012; Zaccolo *et al.*, 2021). PKA signalling is also involved in epithelial homeostasis, including the regulation of skin epithelial stem-cell proliferation (Iglesias-Bartolome *et al.*, 2015). Therefore, even if both CK2 α and PKA are present in infected keratinocytes, they may not act on the same viral substrates at the same time or in the same epithelial context. In this model, CK2 α acts as a broadly supportive factor, preserving E1 stability and maintaining the core replication machinery across several HPV types. PKA, however, produces more type-specific effects by enhancing replication in mucosal α -HPVs by suppressing E8^{E2}, whereas inhibiting cutaneous β -HPVs by promoting E2 degradation. Thus, HPV replication is likely determined by the integration of multiple spatially and temporally organised kinase-dependent signals rather than by a simple on/off effect of individual kinases.

6. CONCLUSIONS

1. Using mass spectrometry-based analysis of HPV5 E2, we identified serine-402 as a conserved, top-scoring phosphorylation residue, whose phosphorylation is required for transcriptional activation and viral replication.
2. We have shown that CK2 α plays an essential role in supporting genome replication of HPV5, HPV11, HPV16, HPV18 and HPV31 primarily by stabilising the E1 helicase. Loss of CK2 α activity, either by siRNA depletion or pharmacological inhibition, led to a proteasome-mediated degradation of E1 in HPV18. Notably, pharmacological inhibition of CK2 had a positive effect on HPV16 and HPV31, suggesting that pharmacological treatment may introduce compound-specific or off-target effects.
3. The investigation of PKA signalling revealed contrasting effects on replication across HPV types. While PKA activation enhanced replication of α -HPVs (HPV11 and HPV18), it inhibited β -HPV5 replication. We found that elevated PKA activity directs HPV5 E2 protein to proteasomal degradation. In contrast, PKA activity stimulates HPV18 replication by downregulating the E8^{E2} transcript.

SUMMARY IN ESTONIAN

Valgukinaasid kui inimese papilloomiviiruse replikatsiooni regulaatorid

Inimese papilloomiviirused (HPV-d) on kaheaheelalise DNA genoomiga viirused, mis nakatavad nii naha- kui ka limaskestast basaalseid keratinotsüüte ning mille elutsükkel on tihedalt seotud rakkude diferentseerumisprogrammiga. Elu jooksul puutub enamik inimesi kokku vähemalt ühe HPV tüübiga. Suurem osa infektsioonidest kulgeb aga kliiniliselt märkamatuks ega põhjusta nähtavaid haigustunnuseid. Siiski võib HPV infektsioon põhjustada naha või limaskestast epiteeli healoomulisi vohandeid, sealhulgas tüükaid ja papilloome. Taolised vohandid taanduvad immuunvastuse tulemusel või vajavad lokaalset ravi, näiteks keemilist destruktiooni hapetega, krüoteraapiat vedela lämmastikuga või kirurgilist eemaldamist. Kuigi enamik HPV tüüpide põhjustatavad nakkused on mööduvad ja healoomulised, võib infektsioon kõrge riski tüüpidega areneda teatud tingimustes halvaloomulisteks kasvajateks, näiteks emakakaela-, anogenitaalpiirkonna- ja kurgu-kaela vähideks.

Enamik HPV-ga seotud vähiuuringud on keskendunud limaskesti nakatavate kõrge riskiga α -HPV-dele. Samas on kliiniliselt olulised ka nahka nakatavad β -HPV tüübid. Kuigi need iseloomustavad enamasti asümptomaatilised või subkliinised infektsioonid, võivad nad immuunpuudulikkusega isikutel põhjustada ulatuslikke ja püsivaid nahakahjustusi. Selliste patsientide hulka kuuluvad HIV-infektsiooniga inimesed, organi transplantatsiooni järgselt immunosupressiivset ravi saavad patsiendid, vähiravi tõttu immuunsupressioonis olevad patsiendid ning pärilike haigustega, näiteks *Epidermodysplasia verruciformis*-ega, isikud. Nendes olukordades võib nahka nakatavate β -HPV perekonna tüüpidega nakatumine põhjustada mittemelanoomse nahavähi tekket, eriti ultraviolettkiirgusega koosmõjus.

Vaatamata märkimisväärsetele edusammudele ennetuses puudub praegu viirusevastane ravi, mis võimaldaks juba kujunenud HPV infektsiooni organismist elimineerida. Kõige olulisemate limaskesti nakatavate HPV tüüpide vastased profülaktilised vaktsiinid on uute infektsioonide ennetamisel väga tõhusad, kuid ei ravi olemasolevaid nakkusi. Tõhusa viirusevastase ravimi väljatöötamise ebaõnnestumise põhiliseks põhjuseks on selle uurimiseks soodsate tingimuste puudumine laboritingimustes. HPV peremeesrakkude, keratinotsüütide kasvamine ja käitlemine on keerukas, ajakulukas ja üpris kallis. Rakkude diferentseerumisprogrammi saavutamiseks kasutatakse osteosarkoomi rakuliini U2OS, milles on võimalik imiteerida väga sarnast geenide ekspressiooni.

HPV püsiva infektsiooni eelduseks on viiruse genoomi replikatsioon ja säilimine nakatunud epiteelirakkudes. HPV kodeerib kahte varajast valku, E1 ja E2. E1 on ainus viiruse kodeeritud ensüüm, mis toimib ATP-st sõltuva helikaasina ning algatab ja juhib viiruse DNA replikatsiooni. E2 on aga multifunktsionaalse regulaatorne valk, mis soodustab E1 värbamist viiruse replikatsiooni alguspunkti, moduleerib viiruse transkriptsiooni ning aitab kaasa viiruse episoomide säilimisele

ja jaotumisele rakkude jagunemise käigus. Mõlemad valgud kuuluvad fosfoproteiinide hulka ning nende funktsionaalset aktiivsust reguleerivad peremeesraku proteiinkinaasid. See osutab võimalusele, et rakuline kinaassignalisatsioon võib toimida olulise regulatoorse mehhanismina, mõjutades viiruse replikatsiooni ja geeniekspressiooni ning võib seeläbi pakkuda lähtekohti uute terapeutiliste sekkumisviiside väljatöötamiseks.

Käesolev doktoritöö näitab, et kinaasivahendatud regulatsioon on tugevalt HPV tüübispetsiifiline. Mass-spektromeetri abil oleme tuvastanud β -HPV5 E2 valgu seriini-402 (S402), mis näitas kõrget konserveeritust ning fosforüleeritust. Lisaks oleme tuvastanud, et S402 on vajalik viiruse transkriptsiooniks ja replikatsiooniks, kuigi ei mõjuta E2 stabiilsust, lokatsiooni ega seondumisafiinsust E1 ja Brd4 suhtes.

Lisaks sellele uurisime kaseiinkinaas 2 (CK2) ja proteiinkinaas A (PKA) osalemist HPV elutsükli regulatsioonis. Oleme tuvastanud, et CK2 α alaühik on hädavajalik HPV5, α -HPV11, α -HPV16, α -HPV18 ja α -HPV31 replikatsiooniks. HPV18 puhul leidsime, et CK2 α aitab säilitada E1 valgu stabiilsust ja tuumalokatsiooni. Seevastu CK2 farmakoloogiline inhibeerimine CX4945 abil võimaldas HPV16 ja HPV31 replikatsiooni, viidates sellele, et CX4945 võib lisaks CK2 α inhibeerimisele mõjutada HPV replikatsiooni ka ühendispetsiifiliste või sihtmärgiväliste mehhanismide kaudu. Sarnase HPV tüübispetsiifilisuse molekulaarse regulaatorina toimib ka PKA-vahendatud signaalirada. HPV11 ja HPV18 puhul soodustab PKA aktiivsus viiruse replikatsiooni, suurendades E1 ja E2 valkude taset ning samal ajal pärssides transkriptsioonilist repressorvalku E8^{E2}. Seevastu HPV5 puhul avaldab PKA aktiveerumine replikatsioonile inhibeerivat mõju, indutseerides E2 valgu kiire proteasomaalse degradatsiooni.

Saadud tulemused näitavad mehhanisme, mille kaudu peremeesraku kinaasid on võimelised avaldama HPV elutsükli tüübispetsiifilist vastassuunalist mõju ja rõhutavad vajadust arvestada viiruse tüübispetsiifiliste eripäradega kinaasidele suunatud viirusevastaste strateegiate väljatöötamisel.

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Lototskaja, Elina; Sahharov, Olga; Piirsoo, Marko; Kala, Martin; Ustav, Mart; Piirsoo, Alla (2021). Cyclic AMP-dependent protein kinase exhibits antagonistic effects on the replication efficiency of different HPV types. *Journal of Virology*. DOI: 10.1128/JVI.00251-21.

Piirsoo, Alla; Piirsoo, Marko; Kala, Martin; Sankovski, Eve; **Lototskaja, Elina**; Levin, Viktor; Salvi, Mauro; Ustav, Mart (2019). Activity of CK2 alpha protein kinase is required for efficient replication of some HPV types. *PLoS Pathogens*, 15 (5), ARTN e1007788. DOI: 10.1371/journal.ppat.1007788.

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