UNIVERSITY OF TARTU

FACULTY OF SCIENCE AND TECHNOLOGY

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Replication of some HPV types is modulated by cAMP-dependent protein kinase activity

Master of Science thesis

30 EAP

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TARTU 2020

ABSTRACT

Replication of some HPV types is modulated by cAMP-dependent protein kinase activity

Human papillomaviruses (HPVs) are dsDNA viruses infecting basal keratinocytes of the cutaneous and mucosal epithelia. Some HPVs are tumorigenic; therefore, studies of the viral life cycle may help to develop novel treatment strategies. Protein kinases are important enzymes that regulate numerous cellular processes. HPV E1 and E2 regulatory proteins have several putative consensus sites for many kinases including cAMP-dependent protein kinase (PKA). The main focus of this thesis was to generate the constructs encoding the FLAG-tagged PKA catalytic subunit α (PKACα) and its two catalytically deficient mutants, and to investigate the influence of the over-expressed PKA proteins on replication of HPV types 5, 11, and 18 in U2OS cells. Additionally, PKA activator IBMX and inhibitor H89 were used to demonstrate the impact of the endogenous PKA catalytic activity on the replication efficiency of the HPV5 genome. Finally, the mechanism of the PKA-mediated stimulation of the HPV18 replication was studied.

Keywords: human papillomavirus (HPV), replication, transcription, protein kinase A (PKA)
CERCS: B230 Microbiology, bacteriology, virology, mycology

Mõnede HPV tüüpide replikatsioon on moduleeritud cAMP-sõltuva proteiinkinaasi aktiivsuse poolt

Inimese papilloomiviirused (HPV) on kaheahelalise DNA genoomiga viirused, mis nakatavad basaalseid keratinotsüüte nahas ja limaskestades. Mõned HPV tüübid omavad kõrget onkogeenset potentsiaali, seetõttu on viiruse elutsükli uurimine tähtis uute ravistrateegiate arendamiseks. Proteiinkinaasid modifitseerivad oma märklaudvalkude aktiivsust ning seega reguleerivad paljusid bioloogilisi protsesse rakkudes. HPV E1 ja E2 on viiruslikud regulatoorsed valgud, mis sisaldavad mitu oletatavat cAMP-sõltuva proteiinkinaasi (PKA) konsensus saiti. Selle töö eesmärk oli luua plasmiidid, mis kodeerivad FLAG-märgisega PKA katalüütilist subühikut α (PKACα) ja tema kahte katalüütiliselt inaktiivset mutanti, ja uurida PKA valkude üle-ekspressiooni mõju HPV tüüp 5, 11, 18 replikatsioonile U2OS rakkudes. Lisaks, testiti PKA aktivaatori IBMX ja inhibiitori H89 mõju HPV5 replikatsioonile ning selgitati välja PKA-st sõltuva HPV18 replikatsiooni aktivatsiooni mehhanism.

Märksõnad: inimese papilloomiviirus (HPV), replikatsioon, transkriptsioon, proteiinkinaas A (PKA)
CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia

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LIST OF ABBREVIATIONS

AKAP - A-kinase anchoring proteins

Amp – ampicillin

AP – alkaline phosphatase

BCC – basal cell carcinoma

BPV – bovine papillomavirus

BS – binding site

C – catalytic (subunit)

CDK – cyclin-dependent kinase

CK2 – casein kinase II

cDNA – complementary DNA

CREB - cAMP-responsive element binding protein

dsDNA – double-stranded DNA

E2Fs - transcription factors involved in cell cycle regulation

E2-TA – full-length E2 protein

E – early (genomic region or protein)

FGFR3 - fibroblast growth factor receptor 3

H89 - n-[2-p-bromocinnamylamino-ethyl]-5-isoquinolinesulfonamide

HPV – human papillomavirus

HR – high-risk (papillomavirus)

HSPG - heparan sulfate proteoglycan

IBMX - 3-isobutyl-1-methyl-xanthene

K72R – lysine residue at position 72 mutated to arginine

KAB – kinase assay buffer

L – late (genomic region or protein)

LR – low-risk (papillomavirus)

MAPK – mitogen activated protein kinase

NGM – normal growth medium

ORF – open reading frame

PDE – phosphodiesterase

PEI – polyethyleneimine

PKA - cAMP-dependent protein kinase

PKC - protein kinase C

PKACα - catalytic subunit α of PKA

PKA C – catalytic subunits of PKA

PKG - cGMP-dependent protein kinase

pRb – retinoblastoma protein

PV – papillomavirus

R – regulatory (subunit)

RT – room temperature

SB – southern blot

SCC – squamous cell carcinoma

T197A - threonine residue at position 197 mutated to alanine

URR – noncoding upstream regulatory region

WB – western blot

WR – working reagent

INTRODUCTION

Human papillomaviruses (HPVs) are double-stranded DNA (dsDNA) viruses infecting epithelial keratinocytes of mucosa or skin. HPV infection is among the most common sexually transmitted diseases worldwide. Different types of HPVs are divided into high- and low-risk, depending on presence or absence of an oncogenic potential (Rosa *et al*., 2013). The medical importance of studying the life cycle of HPVs and their interactions with host cells is attributable to the absence of drugs against HPV infections.

Inhibition of HPV replication is one of the promising therapeutic strategies for the treatment of HPV-related diseases. However, in order to interfere with viral life cycle and avoid damaging the host cells, it is essential to understand the interplay between HPV and the host cell in molecular terms. Protein kinases are important regulators of diverse biological processes in the cells. HPV key replication proteins E1 and E2 are found to be substrates for many cellular kinases, including cAMP-dependent protein kinase (PKA). Many of these kinases are known to regulate HPV replication in a HPV type-dependent manner (Ma *et al*., 1999; Piirsoo *et al*., 2019; Xie *et al*., 2017; Yu *et al*., 2007; Zanardi *et al*., 1997).

The aim of this study was to investigate the impact of the catalytic subunit α of PKA (PKACα) on replication of HPV types 5, 11 and 18. The chosen HPV types belong to different genera and risk groups. In order to prove that PKA acts in a kinase activity-dependent manner, we generated plasmids encoding for PKACα and its two catalytically deficient mutants, PKA(T197A) and PKA(K72R). The first part of this thesis provides an overview of the HPV infection cycle and functions of the viral proteins, and describes the roles of PKA in both host cells and viral life cycle. The experimental part describes the generation of the FLAG-tagged PKA expression constructs and the analysis of the viral genome replication in the presence of the over-expressed PKA proteins, and provides an explanation of the possible mechanisms of the PKA-mediated activities. We show that the over-expressed catalytically active PKA has a different impact on the viral replication depending on the type of the HPV. Furthermore, the present study demonstrates that efficiency of the viral genome replication may be affected by endogenous PKA activity, either potentiated with 3-isobutyl-1-methyl-xanthene (IBMX) or suppressed with n-[2-p-bromocinnamylamino-ethyl]-5-isoquinolinesulfonamide (H89). This study was performed in the molecular virology research group, Institute of Technology, University of Tartu.

1. LITERATURE OVERVIEW

1.1. Papillomaviruses and human papillomaviruses

Papillomaviruses (PVs) are small non-enveloped icosahedral viruses with circular dsDNA genomes approximately 5-8 kb in size typically containing up to eight genes. PVs belong to *Papillomaviridae* family, and according to the International Committee on the Taxonomy of Viruses, there are two subfamilies *Firstpapillomavirinae*, which includes over 50 genera (from *Alpha-* to *Zetapapillomavirus*) and more than 130 species, and *Secondpapillomavirinae* with a single genera and a unique specie (Van Doorslaer *et al*., 2018). The classification is based on pairwise sequence alignment identity across the open reading frame (ORF) of *L1*, which encodes the major capsid protein L1 is one of the most conserved genes among PVs. In order to be classified as a novel PV type, the nucleotide sequence of *L1* must be at least 10% dissimilar from that of any other PV (de Villiers *et al*., 2004).

PVs infect epithelial keratinocytes in large variety of animals, where they can persist asymptomatically or cause neoplasms (Bernard *et al*., 2010). Wide range of vertebrate species can be infected by PVs: mammals, birds, reptiles and fish (Herbst *et al*., 2009; López-Bueno *et al*., 2016; Terai *et al*., 2002; de Villiers *et al*., 2004). HPVs are a diverse group of PVs with over 200 different types being described, and new HPV types being continuously found (Papillomavirus Episteme database). HPV types are phylogenetically organized into five major genera: alpha- (α), beta- (β), gamma- (γ), mu- (μ), and nu- (ν) PVs (de Villiers *et al*., 2004). HPVs infect mucosal and cutaneous epithelia: the members of the α genus are associated with infections of oral and genital mucosal surfaces and external genitalia, while β, γ, μ, and ν genera HPVs infect non-genital mucosa and skin (Rosa *et al*., 2013). HPVs can be additionally divided into high- and low-risk (HR and LR, respectively) types according to their ability to induce benign hyperplasia and trigger their progression to malignancy (Cubie, 2013).

HR HPV types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 68, 73, 82, 83. HR HPVs are found in different invasive cancer specimens (Gillison and Shah, 2003). For example, mucosal types of HPV α genus are a major cause of cervical cancer (Walboomers *et al*., 1999) with HPV16 accounting for over 50% and HPV16 and 18 for >70% found in invasive cervical cancer histological specimens worldwide (Li *et al*., 2011). In addition, types from α genus are associated with head and neck cancers (Leemans *et al*., 2011), increased risk of oral cavity and oropharyngeal cancer (Anantharaman *et al*., 2013). HPVs are also associated with other anogenital cancers such as anal, vulvar and penile cancers with the prevalence of HPV16 and HPV18 (Daling *et al*., 2004; McCance *et al*., 1986; de Sanjosé *et al*., 2013).

Keratinocytes-derived non-melanoma skin cancers (Small *et al*., 2016) can be divided into basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) (El-Abaseri *et al*., 2006). It was previously shown that genus-β HPV infections may contribute to SCC (Iannacone *et al*., 2014) and BCC development (Iannacone *et al*., 2013). DNA of cutaneous HPV types was found in biopsies from patients with SCC of the conjunctiva (Ateenyi-Agaba *et al*., 2010). HPVs from this genus are also associated with skin cancer in patients with epidermodysplasia verruciformis – a rare autosomal recessive skin disorder that increases the risk of developing HPV-induced SCC (Orth, 2006).

HPVs of γ, μ, and ν genera cause warts or cutaneous papillomas (de Villiers *et al*., 2004), and there is little to no evidence of their involvement in tumorigenesis. HPVs can also be found in healthy skin: DNA of β HPV types can be detected in babies after a few days of life (Antonsson *et al*., 2003). One of the articles suggests that the prevalence of β HPVs increases with age in healthy individuals without inducing SCC (de Koning *et al*., 2009).

1.2. HPV genome and viral proteins

Different HPV types have similar genome organization. Schematic representation of HPV genomes used in the present study are shown in Figure 1. Although the viral genome can vary in size between different types, it typically contains around 8000 bp (Doorbar, 2006). The genomes can be divided into three different regions: early (E), late (L) and noncoding upstream regulatory region (URR) also called long control region (LCR) located between them. Most types of HPVs contain eight ORFs coding 6 early and 2 late proteins. Furthermore, HPVs encode several truncated forms of proteins translated from alternatively spliced transcripts (*e.g.* E1^E4 and E8^E2C). The early proteins mostly play regulatory role, for example, participate in viral replication and transcription. The late proteins are involved in virus capsid formation (Graham, 2010).

URR is approximately 500 to 1000 bp region located upstream of the coding region. It contains the replication origin (ori), transcriptional enhancer and promoter elements, binding sites (BSs) for cellular transcription factors and viral proteins E1 and E2, which are all involved in the control of the viral gene expression (McBride, 2008). Polycistronic transcripts are synthesized from only one DNA strand, then undergo alternative splicing and may be subjected to alternative polyadenylation resulting in a huge variety of different functional mRNAs. URR contains also early and late promoters - regions in which the synthesis of early or late transcripts,

respectively, is initiated, and polyadenylation sites for the late transcripts (Baker and Calef, 1996).

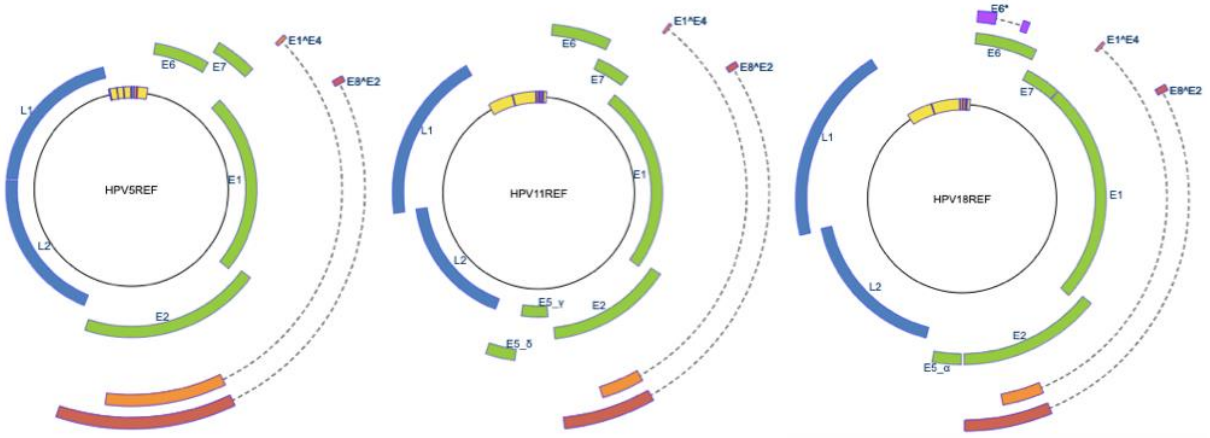

Figure 1. Schematic representation of HPV5, HPV11 and HPV18 reference genomes. Early ORFs (*E1*, *E2*, *E5*, *E6*, and *E7* – indicated by green), late ORFs (*L1* and *L2* - shown in blue) and URR region (yellow). HPVs encode truncated forms of proteins using alternatively spliced pre-mRNAs (*E1^E4* and *E8^E2C* - orange and pink). URR contains regulatory sequences and binding sites (BSs) for viral and cellular proteins (shown in red, blue and purple) (Papillomavirus Episteme database).

1.2.1 Early region proteins

The E1 protein is approximately 70 kDa ATP-dependent helicase that binds specifically to the ori and initiates replication (Yang *et al*., 1993). The E1 protein consist of the N-terminal domain, central domain and the C-terminal domain (McBride, 2008). The N-terminal domain is a regulatory region essential for optimal replication; the central domain recognizes specific sites in the ori and binds to the DNA; the C-terminus is an enzymatic domain, which is necessary for self-assembly into hexamers and for unwinding short DNA duplexes. E1 interacts with E2 and specific host factors to coordinate the assembly of a functional viral replisome (Bergvall *et al*., 2013).

The E2 is around 50 kDa regulatory protein (Rajkumar, 2016). *E2* gene products vary in size as a result of expression from different promoters and/or alternative RNA splicing. These alternatively named proteins (E8^E2, E8^E2C, E1^E2, E1M^E2 and E9^E2) function as repressors of viral transcription and replication (McBride, 2013). The full-length E2 (E2-TA) can influence the transcription of HPV genes both positively and negatively (Steger and Corbach, 1997). The E2 proteins play an important role in the maintenance of the viral genome as an independent episomal element (Ilves *et al*., 1999). E2 directs E1 to the ori region by increasing the E1 ori-binding specificity (Sedman and Stenlund, 1995). It regulates pre-mRNA processing (possibly via interacting with cellular splicing factors); induces cell apoptosis and

suppresses cellular growth through modulation of the *E6* and *E7* expression (Dowhanick *et al*., 1995; Lai *et al*., 1999; Webster *et al*., 2000).

The E4 protein is usually expressed from *E1^E4* spliced transcript. *E4* ORF is located in *E2* gene and also contains few nucleotides and the initiation codon of *E1* (Doorbar, 2013). The E4 protein influences the cell cycle along with E5, E6 and E7 oncoproteins (Davy *et al*., 2002). E4 interacts with E2 and promotes its relocation to the cytoplasm changing the availability at different times during the viral life cycle (Davy *et al*., 2009). The Abovementioned mechanisms are important for effective amplification of viral DNA. E4 also increases the egress of PV virions (Doorbar *et al*., 1991).

E5 is a short membrane-associated protein with transforming activity, typically mediating specific protein-protein interactions (DiMaio and Petti, 2013). The majority of HPV types encodes distinct forms of E5 protein: HR HPVs encode E5 α, which is associated with the progression of tumor development, whereas LR HPVs either lack E5 or express different polymorphic types of the protein (E5 β, -γ, -δ) and most likely lead to non-tumoral outcomes (Bravo and Alonso, 2004). The E5 protein is also associated with modulation of the host immune response (Grabowska and Riemer, 2012), and has the ability to enhance signal transduction from epidermal growth factor to the nucleus resulting in increased cell proliferation (Bouvard *et al*., 1994; Leechanachai *et al*., 1992; Stöppler *et al*., 1996).

The E6 is one of the PV oncoproteins with transforming abilities. E6 consists of approximately 150 amino acids and contains two zinc-finger binding motifs (Lipari *et al*., 2001). The E6 protein promotes cell proliferation through degradation of p53 tumor suppressor protein (Scheffner *et al*., 1990). The E6 proteins produced by HR HPVs has higher affinity to p53 than LR ones (Li and Coffino, 1996), which can be one of the reasons of the high oncogenic activity of HR HPVs. One of the targets of HR E6 is the tumor suppressor protein DLG, which participates in regulation of cell adhesion, polarity, proliferation in epithelial tissues and formation of junctions between cells (Bilder *et al*., 2000). Deregulation of the DLG protein leads to invasive cell growth (Kiyono *et al*., 1997).

E7 is also PV oncoprotein with transforming properties. E7 is approximately 10-14 kDa (Rajkumar, 2016). E7 induces cellular proliferation through association with the tumor suppressor protein pRb (retinoblastoma protein) (Dyson *et al*., 1989). E7 disrupts natural formation of pRb and the cellular transcription factor (E2F) complex, resulting in increased production of active E2Fs (Chellappan *et al*., 1992). This mechanism suggests that HPVs are able to reactivate host DNA replication machinery in already differentiated cells (Cheng *et al*.,

1995). The E6 and E7 oncoproteins were shown to induce mitotic defects, genomic instability and centrosomal abnormalities in host cells by coupling with pRb-related proteins (p107 and p130) (Duensing *et al*., 2000).

The E8^E2 is a fusion protein generated by splicing a short *E8* exon to the major splice acceptor in the middle of the *E2* ORF. As the E2-TA protein, E8^E2 also inhibits transcription of the viral major early promoter but more efficiently: it represses not only the expression of the HPV E6 and E7 oncoproteins, but also E1 and E2 replication proteins (Stubenrauch *et al*., 2000).

1.2.2 Late region proteins

Icosahedral PV virions consist of two proteins: L1 and L2 (Buck *et al*., 2008). L1 is the major capsid antigen, which is displayed on the surface of the virion (Buck *et al*., 2013). The L1 protein can self-assemble into virus-like particles, forming the native structure of PV virions (Kirnbauer *et al*., 1992). L2 or minor capsid protein, is hidden inside the mature capsid, whereas only a part of its N-terminus is exposed on the surface, playing role in the interactions with the host cell (Joyce *et al*., 1999; Liu *et al*., 1997). In addition, L2 is essential in the process of virion assembly (Holmgren *et al*., 2005).

1.3. HPV infection cycle

HPV infection occurs in basal keratinocytes of the stratified epithelium, which is able to proliferate constantly. Therefore, HPV cycle is tightly linked to the normal differentiation process of the host keratinocytes. The viral particle invades the basal layer through micro-wounds of the skin surface or via hair follicles (Kines *et al*., 2009; Schmitt *et al*., 1996). Virions interact with the heparan sulfate proteoglycans (HSPGs) and yet uncharacterized secondary specific receptors on the surface of basal keratinocytes, and then enter the cell (Kines *et al*., 2009). The hypothetical model suggests that the initial interaction between L1 and the host cell surface HSPGs causes conformational change in L1 and exposure of the N-terminus of L2, allowing its cleavage and interaction with the host cell secondary and, probably, entry receptors (Day *et al*., 1998; Joyce *et al*., 1999). Conformational changes in the viral capsid allow viral DNA to be transported to the nucleus (Kämper *et al*., 2006; Li *et al*., 1998), where the HPV genome is amplificated, then maintained at a low-copy number, and later, in the vegetative replication phase, is amplified to a high-copy number, in order to be packed into new virions and egress from the host cell (Figure 2) (Fisher, 2015).

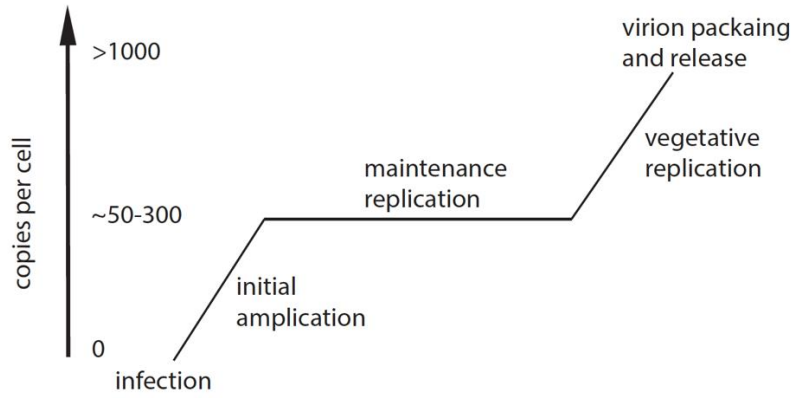

Figure 2. The phases of the replication of HPV (Fisher, 2015).

1.3.1 Initiation of viral DNA replication and genome maintenance

The E1 protein has an ATPase and helicase activity: it unwinds dsDNA, recruits host replication factors (DNA polymerase α/primase, replication protein A and topoisomerase I) and initiates viral replication (Burley *et al*., 2020; Wilson *et al*., 2002). E2-TA binds to BSs - 12 bp ($ACCGN_4CGGT$) palindromic sequences, which are present in multiple copies within the URR (Hirochika *et al*., 1988) and loads E1 protein onto the ori site; however, E2 must dissociate from the complex for the viral DNA replication to start (Sanders and Stenlund, 1998). Oligomerization of E1 and dissociation of E2 from the E1-E2-ori complex is stimulated by ATP (Titolo *et al*., 2000). The replication is initiated bidirectionally via theta model (Flores and Lambert, 1997).

E2-TA can act as an activator of HPV gene transcription or as a repressor depending on the dose of *E2* product, context of BS and nature of associated cellular factors, which are recruited by the virus (Muller and Demeret, 2012; Steger and Corbach, 1997). Low amounts of E2 bind to the most distal BS with high-affinity (BS-4) and activate the early promoter of HPV18 (p105) generating early proteins. In high concentrations, E2 binds also to the sites with the reduced affinity (BS-1, 2, 3) resulting in early promoter repression (Steger and Corbach, 1997). Binding of E2 proteins to its multiple BSs and chromatin is required for maintenance of the viral DNA as an episome or independent cell element and segregation of the HPV genome evenly among daughter cells during mitosis (Ilves *et al*., 1999).

The E8^E2 protein is one of the proteins, which regulate viral cycle and is also important for the maintenance of low-copy number strategy. It inhibits transcription of the viral major early promoter, additionally recruiting cellular corepressors such as histone deacetylases (Ammermann *et al*., 2008; Stubenrauch *et al*., 2000). E8^E2 is able to form a complex with E2-TA and to bind to E2 BS in the URR (Kurg *et al*., 2010). Formation of the E8^E2/E2 complex

excludes the E1 protein from the ori, which strongly represses HPV replication. The *E8* promoter region can be either inhibited by E8^E2 or weakly activated by E2 suggesting that this promoter can act as a modulator for viral copy number (Straub *et al*., 2015).

1.3.2 Viral genome amplification

After leaving the basal membrane, cells initiate the differentiation program, exit from the cell cycle and suppress DNA replication activity. Since the virus does not encode DNA polymerase, it requires host DNA replication machinery to replicate its DNA, and therefore HPV must reactivate cell division (Figure 3).

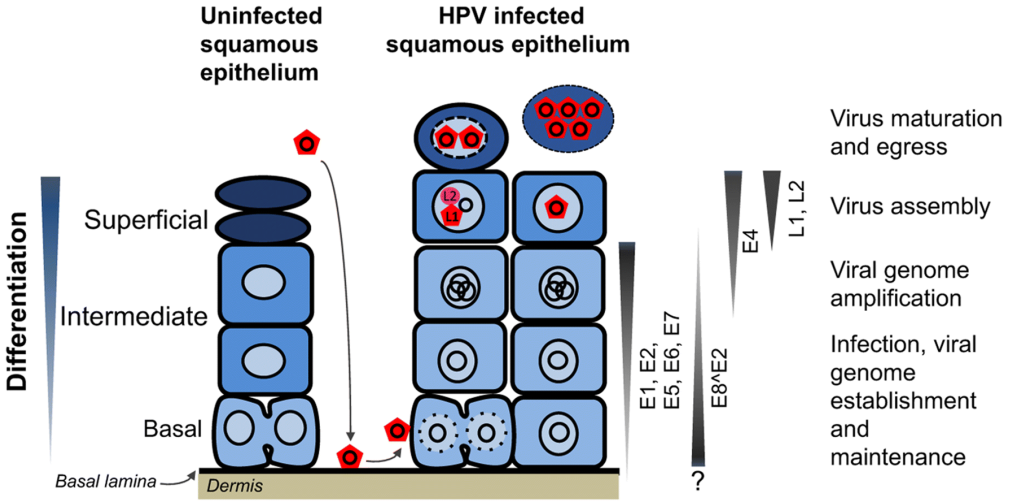

Figure 3. The key events and viral gene expression in a differentiating epithelium during the life cycle of HPV. Virus is shown as red pentagon and its genome as black circle. Shading on the arrows represents the quantity of expression of each protein during the viral replication cycle (Burley *et al*., 2020).

Active HPV DNA amplification occurs not only in the S but also in the G2 phase of cell cycle, to avoid competing with host DNA replication during S phase. The E4 protein targets the host cell machinery arresting the cell cycle progression in G2 phase (Davy *et al.*, 2002). In addition, E7 activates DNA damage repair pathways to use it for its own genome replication purposes (Moody and Laimins, 2009). Vegetative replication phase progresses to active phase and theta structures change to unidirectional rolling-circle mode, which allows to generate large amounts of viral DNA copies (Flores and Lambert, 1997).

1.3.3 Genome packaging and egress of the virions

The late stages occur in the upper layers of the stratified epithelium, where the viral copy number increases to several thousand copies per cell (Bedell *et al*., 1991). The E7 proteins are important for HPV late promoter activation (Bodily *et al*., 2013). The expression of genes involved in replication is accelerated in order to provide high number of viral copies to be packed in newly synthesized virions (Hummel *et al*., 1992; Ozbun and Meyers, 1997). The *E8*

promoter, however, remains active in the late stages of viral cycle, which means that the E8^E2 protein can still act as a repressor and modulate HPV genome amplification (Dreer *et al*., 2017).

The whole cascade of events triggers the synthesis of capsid proteins, while L2 synthesis occurs prior to L1 (Becker *et al*., 2003). At the same time, E2 recruits viral genomes to the sites of virus assembly (Day *et al*., 1998). The evidence suggests that L2 affects the viral DNA encapsidation process and recruits L1 protein for efficient packaging (Holmgren *et al*., 2005). Then, the progenitor virions are released externally with peeled keratinocytes. The E4 protein increases the egress of PV virions by remodeling the cytokeratin network of the cell and even inducing disruption, when overexpressed (Doorbar *et al*., 1991).

1.3.4 Phosphorylation of HPV proteins and host-provided viral cycle regulation

Protein kinases are enzymes that modify other proteins in the cell by transferring the phosphate from ATP to amino acid side chains. In eukaryotes, most protein kinases phosphorylate either Ser/Thr or Tyr, and a few of them phosphorylate all three amino acids. Phosphorylation usually triggers functional changes in the target proteins (referred in literature as substrates) such as changes of their enzymatic activity, cellular location, or their ability to interact with other proteins. The catalytic domain contains an active site with ATP binding pocket, where ATP is bounded and then hydrolyzed. In addition, most protein kinases have other domains required for the regulation of catalytic activity, interactions with other proteins or subcellular localization (Pollard *et al*., 2017).

Both E1 and E2 are phosphorylated by host protein kinases. Casein kinase II (CK2) plays a role in regulation of cell cycle, cellular division, survival and apoptosis (Litchfield, 2003). Interestingly, CK2 phosphorylates the N-terminal domain in the E1 protein and the hinge region in the E2 protein. Phosphorylation of E1 of HPV11 and HPV31 and E2 of BPV1 (bovine papillomavirus) resulted in inactivation of their DNA binding activity that imply CK2 as a negative regulator of viral replication (Schuck *et al*., 2013). In contrast, CK2-mediated phosphorylation of BRD4 (cellular chromatin-binding factor) is required for its interaction with HR HPV E2 proteins being critical for viral life cycle: replication initiation, gene transcription, viral genome segregation and maintenance during mitosis (Iftner *et al*., 2017). Also, knockdown of CK2α or inhibition of its catalytic activity was shown to downregulate HPV5, HPV11 and HPV18 replication indicating that CK2 activity is required for the life cycles of some HPV types (Piirsoo *et al*., 2019).

Mitogen-activated protein kinases (MAPKs) participate in different signal transduction pathways that control numerous intracellular events (Pearson *et al*., 2001). Phosphorylation of

the HPV11 E1 nuclear localization sequences by MAPK is required for the protein import to the nucleus to support viral DNA replication. This mechanism is suggested to be important for the establishment of persistent HPV infection (Yu *et al*., 2007).

Cyclin-dependent kinases (CDKs) regulate cell division and transcription in response to extra- and intracellular stimuli, and are characterized by requirement of cyclin – a separate unit providing domains for enzymatic activity (Malumbres, 2014). The E1 protein was previously shown to be able to interact with cyclins and therefore with CDK. E1 nuclear export signal is inactivated while phosphorylated by CDK, and therefore the protein remains in the nucleus, allowing for regulation of viral DNA replication (Deng *et al*., 2004; Ma *et al*., 1999).

Fibroblast growth factor receptor 3 (FGFR3) regulate cell growth, differentiation and migration, depending on cell type and its developmental stage. Interestingly, mutations activating FGFR3 kinase activity are associated with different tumor types including skin and cervix (Logié *et al*., 2005). FGFR3 colocalize with HPV E2 protein in the nucleus and phosphorylates it, restricting viral replication during early stages of infection. As cell differentiates, FGFR3 expression decreases, permitting viral genome amplification (Xie *et al*., 2017).

The AGC group of kinases and in particular PKA was shown to have an effect on PV proteins. The serine residue 109 of BPV E1 protein is a target for PKA, and mutation of this position reduces viral replication. However, the exact mechanism remained unclear (Zanardi *et al*., 1997). The serine 253 of the forced-expressed HPV8 E2 protein is most likely phosphorylated by PKA. The Phosphorylated protein has longer half-life, being stabilized by binding to host chromatin, from S-phase and through mitosis (Sekhar and McBride, 2012). However, this phosphorylation seemed to be useless for HPV8 replication in U2OS cells.

1.4. Protein kinase A

The AGC group is a subgroup of the Ser/Thr kinases named after 3 representative families: the cAMP-dependent protein kinase (PKA), the cGMP-dependent protein kinase (PKG) and the protein kinase C (PKC) (Hanks and Hunter, 1995). In addition, each family has multiple isoforms and splice variants, making the system of regulatory kinases even more complex.

1.4.1 PKA structure and regulation of activation

The PKA holoenzyme exists as a tetramer, comprised of two regulatory (R) subunits and two catalytic subunits (C) (Krebs and Beavo, 1979). The catalytic subunits Cα and Cβ are encoded by *PRKACA* and *PRKACB* genes, while Cγ (encoded by *PRKACG*) is expressed only in germ

cells in the human testis (Reinton *et al*., 1998; Søberg *et al*., 2013). The PKA subunits have several isoforms due to alternative splicing. There are two distinct types of PKA, type I and II, each of them contains either RI or RII type of R subunit, respectively. In addition, each of type of R subunit has two isoforms: RIα, RIβ, RIIα, and RIIβ (Corbin *et al*., 1977, 1978). The RI and RII classes have different sensitivity to cAMP, phosphorylation patterns and subcellular localization (Cadd and McKnight, 1989).

A-kinase anchoring proteins (AKAP) are functionally related family of proteins, containing "anchoring motifs", which binds R subunit of PKA, and "targeting domain", which associates PKA-AKAP complex with cellular organelles, membranes or structural proteins. AKAPs allow to place PKA closely to target substrates and enzymes, which regulate its activation and inactivation (Dell'Acqua and Scott, 1997). AKAP function is essential for controlling PKA function as PKA itself lacks localization domains.

As it is described in the name, PKA activity depends on cellular levels of cAMP, a second messenger synthesized by adenylate cyclase from ATP, which is able to modulate many cellular functions and the activation of PKA in particular (Kamenetsky *et al*., 2006). The release of cAMP occurs in response to stimulation of the receptor by a first messenger. The cAMP binds to the R subunits of the enzymatically inactive PKA holoenzyme localized in the cell cytoplasm, thereby liberating the catalytically active C subunits for phosphorylation of the protein substrates in cytosol and nucleus (Hunter, 2000). Activity of the PKA is regulated by the level of cAMP in the cytosol, and any change in concentration directly influences the PKA activity. Modulation of PKA activity occurs by a complex feedback mechanism. The level of cAMP can be decreased by phosphodiesterases (PDEs), which hydrolyze cAMP and therefore have a negative impact on PKA activity (Plattner and Bibb, 2012). Phosphatases have an ability to modulate both the phosphorylation of PKA subunits and counteract to PKA by dephosphorylating the target proteins (Burdyga *et al*., 2018). In addition, activation of PKA is regulated by complex phosphorylation events, such as autophosphorylation and phosphorylation by other regulatory kinases (for example, phosphoinositide-dependent kinase - PDK1) (Byrne *et al*., 2016; Cheng *et al*., 1998).

PKA core consists of N-lobe and C-lobe, forming a cleft for both ATP and target substrate binding (Figure 4). The N-terminus accommodates the ATP molecule, and conserved lysine 72 (K72) was shown to be important for stabilization of both the hydroxyl group of the substrate and the phosphate. Highly conserved DFG motif forms contact with all three ATP phosphates through Mg^{2+} ions and conserved D184 position. The DFG phenylalanine forms contact with HRD motif in the C-lobe, where conserved D166 position corrects the orientation of peptide

substrate. One of the main conserved positions in the core is phospho-threonine 197 (T197), which helps to secure the contact of the substrate and ATP molecule (Johnson *et al*., 2001; Kornev *et al*., 2006; Meharena *et al*., 2013).

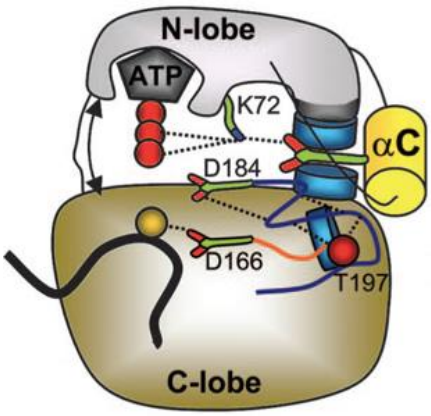

Figure 4. PKA core structure with associated ATP and substrate. The right part of the picture can be referred as the activation or catalytic segment. The phosphates are shown in red; hydroxyl group of the protein substrate is shown in brown; important contacts are shown by dashed lines (Kornev *et al*., 2006).

The cleft also contains glycin-rich loop, which is essential for γ-phosphate transfer from ATP to the substrate (Kornev *et al*., 2006). Typically, each protein kinase has a strict number of substrates. All of them have a consensus target sequence, which consists of similar residues surrounding the target Ser/Thr. The PKA C subunits phosphorylate these residues in the sequence context of R-R/K-X-S/T (X represents any amino acid) (Kemp *et al*., 1977; Kennelly and Krebs, 1991). Hydroxyl group of the Ser/Thr residue of the target peptide should be facing the γ-phosphate of the ATP. Lastly, the terminal phosphate is transferred to the recipient substrate, and both phosphorylated substrate and ADP are released from the kinase *(Kornev et al*., 2006).

1.4.2 PKA role in cell

Major physiological functions of PKA include glucose homeostasis and triglyceride storage (Czech *et al*., 2013). PKA pathway interacts with other pathways and influences different signal mechanisms (Robinson-White and Stratakis, 2002), for example, PKA acts as a feedback inhibitor for cAMP through phosphorylation of cAMP-responsive element binding protein (CREB) in the nucleus, activating CREB pathway (Shabb, 2001; Xia *et al*., 2009). PKA pathway influences proliferation of different cell types (Bacallao and Monje, 2013; Cheadle *et al*., 2008; Kim *et al*., 2012) and regulates cell migration due to the ability of PKA to interact with cell factors that control Ca^{2+} flux (Howe, 2011). Also, that the inflammatory response is regulated PKA-dependently through localization and coordination of macrophages (Wall *et al*., 2009).

1.5. Reasons to study

HPV is considered to be one of the most widespread sexually transmitted infections worldwide, suggesting that more than 80% of men and women acquire HPV by age 45 years (Chesson *et al*., 2014; de Sanjosé *et al*., 2007). Pap test (Papanicolau test) is routinely performed screening method, which can be used to detect atypical cells in cervicovaginal smears, and is considered to be the most important preventive measure for cervical carcinoma. However, co-testing with other methods is recommended in order to decrease the rate of false-negative results. Therefore, molecular tests to detect HPV DNA, RNA or proteins are being developed and becoming available (Kroupis and Vourlidis, 2011).

Nowadays, vaccination is the main strategy for prevention of the HPV-related cancers. The vaccine is produced by inserting the *L1* gene into a host (either yeast or baculovirus vector) to produce L1 major capsid proteins, which then self-assemble into virus like particles. Several prophylactic vaccines are available and approved for use, such as bivalent vaccine produced by GlaxoSmithKline (Cervarix) containing HPV16 and HPV18 antigens, and the quadrivalent vaccine produced by Merck (Gardasil) containing HPV6, HPV11, HPV16, and HPV18 antigens. Both Gardasil and Cervarix were shown to provide nearly 100% protection against persistent cervical infections with HPV16 and HPV18. Expanded nonavalent vaccine Gardasil 9 was recently approved by FDA. The vaccine is preventive against HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58 (Herrero *et al*., 2015).

At the moment, very little is known about the role of PKA in the life cycle of different HPV types; however, the information obtained from various articles suggests that PKA may somehow modulate HPV replication and, if proceed from this premise, this study can contribute to the better understanding of this interplay.

2. EXPERIMENTAL PART

2.1. Aims of the study

The general aim of this thesis was to investigate the influence of the PKACα on replication of HPV types 5, 11, 18 in U2OS cells. In order to achieve this purpose, the following step-by-step goals were set:

- generation of the plasmids encoding the FLAG-tagged wild-type PKACα and its two mutants carrying the point mutations in the amino acid residues T197 and K72;
- control of the kinase activities and the expression levels of the PKA C proteins;
- analysis of the replication efficiencies of HPV types 5, 11 and 18 in the presence of the over-expressed PKA C proteins in the U2OS cells;
- testing the influence of potentiation or inhibition of the endogenous PKA catalytic activity on the replication efficiency of HPV5;
- investigation of the possible mechanisms involved in the PKA-mediated alterations of the HPV18 replication.

2.2. Materials and methods

2.2.1. Plasmids

- **pJET1.2/blunt vector** (2974 bp) – linearized cloning vector, which is suitable for cloning of DNA fragments with blunt ends (CloneJET PCR Cloning Kit, *Thermo Fisher Scientific*). The vector contains multiple cloning sites (MCS), *eco47IR* (lethal gene, enables positive selection of the recombinants) and β-lactamase gene containing resistance to ampicillin (Amp).
- **pFLAG-CMV-4 vector** (6271 bp) - FLAG epitope (DYKDDDDK) containing vector (*Sigma-Aldrich*).
- **pFLAG-PKAwt –** a plasmid encoding for the protein kinase A catalytic subunit α transcript variant 1 (PRKACA, Gene ID 5566, NCBI accession number NM_002730.4, referred later as PKA). The coding region of the gene was amplified using RT-PCR, U2OS cells derived complementary DNA (cDNA) and primers 1 and 2 (Appendix 1. Primers used in the study).
- **pFLAG-PKA(T197A) -** a plasmid encoding the protein kinase A catalytic subunit α transcript variant 1 with the threonine residue at position 197 mutated to alanine.
- **pFLAG-PKA(K72R) -** a plasmid encoding the protein kinase A catalytic subunit α transcript variant 1 with the lysine residue at position 72 mutated to arginine.

Numbering of the mutated amino acid residues of the pFLAG-PKA(T197A) and -PKA(K72R) constructs is given relative to the 1st methionine of PKACα (NCBI accession number NP_002721.1). The mutations were introduced using PCR mutagenesis and oligonucleotides 3 and 4 (Appendix 1. Primers used in the study). All above-mentioned PKA encoding sequences were cloned into pFLAG-CMV-4 vector between HindIII and NotI restriction sites.

- **pCI-GFP** – a plasmid expressing Green Fluorescence Protein (GFP) (*Promega*, kind gift of Lagle Kasak, Tallinn University of Technology).
- **HPV5 minicircle (m.c.)** (7756 bp) – HPV5 genome containing plasmid (Sankovski *et al*., 2014).
- **HPV11 m.c.** (7931 bp) – HPV11 genome containing plasmid (Orav *et al*., 2013).
- **HPV18 m.c.** (7893 bp) – HPV18 genome containing plasmid (Orav *et al*., 2013).
- **HPV5-Nluc m.c.** was generated on the basis of the HPV5 wt genome by inserting the sequences encoding the codon optimized Nluc and the self-processed 2A region of the foot-and-mouth disease virus (FMDV) after the 59nd nucleotide of the *E2* ORF, which

corresponds to the *E1* stop codon. The full-length wt *E2* ORF begins next to the 2A sequence (Piirsoo *et al*., 2019).

- **HPV18-Nluc m.c.** was generated on the basis of the HPV18 wt genome by inserting the sequences encoding the codon optimized Nluc and the 2A region of the FMDV after the 72nd nucleotide of the *E2* ORF, which corresponds to the *E1* stop codon. The full-length wt *E2* ORF begins next to the 2A sequence (Piirsoo *et al*., 2019).
- **HPV18E1^{-} m.c.** – a plasmid encoding HPV18 wt genome containing a point mutation in the *E1* first AUG and therefore being deficient for the *E1* expression (Geimanen *et al*., 2011).
- **HPV18E8^{-} m.c.** - a plasmid encoding HPV18 wt genome containing a point mutation in the *E8* first AUG and therefore being deficient for the *E8^E2* expression (Geimanen *et al*., 2011).

The abovementioned HPV genomes come from the laboratory collection of the Chair of Virology, University of Tartu. The genomes were generated as covalently closed minicircle plasmids in *E. coli* strain ZYCY10P3S2T using minicircle DNA technology and pMC.BESBX vector for generation of the respective parental plasmids (Kay *et al*., 2010; Orav *et al*., 2013; Sankovski *et al*., 2014). The pMC.BESBX vector contains *attB* and *attP* sites for binding of bacteriophage ΦC31 integrase, 32 restriction sites of SceI endonuclease, MCS, kanamycin resistance gene, and ColE1 origin of replication.

2.2.2 Isolation of total RNA and synthesis of cDNA

Total RNA was isolated from U2OS cells using the Quick RNA MiniPrep Kit (*Zymo Research*). Cells were lysed in the RNA Lysis Buffer. Majority of genomic DNA was removed from the sample using a column containing DNA-binding resin. RNA was washed with Wash Buffer and eluted in DNase/RNase free water. Approximately 10 µg of the total RNA was treated with 8 U of Turbo DNase (*Thermo Fisher Scientific*) for 2 h at 37 ˚C. Turbo DNase was inactivated by incubation at 75 ˚C for 10 min in the presence of 15 mM EDTA. The total RNA was precipitated with 7.5 M LiCl by centrifugation at 4 ˚C and 15000 rpm for 15 min using MicroCL 21R Microcentrifuge (*Thermo Fisher Scientific*). The samples were washed with ice-cold 75% ethanol and centrifuged at 4 ˚C 15000 rpm for 5 min. The pellets were resuspended in 15 µl of nuclease-free water.

cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (*Thermo Fisher Scientific*), oligo(dT) and either 5 µg of total RNA in the case of subsequent amplification of the sequence encoding for PKA or 2 µg of total RNA for analysis of the HPV18 transcripts.

The appropriate amount of the total RNA was mixed with 1 µl of oligo(dT), incubated for 5 min at 65 ˚C and chilled on ice. cDNA was synthesized at 45 ˚C for 1 h in total volume of 20 µl in the presence of 4 µl of 5x Reaction Buffer, 20 U of RiboLock RNase Inhibitor, 2 µl of 10 mM dNTP Mix and 200 U of RevertAid M-MuLV RT. The cDNA synthesis was terminated by heating the mix at 70 ˚C for 5 min.

2.2.3 PCR and gel extraction of PCR products

Reactions for qPCR were held in total 10 µl containing 0.5 µl of cDNA, 1 µM primers 5-9 (Appendix 1. Primers used in the study) and 2 µl of 5x EvaGreen (*Solis Biodyne*) on MicroAmp 96-well plates (*Thermo Fisher Scientific*). The qPCR was performed in triplicates using QuantStudio Real-Time PCR System (*Thermo Fisher Scientific*) and the following program: denaturation at 95 ˚C for 12 min, 40 cycles of amplification (denaturation 95 ˚C 15 s, annealing 60 ˚C 15 s, synthesis 72 ˚C 15 s).

The PKA encoding sequences were amplified using reverse transcription PCR method (RT-PCR) and Phusion High-Fidelity DNA Polymerase Kit (*Thermo Fisher Scientific*). Reagents for the PCR included 28.5 µl of nuclease-free water, 5 µl of 10 mM dNTP mix, 10 µl of 5x Phusion HF or GC buffer, 1 µl of cDNA template, 0.75 µl of Phusion DNA polymerase and 1 µM primers. Expression of the different C subunits of PKA was analyzed using primers 10-15 (Appendix 1. Primers used in the study). Sequence encoding the PKA was amplified using primers 1 and 2 (Appendix 1. Primers used in the study). The constructs encoding PKA mutants (T197A, K72R) were generated using oligonucleotides 3 and 4 (Appendix 1. Primers used in the study) on the basis of the construct encoding for the PKA.

Reaction was performed with PCR Mastercycle (*Eppendorf Scientific*) using the following program: initial denaturation of template DNA for 30 s at 98 ˚C, 32 cycles of the synthesis: (DNA denaturation at 98 ˚C for 8 s, annealing of the primers at 59 ˚C for 20 s, and extension at 72 ˚C for 45 s), final extension at 72 ˚C for 5 min and cooling to 10 ˚C. The program of the PCR mutagenesis for generation of the sequences encoding the PKA mutants involved 20 cycles of the synthesis with extension time 6 min.

The PCR products were separated using the gel electrophoresis method and 0.8% agarose gel containing 0.3 µg of ethidium bromide in 1xTAE buffer (40 mM Tris-acetate, 1 mM EDTA) and in the presence of 0.6 µg of O'GeneRuler 1kb DNA ladder (*Thermo Fisher Scientific*). The required fragments were cut out from the gel with a scalpel under UV (260 nm).

The DNA was extracted from the gel using Zymoclean™ Gel DNA Recovery Kit (*Zymo Research*) according to manufacturer`s instructions. The gel pieces with DNA were mixed with

ADB Buffer Gel in ratio 1:3 and incubated at 55 ˚C until the gel slices were completely dissolved. Melted agarose solution was transferred to the DNA-binding columns, centrifuged at 13000 rpm for 1 min and the flow-through was discarded. The DNA was washed two times with 200 µl of DNA Wash Buffer and centrifugated for 30 seconds at 13000 rpm. The column-bound DNA was eluted in 20 µl of pure water. The DNA was additionally precipitated with 2 µl of 5 M NaCl and 40 µl of 96% ethanol with subsequent centrifugation at 4 ˚C 15000 rpm for 15 min. The pellets were washed with cold (−20 ˚C) 75% ethanol, centrifuged at 4 ˚C 15000 rpm for 5 min and resuspended in 15 µl of nuclease-free water. The concentrations of nucleic acids were measured using a Nanodrop-1000 spectrophotometer (*Thermo Fisher Scientific*) at 260 nm wavelength.

2.2.4 Cloning of the PCR products into pJET1.2/blunt vector and transformation

The obtained RT-PCR product was cloned into pJET1/2 cloning vector (CloneJET PCR Cloning Kit (*Thermo Fisher Scientific*)) between HindIII and NotI restriction sites. Reagents for the reactions included 4 µl of 5x Reaction Buffer, 350 ng of the gel-purified PCR product, 9 U of T4 DNA Ligase and 35 µg of pJET1.2/blunt vector. The reactions were incubated at room temperature (RT) for 3 h.

Competent cells of nonpathogenic *E. coli* strain DH5α were used for the transformation. The bacteria were thawed on ice for 20 min. Ligation mixes were added to 200 µl of the competent cells and incubated on ice for 30 min. After that, the reaction mixes were incubated at 37 ˚C for 4 min and then transferred on ice for 1 min. Next, 800 µl of LB broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) was added to each tube, and mixes were incubated at 37 ˚C for 40 min. The tubes were centrifuged at RT and 5000 rpm for 3 min. The pellet was resuspended in 100 µl of LB broth and plated using the spread plate technique on the LB agar supplemented with 50 µg/ml of Amp. The plates were incubated at 37 ˚C for 24 h. The growth of colonies was observed.

The presence of the insert in the cloning vector was verified using the colony PCR method. The reaction mix consisted of 2 µl of 5x HOT FIREPol PCR mix (*Solis BioDyne*), 6.5 µl of nuclease-free water and 1 µM of primers 1 and 2 (Appendix 1. Primers used in the study). Bacteria from each separate colony were transferred to the LB agar supplemented with Amp and then into the PCR mix using pipette tips.

Reaction was performed using PCR Mastercycle (*Eppendorf Scientific*) according to the following program: the polymerase activation at 96 ˚C for 12 min 30 s, 25 cycles of the

synthesis: (denaturation at 96 ˚C for 15 s, annealing of the primers at 59 ˚C for 15 s and extension at 72 ˚C for 45 s).

The obtained PCR products were analyzed on 1.2% agarose gel as described in 2.2.3. The correct colonies were transferred from the plates to 3 ml of LB broth containing 200 μg/ml of Amp and incubated at 37 ˚C and 220 rpm for 18 h in Orbital Incubator MIR-220RU (*Sanyo*).

2.2.5 Plasmid DNA extraction and control with restriction and sequencing

Plasmid DNA was extracted from bacteria using Plasmid Extraction Mini Kit *(Flavoprep*) or using Endotoxin-free Plasmid DNA Purification Midi Kit (*Thermo Fisher Scientific*) according to manufacturers' protocol and using 3 ml or 200 ml of bacteria culture, respectively. All procedures were performed at RT. First, bacteria culture was centrifuged at 5000 rpm for 5 min and the supernatant was removed. The pellet was resuspended in the appropriate buffer (FAPD1 or RES-EF) containing RNase A and lysed in the respective lysis buffer (FAPD2 or LYS-EF) for 5 min. Reactions were neutralized with FAPD3 or NEU-EF buffers, respectively, and centrifuged at 13000 rpm for 5 min. Supernatant containing DNA was transferred to the DNA-binding columns. Beforehand, the NucleoBond Xtra Column (Midi kit) was equilibrated with EQU-EF Buffer. The column-bound DNA was washed with the appropriate washing buffers, eluted either with nuclease-free water or ELU-EF buffer (Midi kit). Next, the DNA extracted using Midi kit was precipitated with 3 ml of isopropanol, washed with 75% ethanol and dissolved in 1 ml of nuclease-free water.

The obtained constructs were verified by restriction analysis using restriction endonucleases HindIII and BamHI (*Thermo Fisher Scientific*) with subsequent sequencing of the correct clones using pJET1/2 primers 16 (Appendix 1. Primers used in the study). For each restriction reaction, the following components were used: 0.5 μl of each restriction enzyme (10 U/μl), 400 ng of each DNA sample and 2 μl of 10x FastDigest green Buffer. Total volume of a reaction was 20 μl. The restriction reactions were incubated at 37 ˚C for 1 h. The DNA was visualized under UV (260 nm) using the gel electrophoresis method as described in section 2.2.3.

DNA sequencing was performed in Estonian Institute of Genomics Core Facility, Tartu, Estonia.

2.2.6 Generation of the pFLAG-PKA constructs

The coding sequences the PKAwt and its mutants were excised from pJET1.2/blunt vector using HindIII and NotI restriction enzymes, purified from agarose gel and ligated with the pre-cleaved with the same enzymes and gel-purified pFLAG-CMV-4 vector. The ligation reactions

containing 70 ng of the pFLAG-CMV-4 vector, 500 ng of the gel-purified PCR product, 1.2 µl of 10x reaction Buffer and 1.2 µl of T4 DNA Ligase were incubated at 16 ˚C overnight. The ligation mixes were transformed to competent DH5α as described in section 2.2.4.

2.2.7 Cell culture and transfection

The human osteosarcoma cell line U2OS were propagated in the normal growth medium (NGM): Iscove's Modified Dulbecco's Medium (IMDM, *Pan Biotech*), 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (PEST, *Sigma-Aldrich*). A derivative of human embryonic kidney cell line HEK293 - 293T cells were maintained in the NGM containing Dulbecco's Modified Eagle Medium (DMEM, *Pan Biotech*), 10% FCS and 1% PEST. The cells were propagated in incubator at 37 ˚C and 5% CO_2.

U2OS cells were transfected by electroporation using approximately 10^6 cells for each transfection. Confluent U2OS cells were subcultured on 10 cm cell culture dishes (*Corning Inc*) approximately 24 h before transfection. Prior electroporation, NGM was removed; the cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO_4) and detached using 0.25% Trypsin-EDTA (*Sigma-Aldrich*). The detached cells were transferred into 8 ml of fresh NGM, centrifuged at 1000 rpm and RT for 5 min using Eppendorf Centrifuge 5810R (*Thermo Fisher Scientific*) and then resuspended in the fresh NGM using 250 µl of medium per one transfection. Approximately 10^6 U2OS cells were mixed with 50 µg of salmon sperm DNA and the following amounts of the HPV genomes and PKA encoding plasmids: HPV5 and HPV5-Nluc (1500 ng), HPV11 (750 ng), HPV18, HPV18E1$^-$, HPV18E8$^-$ and HPV18-Nluc (1000 ng), pFLAG-PKAwt (240 ng), pFLAG-PKA(T197A) (1000 ng), pFLAG-PKA(K72R) (1500 ng). Transfection mixtures containing pFLAG-PKAwt and mutant pFLAG-PKA(T197A) were compensated up to 1500 ng with the respective amounts of the pFLAG-CMV-4 vector. Suspension of the cells with DNA was transferred into an electroporation cuvette with gap size of 4 mm. The electroporation was performed using a Gene Pulser XCell machine (*Bio-Rad Instruments*) at 220 V and 975 µF. After electroporation, approximately 260 µl of NGM was immediately added to the cells. The transfected cell suspensions and NGM were transferred to appropriate plates. The cells were incubated for 2, 3 and 4 days. For the experiment with IBMX and H89 (both purchased from *Sigma-Aldrich*), the cells were incubated for 2 or 3 days after transfection and then treated with 500 µM IBMX or 5 µM H89 for 2 days in NGM. The NGM containing IBMX, H89 or DMSO (used as a vehicle in dilution 1:1000), was replenished every 24 h.

The 293T cells were transfected with using polyethyleneimine (PEI) (*Inbio*). The cells were plated on 10 cm cell culture dishes approximately 24 h prior transfection. Different amounts of the PKA encoding constructs were used for transfection of 293T cells: pFLAG-PKAwt (2500 ng), pFLAG-PKA(T197A) (8000 ng), pFLAG-PKA(K72R) (16000 ng per two plates) and pCI-GFP (8000 ng) for control. Transfection mixture containing pFLAG-PKAwt was compensated with 6500 ng of the pFLAG-CMV-4 vector. PEI stock solution was diluted in sterile water to final concentration 1 mg/ml and mixed with DNA in ratio 3:1 in 100 µl of pure DMEM. The mixture was incubated for 5-10 min at RT. The cells were washed with PBS, and 8 ml of pure DMEM and DNA/PEI complexes were added to the cells followed by incubation for approximately 2 h. Afterwards, 12 ml of NGM was added to the cells, and the cells were incubated for 48 h.

2.2.8 Isolation of total DNA from U2OS cells

U2OS cells were washed with PBS and lysed in 500 µl of Sol IV buffer (20 mM Tris pH 8.0, 100 mM NaCl, 0.1 g/L mM EDTA, 0.2% SDS). The lysates were homogenized using insulin syringe and 24G needle and incubated in the presence of 200 µg/ml of Proteinase K at 56 ˚C overnight. Next, 500 µl of phenol-chloroform mixture (1:1) was added to each lysate. The samples were mixed by vortexing for 10-15 s and centrifuged at RT and 13000 rpm for 3 min. Upper phase containing DNA was transferred to a new microtube. Two volumes of 96% ethanol were added to each tube for DNA precipitation. The samples were centrifuged at 4 ˚C 15000 rpm for 15 min using MicroCL 21R Microcentrifuge (*Thermo Fisher Scientific*). Supernatant was removed, and dry precipitate was resuspended in 100 µl of TE (10 mM Tris, 1 mM EDTA pH 8.0) containing 40 ng of RNase A. The mixtures were incubated at 37 ˚C for 1 h. Total DNA was additionally precipitated using 10 µl of 5 M NaCl and 200 µl of 96% ethanol as described in 2.2.3 and resuspended in 30 µl of nuclease-free water.

2.2.9 Luciferase assay

U2OS cells were washed with PBS solution and lysed in 40 µl of Passive Lysis Buffer (*Promega*) for 10 min at RT. The lysates were transferred in two replicates to different 96-well plates for analysis of Nluc and alkaline phosphatase (AP) activities. Nluc activity was measured using Nano-Glo Luciferase Assay System Kit (*Promega*) and 20 µl of the furimazine substrate diluted with the Luciferase Assay Buffer in ratio 1:300. AP was measured by adding 20 µl of CSPD substrate (*Applied Biosystems, Tropix*) to 10 µl of the lysates with further incubation for 10 min. Chemiluminescence of both substrates was measured using GloMax 96 Microplate Luminometer (*Promega*). For each sample, Nluc activity was normalized by AP activity.

2.2.10 Southern blot and hybridization

The following amounts of the total DNA were used for analysis of different HPV replication efficiencies: 6 µg for HPV5, 3 µg for HPV11, and 5 µg for HPV18. The total DNA was digested with 0.8 µl of DpnI enzyme to cut the palindromic sequence (5'-Gm^6A^TC-3') of the bacterially methylated input DNA and 1.5 µl of SacI, HindIII and BglI enzymes (*Thermo Fisher Scientific*) to linearize the HPV5, HPV11 and HPV18 genomes, respectively. Reactions were held in total volume 30 µl in the presence of 3 µl of 10x FastDigest Green Buffer per one reaction at 37 ˚C overnight. The DNA fragments produced during the restriction reaction were separated on 0.8% agarose gel.

Afterwards, the gel was incubated in Solution A (0.5 M NaOH, 1.5 M NaCl) at RT for 40 min, washed with distilled water and neutralized in Solution B (1 M Tris pH 8.0, 1.5 M NaCl) at RT for 30 min. The DNA was transferred from the gel to a nylon membrane (*Millipore*) using upward capillary transfer method in 10x saline-sodium citrate buffer (10x SSC: 1.5 M NaCl, 150 mM $Na_3C_6H_5O_7$) for 18 h.

After transfer, DNA was cross-linked to the membrane using UV light (*Stratalinker*). In order to prevent nonspecific binding, the membranes were incubated in prehybridization solution at 67 ˚C for 45 min in rolling tubes. Prehybridization solution for one membrane consisted of 16 ml of water, 9 ml of 20x SSC, 3 ml of 50x Denhardt's solution, 1.5 ml of 10% SDS and 6000 µg of salmon sperm DNA denaturated at 100 ˚C.

For synthesis of hybridization probes, 150 ng of the linearized and gel purified HPV5, HPV11 and HPV18 m.c. DNAs and DecaLabel TM DNA Labeling Kit (*Thermo Fisher Scientific*) were used. The linearized genomes were obtained as described in section 2.2.3. For the synthesis, 10 µl of decanucleotide primers were added to the DNA template in a total volume of 30 µl. The samples were incubated at 100 ˚C for 10 min and placed on ice. Then, 3 µl of mixC (mixture of all dNTPs except dCTP), 4 µl of α-32P-dCTP isotope (*Hartman Analytics*) and 1 µl of Klenow Fragment lacking 5'-3' exonuclease activity were added. After incubation of the samples at 37 ˚C for 15 min, 4 µl of dNTP were added. The reaction mixtures were incubated at 37 ˚C for additional 15 min. Denaturation was performed at 100 ˚C for 10 min.

Hybridization of the membranes with the radiolabeled probes was performed in the prehybridization solution at 67 ˚C overnight. Next day, the membranes were washed with pre-heated washing solutions I, II and III at 67 ˚C. The wash step was repeated twice with solution I (10x SSC, 0.1% SDS) for 5 min each, then once with solution II (5x SSC, 0.1% SDS) for 15 min and twice with solution III (0.5x SSC, 0.1% SDS) for 10 min. Thereafter, the membranes

were exposed to the X-ray film at −80 ˚C for about 10 h. The X-ray films were developed and fixed using AGFA Developer and Rapid Fixer solutions.

2.2.11 Western blot and immunoprecipitation

U2OS cells were lysed in 500 µl of RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.1% SDS and 0.1% Triton X-100) supplemented with protease inhibitor cocktail (PIC, *Sigma-Aldrich*) for 10 minutes on ice. The amount of total protein was measured using BCA Protein Assay Kit (*Pierce*). The working reagent (WR) was prepared by mixing Reagent A with Reagent B in ratio 50:1, and 40 µl of the WR was added to 4 µl of each BSA standard or a sample of interest in the microplate. The plate was incubated at 37 ˚C for 30 min. The absorbance was measured at 562 nm on an absorbance microplate reader Sunrise using Magellan data analysis software (*Tecan*).

293T cells were lysed in 5 ml of Sigma Lysis Buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and PIC) on ice for 15 min. The lysates were cleared by centrifugation at 4 ˚C 15000 rpm for 5 min, and supernatant was transferred to the new tubes. The over-expressed proteins containing the FLAG epitope were precipitated using 20 µl of ANTI-FLAG M2 Affinity Gel (*Sigma-Aldrich*), previously washed 2 times with PBS solution and one time with Sigma Lysis Buffer. Precipitation was carried out at 4 ˚C under slow end-to-end rotation overnight. The immunocomplexes were washed with 2 ml of the Sigma Lysis buffer 3 times for 10 min and resuspended in 100 µl of PBS.

Next, 15 µg of total proteins obtained from U2OS cells or 10 µl of immunoprecipitated proteins from 293T were mixed with the respective amount of 3X Laemmli Sample Buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 100 µM DDT, 0.004% bromophenol blue). The samples were denaturated at 100 ˚C for 5 min.

Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (*Millipore*). The membrane was blocked in PBS solution containing 0.1% Tween-20 (PBS-T) and 5% of non-fat dry milk for 30 min at RT. Afterwards, the membrane was incubated with a primary antibody at 4 ˚C overnight. The following procedures were performed at RT. The membrane was washed in PBS-T 3 times for 15 min, incubated with a secondary antibody (if appropriate) for 1 h with subsequent washing with PBS-T 3 times for 15 min. The following antibodies were used: Monoclonal ANTI-FLAG M2−HRP antibody (*Sigma-Aldrich*, 1:3000), anti-GAPDH (*Sigma-Aldrich*, 1:5000) and goat anti-mouse IgG conjugated with HRP (*Invitrogen*, 1:10000). Antibodies were diluted in PBS-T solution containing 2.5% of non-fat dry milk. Enhanced chemiluminescence substrate for HRP (SuperSignal™ West Dura

Extended Duration kit, *Pierce*) was added to membrane for 1 min. The membrane was rinsed in PBS-T, exposed to X-ray film and developed as described in 2.2.10.

2.2.12 *In vitro* kinase assay

For the assay, 10x PKA kinase assay buffer (KAB) containing 400 mM Tris-HCl pH 7.5, 200 mM $MgCl_2$, 500 µM DTT in nuclease-free water was prepared. Solutions containing 200 µM "cold" ATP and 400 µM γ^{32}-ATP in 1x KAB were made. Next, 10 µl of PKAwt and PKA(T197A), PKA(K72R) immunoprecipitants and bacterially purified PKA substrate Nth1 (86 kDa, kind gift from M. Loog laboratory) were mixed in 40 µl of 1X KAB on ice.

Ten µl of each reaction was immediately transferred to another microtube for running a separate SDS-PAGE gel and subsequent staining of the gel with Coomassie Brilliant Blue to visualize the Nth1 substrate.

Then, 3.4 µl of the γ^{32}-ATP solution (0.4 µl of γ^{32}-ATP per sample) was added to the "cold" ATP solution, and 10 µl of the mixture was transferred to each immunocomplex. *In vitro* kinase reactions were carried out at RT for 30 min and stopped by adding the Laemmli Sample Buffer and incubation at 100 ˚C for 5 min. After this, the proteins were separated using SDS-PAGE on 12% gel. The gel was transferred to filter paper and dried using Gel Vacuum Dryer Heto Dry GD-2 (*HETO*) for 45 min at 70 ˚C. Autoradiogram of the gel was obtained using the cassette with intensifying screen and Typhoon FLA 9500 (*GE Healthcare*) after overnight exposure at RT.

2.3. Results

2.3.1 Selection of PKA subunit

It has been established that at least three different PKA catalytic subunits (*PKA C*) are encoded by human genome: *PRKACA* (NCBI accession numbers for different isoforms NM_001304349.1; NM_002730.4; NM_207518.3), *PRKACB* (NCBI accession numbers for different isoforms NM_001242857.2; NM_001242858.2; NM_001242859.2; NM_001242860.2; NM_001242861.2; NM_001242862.2; NM_001300915.2; NM_001300916.2; NM_001300917.2; NM_002731.3; NM_182948.4; NM_207578.3) and *PRKACG* (NCBI accession number NM_002732.3). An expression pattern of different PKA C was analyzed in two different human cell lines capable for supporting HPV replication: HPV31-positive human primary keratinocytes CIN612E and osteosarcoma cell line U2OS. Expression of *PKA C* mRNAs was analyzed using RT-PCR and 2 independent pairs of primers for each gene (primers 10-15, Appendix 1. Primers used in the study). Also, mRNA expression of the house keeping gene *GAPDH* was analyzed as a positive control. Our analysis (Figure 5) revealed that *PRKACA* and *PRKACB* mRNAs were expressed in both cell lines. However, expression of *PRKACG* mRNA was detected only in U2OS cells using one pair of primers. Therefore, feasibility of the *PRKACG*-encoded protein to be involved in regulation of HPV replication proteins was considered as insignificant.

Bioinformatic analysis of the PKA C proteins was performed using NCBI blast algorithm. PKACα and PKACβ proteins were approximately 92% identical, while PKACα and PKACγ had 84% identity; PKACβ and PKACγ had approximately 79% identity. Between the two isoforms, PKACα was chosen for further research because of its evident expression level in the target cells and high identity percent with PKACβ protein, suggesting possible similarity of the biological activities of these proteins.

Next, the sequence of the *PRKACA* transcript variant 1 (NCBI accession number NM_002730.4) encoding the PKACα1 protein (NCBI accession number NP_002721, further referred as PKAwt) was amplified using RT-PCR and primers 1-2 (Appendix 1. Primers used in the study) and cloned into the pFLAG-CMV-4 vector. The resulted construct is further referred as pFLAG-PKAwt. On the basis of the pFLAG-PKAwt construct, the constructs encoding PKACα mutants (referred as pFLAG-PKA(T197A) and pFLAG-PKA(K72R)) were generated using PCR mutagenesis.

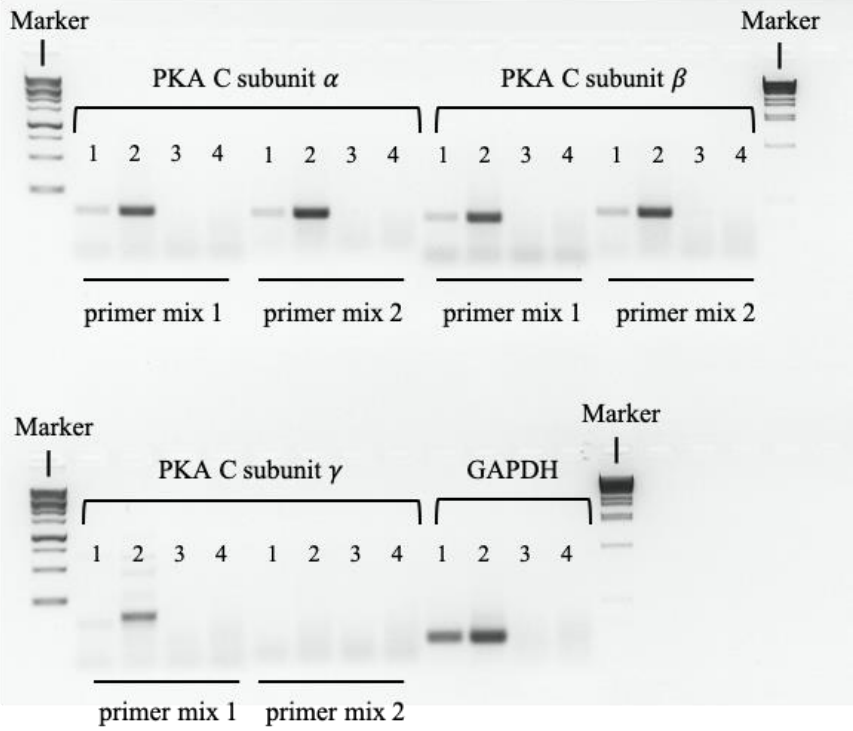

Figure 5. RT-PCR analysis of the expression of *PKA C* in CIN612E and U2OS cells. Expression of the housekeeping gene *GAPDH* was analyzed as a positive control. (1) CIN612E cDNA; (2) U2OS cDNA; (3) CIN612E-RT*; (4) U2OS-RT*. Probes (3) and (4) were identical to the samples (1) and (2), respectively, but they did not contain reverse transcriptase (RT) during cDNA synthesis; these samples were analyzed to confirm absence of contamination with genomic DNA. To estimate the size of the obtained PCR products, O'GeneRuler 1kb DNA ladder was used.

2.3.2 Control of expression level of PKA C proteins in U2OS cells

It has been suggested that the proteins can be stabilized through (auto)phosphorylation processes, which may influence their expression levels and biological activity in cells. Therefore, immunoblotting analysis was required to determine the amounts of the PKA-encoding constructs expressing similar levels of the wt and mutant PKA C proteins in the U2OS cells in order to further compare their biological effects.

Approximately 10^6 U2OS cells were transfected with different amounts of the generated PKACα-encoding constructs. Expression levels of the PKAwt and PKA(T197A), PKA(K72R) were analyzed 48 h post-transfection using Western blot (WB) and Monoclonal ANTI-FLAG M2−HRP antibody. Eventually, the following ratio of the constructs were found to be optimal to produce similar levels of the wt and mutant PKA C proteins: pFLAG-PKAwt 240 ng, pFLAG-PKA(T197A) 1000 ng, and pFLAG-PKA(K72R) 1500 ng (Figure 6, upper panel). The total amount of the transfected DNA was compensated with the respective amount of the empty vector to achieve similar transfection conditions. Amount of total protein was measured prior to SDS-PAGE to ensure the equal loading of the lysates, which was additionally controlled via immunoblotting analysis of the housekeeping protein GAPDH (Figure 6, lower panel). The WB

showed that the generated constructs encoded approximately 42 kDa proteins. Also, the analysis revealed that the levels of PKAwt and mutant proteins expressed using the indicated amounts of the plasmids were similar. Therefore, this ratio of the PKA-encoding plasmids was used in the further experiments.

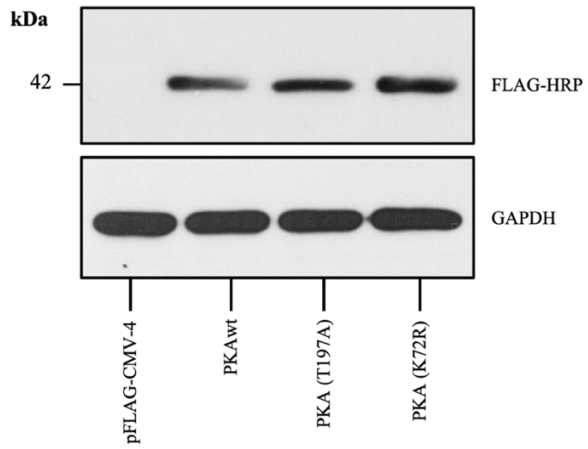

Figure 6. Over-expression studies of the FLAG-tagged proteins PKAwt, PKA(T197A), and PKA(K72R). U2OS cells were transfected with the following amounts of the plasmids: 240 ng pFLAG-PKAwt, 1000 ng pFLAG-PKA(T197A), 1500 ng pFLAG-PKA(K72R) and 1500 ng pFLAG-CMV-4 vector as a negative control. The cells were incubated for 2 days, lysed and subjected to immunoblotting assay. Detection of FLAG-tagged proteins was made using ANTI-FLAG antibody. GAPDH was analyzed as a loading control and detected with anti-GAPDH and goat anti-mouse IgG conjugated with HRP antibodies.

2.3.3 Control of kinase activity of the subunits encoded by the pFLAG-PKA constructs

The mutated PKACα subunits encoded by the generated pFLAG-PKA(T197A) and pFLAG-PKA(K72R) constructs should have severely reduced catalytic activity in contrast to pFLAG-PKAwt. Mutation of T197 to alanine (referred as dephosphorylated mutant) was previously shown to reduce PKA catalytic function, suggesting that the hydroxyl group of the threonine residue may play a significant role in stabilizing the active site and promoting the phosphoryl transfer reaction (Adams *et al*., 1995; Cheng *et al*., 2006). Mutation of K72 to arginine with so-called "bulkier residue", prevents the catalytic cleft from assuming its proper conformation needed for the kinase activity (Iyer *et al*., 2005). *In vitro* kinase assay with γ^{32}-ATP was performed in order to analyze the ability of the PKAwt and mutant subunits to catalyze the phosphorylation reaction.

In order to obtain the target FLAG-tagged proteins, 293T cells were transfected with pFLAG-PKAwt (2500 ng), pFLAG-PKA(T197A) (8000 ng), pFLAG-PKA(K72R) (16000 ng per two 10 cm plates) and pCI-GFP (8000 ng) for negative control. Proteins PKAwt, PKA(T197A), and PKA(K72R) were immunoprecipitated 48 h after the transfection and used for subsequent *in vitro* kinase assay and immunoblotting. The first set of the kinase reactions contained only the

target PKA C proteins. The second set of the reaction mixes (lanes 5-8) contained also the Nth1 protein (86 kDa) - one of the substrates for PKA. The kinase assay reactions were loaded on two separate acrylamide gels. One of the gels was exposed to phosphor imager to detect the radioactive signals (Figure 7, upper panel). Another gel was stained with Coomassie Brilliant Blue to visualize the Nht1 protein (Figure 7, lower panel).

The results of the assay demonstrate that, in contrast to the PKAwt, both PKA(T197A) and PKA(K72R) have hardly visible residual catalytic activity: very faint signals of Nth1 phosphorylation were detected. Therefore, the PKA C mutants can be considered as "kinase-dead" ones. These data ensure that the effects observed using these mutant proteins are associated with their inability to catalyze the phosphorylation reaction. Besides, only PKAwt was able to phosphorylate itself generating a radioactive signal at approximately 42 kDa that corresponds to the molecular mass of the PKA C (Figure 6).

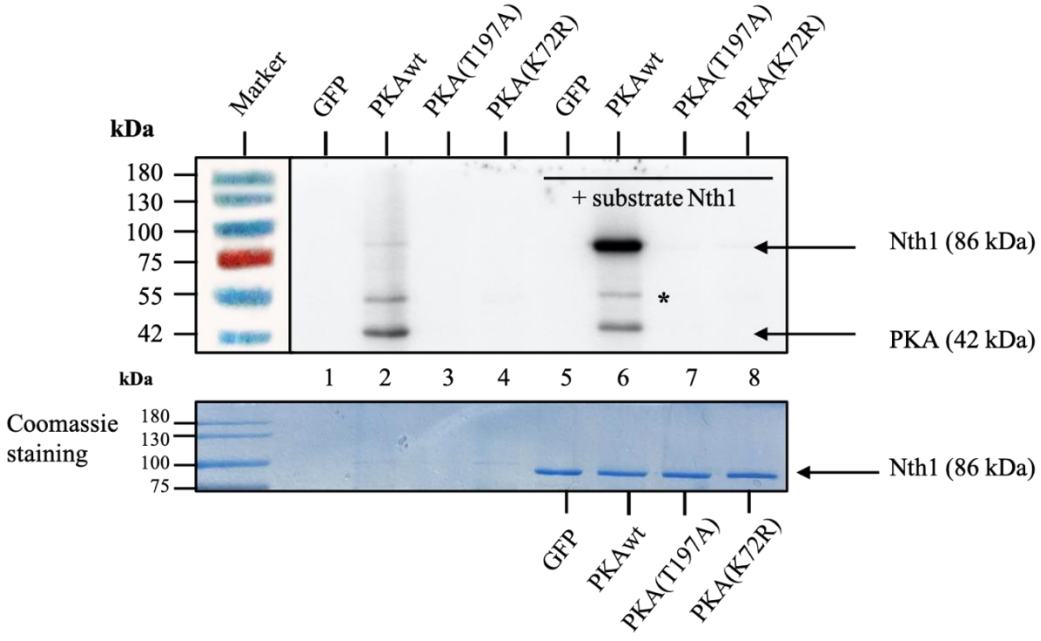

Figure 7. *In vitro* kinase activity of FLAG-tagged proteins PKAwt, PKA(T197A), and PKA(K72R). The proteins were immunoprecipitated 48 h after the transfection of 293T cells and subjected to *in vitro* kinase assay (upper panel). The bottom panel depicts the PKA substrate Nth1 stained with Coomassie Brilliant Blue. Bands marked with an asterisk show an unknown substrate phosphorylated by PKAwt in the cell lysate.

2.3.4 Effects of the over-expressed PKA C proteins on the replication of HPV5, HPV11, HPV18

The association between the phosphorylation activity of kinases and the replication of different HPV types has been previously established. Catalytic activity of CK2 subunit is required for the stability of E1 regulatory protein of HPV11 and 18, while MAPKs, CDKs, FGFR3 kinases play an important role in E1 and E2 nuclear localization of different HPV types, thereby

participating in regulation of the viral genome replication (Deng *et al*., 2004; Ma *et al*., 1999; Piirsoo *et al*., 2019; Xie *et al*., 2017; Yu *et al*., 2007). Considering that several HPV proteins contain putative phosphorylation sites for PKA, it has been hypothesized that PKA activity influences the replication efficiency of different HPV types.

In order to examine this hypothesis, the U2OS cells were transfected with wild type HPV genomes (LR β type HPV5, LR α type HPV11 and HR α type HPV18), pFLAG-PKA constructs in the optimal concentrations, as described above, and pFLAG-CMV-4 vector as a control. Total DNA was isolated 48 h, 72 h and 96 h after the transfection and treated with the respective linearizing restriction enzyme (SacI for HPV5, HindIII for HPV11, and BglI for HPV18) and DpnI for the digestion of the input HPV DNA purified from bacteria. The DNA was subsequently analyzed using southern blot (SB). All the experiments were repeated three times. Data of representative experiments are shown in Figure 8 for HPV5 (A), HPV11 (B) and HPV18 (C). SB signals of three independent experiments were quantified using GelQuantNet program. The signals obtained in the samples transfected with the respective genome and empty vector and incubated for 48 h were set as 100%. Data for other samples were calculated relative to the control (Figure 8A-C, right panels).

Eight kb bands detected on the autoradiograms correspond to the replicated HPV5, HPV11 and HPV18 DNA. Over-expression of PKAwt was accompanied with strong (approximately 29-fold) reduction of HPV5 replication (Figure 8A, lanes 4-6). In contrast to PKAwt, the mutant PKA C proteins demonstrated reduced ability to down-regulate HPV5 replication, approximately 1.5- and 3-fold for PKA(T197A) and PKA(K72R), respectively (Figure 8A, lanes 7-12). These data suggest that PKA down-regulates HPV5 replication in a kinase activity-dependent manner. Results of HPV18 replication assay were the opposite to the ones observed with HPV5: over-expression of PKAwt resulted in increased HPV18 DNA amount, more than 12-fold in 48 h and up to 3-fold in 72 h (Figure 8B, compare lanes 1-3 and 4-6). Quantification showed that replication signal of HPV18 in the presence of PKAwt reached its maximum level in 48 h and did not change substantially during the prolonged incubation (Figure 8B, right panel). Compared to "kinase-dead" mutants (Figure 8B, lanes 7-12), it is clearly visible that any impairment in PKA catalytic activity eventuated in reduced ability of HPV18 for replication.

The results in Figure 8C, otherwise, showed only slight increase in the amount of the replicated HPV11 DNA in the presence of the over-expressed kinase-competent or kinase-deficient PKA C proteins. These data indicate that in contrast to HPV5 and HPV18, HPV11 replication cycle was not extensively affected by PKA kinase activity. Therefore, study of HPV11 replication

was excluded from the further research. The obtained results demonstrate that PKA activity influences differently the replication efficiency of different HPV types.

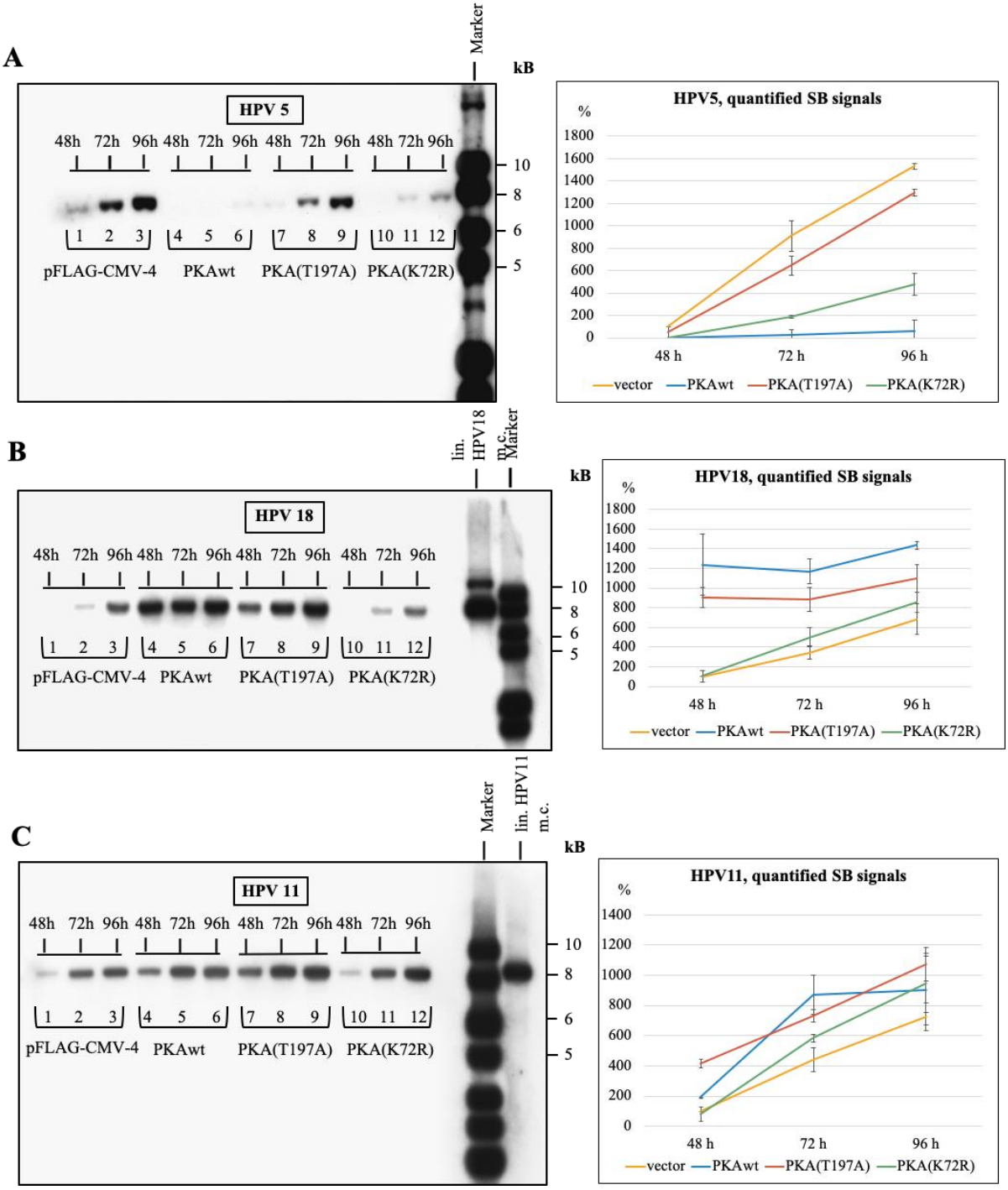

Figure 8. Replication signal of HPV types 5, 18, 11 in the U2OS cells transfected with 1500 ng pFLAG-CMV-4 vector, 240 ng pFLAG-PKAwt, 1000 ng pFLAG-PKA(T197A), 1500 ng pFLAG-PKA(K72R) constructs. Total DNA was extracted 48 h, 72 h and 96 h after the transfection, treated with restriction enzymes DpnI and SacI/BglI/HindIII linearizing HPV5, HPV18 and HPV11 genomes, respectively, and subjected to SB. Quantified signals are shown in the right panels. The signal of the control sample transfected with HPV genome and empty vector and extracted at 48 h was set as 100%. Data from other samples are shown as a percentage of the control (the average mean +/- SD (n=3)). **A**: co-transfection with 1500 ng of HPV5; **B:** co-transfection with 1000 ng of HPV18; **C:** co-transfection with 750 ng of HPV11.

2.3.5 IBMX, H89 and over-expressed pFLAG-PKAwt construct effects on HPV5 replication

The results of our over-expression experiments showed that HPV5 replication was inhibited in the presence of PKAwt. However, it was restored to some extent when PKA(T197A) and PKA(K72R) were added instead of PKAwt. These data suggest that PKA regulates HPV5 replication in a kinase activity-dependent manner. PKA activity can be regulated by special agents, which can affect the enzyme activity or the function of other essential compounds of the PKA-activating signaling cascade. One of the PKA activators is IBMX, which increases cellular cAMP levels by inhibiting PDE activity (Chen *et al*., 1998). H89 is an inhibitor of PKA, competitive for ATP binding site on the C subunit, and thereby preventing phosphorylation reaction of target proteins (Murray, 2008). In order to support our findings, it was decided to conduct bioluminescent reporter assay with subsequent SB analysis to test the effects of IBMX and H89 on the replication of the HPV5-Nluc genome.

For the first part of the experiment, U2OS cells were transfected with the HPV5-Nluc genome, pFLAG-CMV-4 empty vector and the pFLAG-PKA constructs. Next day, H89 or DMSO were added to the cells. For the second part of the experiment, U2OS cells were transfected with the HPV5-Nluc plasmid only and treated with IBMX, H89 and DMSO (as a negative control). The cells were incubated for 3 days and analyzed using Nluc assay and SB. It has been established that the amount of emitted light is proportional to *Nluc* gene expression levels and also it correlates well with HPV5-Nluc copy number in cells. Nluc activity was normalized to AP levels in order to correct for probable variations caused by factors such as possible toxicity of the used chemicals resulting in reduced amount of the treated cells. For SB assay, 5 μg of total DNA was linearized with SacI and treated with DpnI. Filter with the transferred from agarose gel DNA was hybridized with the radioactively labelled HPV5 probe and exposed to the X-ray film. The experiment was repeated twice.

The results from the first part of the experiment are shown in Figure 9A. Over-expression of PKAwt suppressed HPV5-Nluc genome replication more than 90%, while PKA(T197A) and PKA(K72R) were less efficient inhibiting HPV5-Nluc replication approximately 50% probably due to their residual catalytic activity. Interestingly, H89 slightly stimulated the replication of HPV5-Nluc inhibited by the mutant PKA C proteins but could not overcome the influence of the over-expressed catalytically active PKA C protein. However, inhibition of the endogenous PKA catalytic activity with H89 resulted in increased HPV5-NLuc replication (Figure 9A, column 1 and 2). Generally, the levels of the normalized Nluc activity correlated with the results obtained using HPV5 genome and SB assay (Figure 8A).

The second part of the experiment allowed to evaluate the influence of the endogenous PKA activity on HPV5-Nluc replication efficiency. The results of Nluc assay showed approximately 2-fold decrease in HPV5-Nluc copy number, when endogenous PKA catalytic activity was potentiated with IBMX (Figure 9B). In contrast, endogenous PKA activity inhibition with H89 resulted in increased replication of the viral genome. These results demonstrate that the absence of active PKA benefits successful HPV5 replication.

In order to additionally check, if Nluc activity measurements correlate with the replicated HPV5 DNA amount in cells, SB analysis was conducted. The obtained SB results are entirely congruent with Nluc assay findings (Figure 9C).

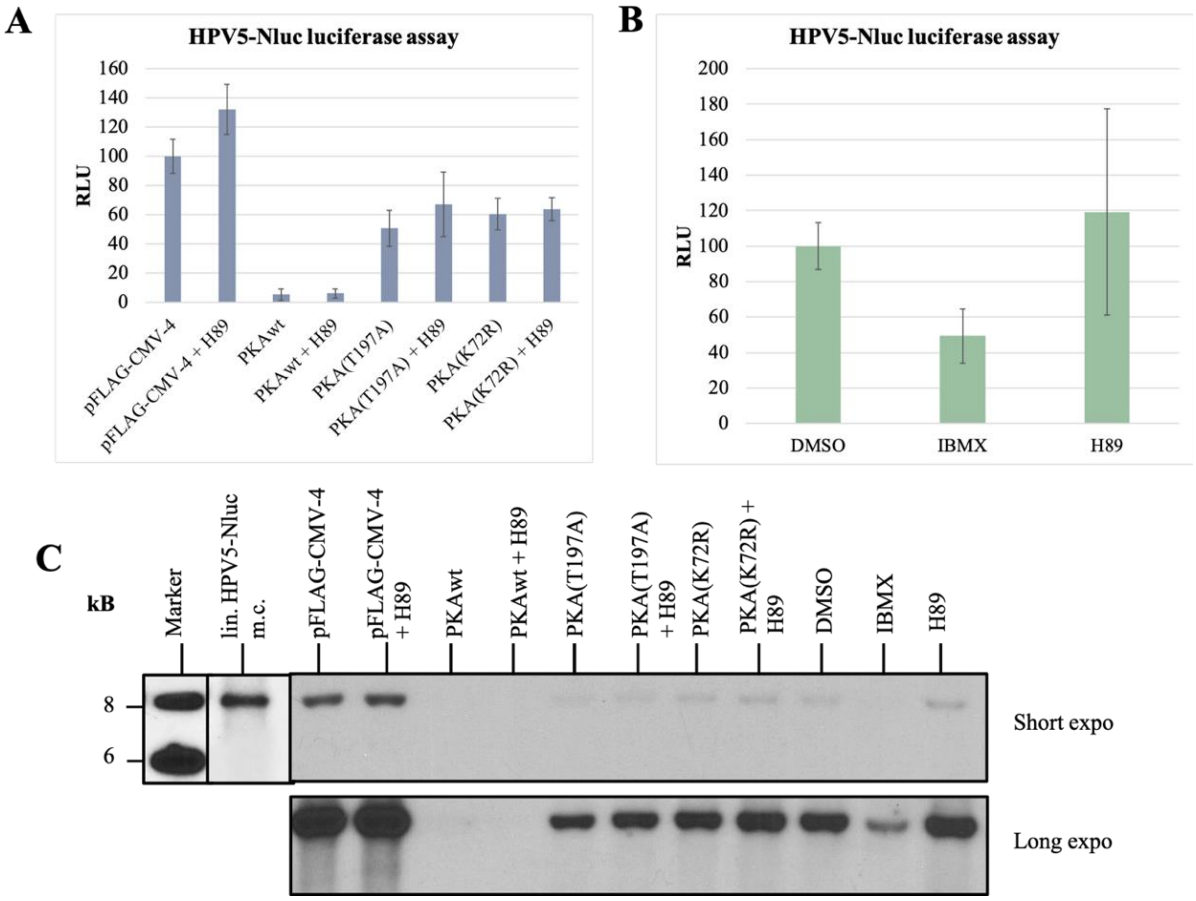

Figure 9. Replication signal of HPV5-Nluc. U2OS cells were transfected with HPV5-Nluc genome and pFLAG-CMV-4 vector, pFLAG-PKA, pFLAG-PKA(T197A), or pFLAG-PKA(K72R) constructs, if indicated. Next day after transfection, the cells were treated with DMSO, 5 μM H89 or 500 μM IBMX. The treatment was repeated every day. The cells were incubated for 3 days.

A, B: Nluc activity was measured in triplicates and normalized to AP phosphatase activity. Normalized Nluc activity of the control cells was set as 100%, and the data of other samples are presented as percentage of the control (the average mean +/- SD (n=2)). RLU: relative luminescence unit.

C: DNA was extracted, linearized with SacI and treated with DpnI. Pictures were captured with short and long exposure time to better distinguish the differences in the signals.

The results obtained in these experiments suggest that both treatments, either activation of endogenous PKA with IBMX or PKAwt over-expression, inhibited HPV5 genome replication in U2OS cells, which can be observed as decreased Nluc signals or decreased replicated HPV5 DNA amount. It can be concluded that PKA kinase activity regulates negatively HPV5 replication. Taken together, our data suggest the PKA activators as a potential tool for modulating HPV5 infection.

2.3.6 Mechanism of activation of HPV18 replication in response to over-expression of PKAwt protein

It is known that HPV itself encodes protein for the repression of viral replication, such as regulatory protein E2 and its truncated form E8^E2, which is a powerful transcriptional repressor, with even stronger repressive effect than E2-TA protein (Stubenrauch *et al*., 2000). Taking into account the data of the previous studies showing that some cellular kinases are able to regulate the viral replication process and the results of our SB assay showing the increase in the amount of HPV18 DNA in U2OS cells in response to PKAwt over-expression, one of the possible mechanisms of the over-expressed PKAwt-mediated activation of HPV18 replication could be linked to a down-regulation of *E8^E2* transcript.

To test this hypothesis, it was essential to analyze the level of the viral transcripts synthesized in response to over-expressed PKA C proteins. U2OS cells were transfected with the appropriate amount of pFLAG-CMV-4 vector, pFLAG-PKA constructs and the HPV18E1$^-$ genome. Due to deficiency of the *E1* expression, HPV18E1$^-$ genome is transcriptionally active but not able to replicate and produce replicons that can later interfere with RT-PCR. In 48 h after the transfection, total RNA was extracted, treated with Turbo DNase and used for cDNA synthesis. The quantitative RT-PCR (qPCR) was performed using primers 6-9 (Appendix 1. Primers used in the study). The mRNA expression levels of the viral *E1, E2, E1^E4* and *E2^E8* transcripts were analyzed in triplicates and normalized to *GAPDH* mRNA expression levels measured using primers 5 (Appendix 1. Primers used in the study). The experiment was repeated two times.

The results of qPCR are shown in Figure 10. Expression levels of the transcripts encoding the full-length viral replication proteins E1 and E2 remained similar to the control in all samples. In addition, the amount of *E1^E4* - alternative transcript shown to be essential for effective amplification of viral DNA (Davy *et al*., 2002; Doorbar *et al*., 1991) is increased in the presence of the over-expressed PKAwt. In addition, the amount of *E8^E2* transcript decreased more than 95%, while PKAwt was over-expressed. It can be concluded that PKA activity may influence

the splicing, stability or production of *E8^E2* transcript, which encodes the powerful transcriptional repressor, and the lack of the E8^E2 repressor results in increased HPV18 replication.

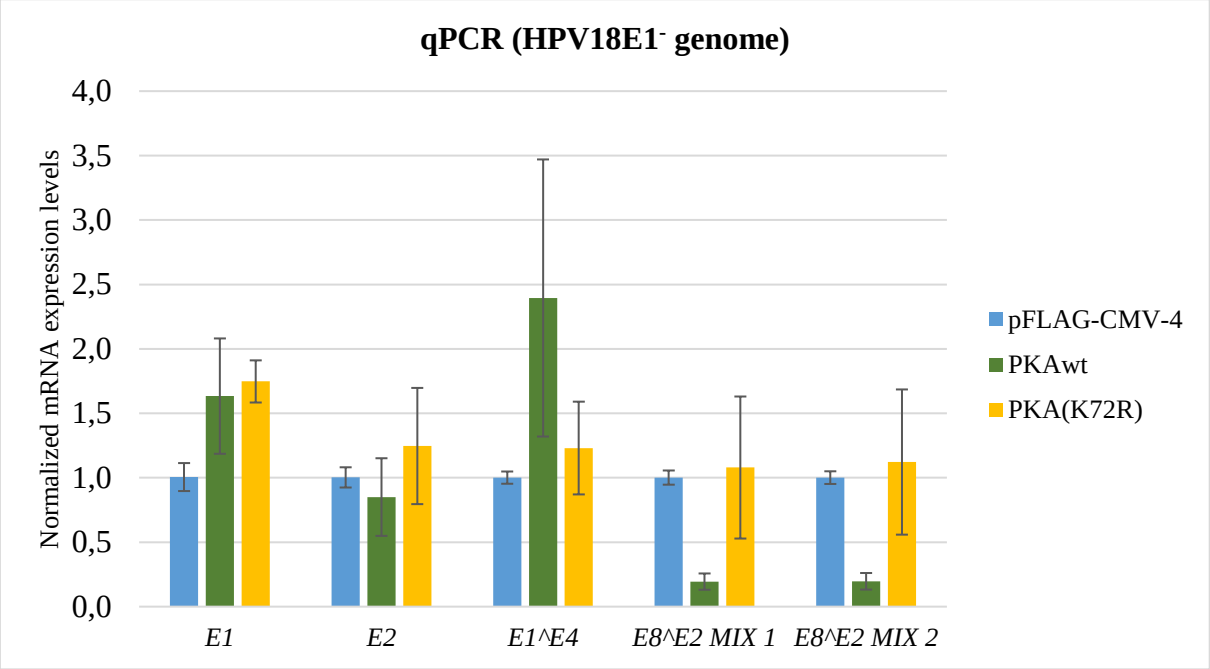

Figure 10. HPV18 transcripts synthesized in 48 h in U2OS cells. U2OS cells were transfected with HPV18E1$^-$ genome and pFLAG-CMV-4 vector, pFLAG-PKAwt and pFLAG-PKA(K72R) plasmids. Total RNA was extracted, and cDNA was synthesized. Expression levels of HPV18E1$^-$ transcripts were analyzed using qPCR in triplicates and normalized to *GAPDH* mRNA expression levels. Expression level of each transcript was set as 1 in the control cells transfected with the empty vector, and the data from other samples were calculated relative to the control. The data are presented as the average mean +/- SD of two independent experiments.

To test, whether the reason for the increased HPV18 replication in response to PKAwt over-expression is only due to down-regulation of the *E8^E2* transcripts or it is a combined effect of some independent mechanisms, the replication efficiency of HPV18E8$^-$ genome in the presence or absence of over-expressed PKAwt was analyzed and compared with that of the HPV18 genome. U2OS cells were transfected with different amounts of HPV18 or HPV18E8$^-$ minicircles (250 and 400 ng) and pFLAG-PKAwt plasmid or empty vector. The HPV18E8$^-$ genome contains a point mutation in the *E8^E2* ATG and therefore it is deficient for E8^E2 expression and allows to monitor the viral replication in the absence of E8^E2 repressor. Total DNA was extracted from U2OS cells 3 d after the transfection, linearized with BglI, treated with DpnI restriction enzymes and subjected to SB analysis (Figure 11A). The SB signals were quantified (Figure 11B). The experiment was repeated twice.

The obtained results showed that PKAwt over-expression up-regulated the HPV18 replication approximately 22-fold. HPV18E8$^-$ replication signal was approximately 30-fold higher than that of HPV18. Interestingly, PKAwt over-expression was able to enhance HPV18E8$^-$ genome

replication, but only 1.5-fold. Taken together, it can be concluded that PKA activity may be involved in positive regulation of HPV18 replication because it decreases the amount of *E8^E2* transcript. However, it appears to be a mixed-type mechanism because of additional PKA-mediated positive effect on HPV18E8$^-$ replication. Further research is needed for the better understanding of the PKA-mediated regulation of HPV18 replication.

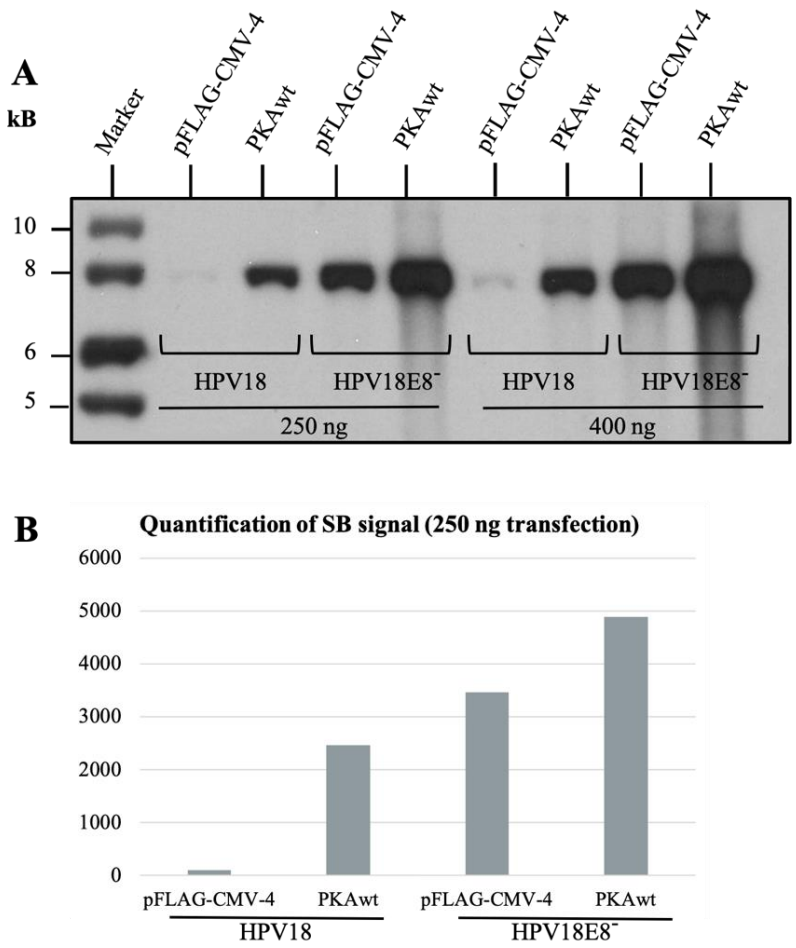

Figure 11. A: Replication signal of the HPV18 and HPV18E8$^-$ in the U2OS cells transfected with 250 and 400 ng of HPV18 or HPV18E8$^-$ genomes, pFLAG-CMV-4 vector or pFLAG-PKAwt construct. DNA was extracted 3 d after the transfection, linearized with BglI and treated with DpnI, and analyzed using SB. **B:** SB signals shown in the panel A were quantified. The signal of the control sample transfected with HPV18 and empty vector was set as 100%. Data from other samples are shown as a percentage of the control.

2.4. Discussion

HPV infections cause approximately 600000 cases of cervical, vulvar, vaginal, penile, anal and oropharyngeal cancers every year, as well as benign diseases such as genital warts (Arbyn *et al*., 2012). HPV infections are strongly associated with sexual transmission but the nonsexual modes (*e.g.* skin-to-skin contacts) are also common (Sun-Kuie *et al*., 1990). The connection between host cell protein kinases and HPV life cycle has been previously demonstrated (Piirsoo *et al*., 2019; Sekhar and McBride, 2012; Xie *et al*., 2017). It was shown that phosphorylation may influence viral replication both negatively and positively. An ubiquitously expressed kinase PKA has many targets for phosphorylation. Bioinformatic analysis suggests that replication proteins E1 and E2 of different HPV types has several putative PKA consensus sites R-R/K-X-S/T (Kennelly and Krebs, 1991). These data suggest that PKA can affect activity of viral proteins and participate in regulation of HPV replication. The characterization of potential association between PKA and HPV proteins may help to learn more about the regulation of the HPV life cycle in host cells and give a clue for developing new drugs against HPV infections. Uncovering the correlation between PKA activity and viral replication, and particularly, understanding if there is any HPV type-specificity, can help to get deeper insight of replication strategies of LR and HR HPVs.

The research of viral replication generally requires sophisticated, expensive and time-consuming methods; therefore, obtaining that kind of information is complicated. This study uses the advantages of easily cultured U2OS cells that support replication of many HPV types (Geimanen *et al*., 2011). In the present study, we have found that two different *PKA C*, *PRKACA* and *PRKACB*, are expressed in the U2OS cells as well as in HPV31-positive human keratinocytes. Since the proteins encoded by these genes share very high rate of homology (92%) that suggest their functional similarity, only one of them, PKACα, has been chosen for further experiments.

This study introduces plasmids, to express FLAG-tagged PKACα wild type and its two catalytically deficient mutants (T197A, K72R), and compares the influence of their over-expression on replication levels of different HPV types. The following HPVs have been chosen for analysis: HPV5 as an oncogenic β HPV associated with different types of skin cancers, HPV11 as one the most frequent LR α-HPVs responsible for development of genital warts, and HPV18 as a widespread oncogenic or HR α-HPV associated with cancers of mucosal epithelia.

As a result, the replication of HPV5 and HPV18 was found to be PKA-dependent, while the HPV11 replication was found to be independent of PKA activity in U2OS cells. HPV5

replication level was severely decreased, while PKAwt was over-expressed or endogenous PKA activity was potentiated with PKA activator IBMX. We have also described that suppression of PKA activity with H89 resulted in increased level of the HPV5 replication. Therefore, PKA activators and inhibitors can be considered as a possible tool for the regulation of the HPV5 replication.

According to Sekhar and McBride (Sekhar and McBride, 2012), PKA may be a positive regulator of the HPV8 E2 protein phosphorylating it in the highly conserved serine residue at position 253 (S253). HPV8 and HPV5 both belong to β genus and, according to the Multiple Sequence Alignment, both have conserved R-XX-S kinase motif with the serine in 253rd position. Therefore, the results of this article somewhat contradict our findings. HPV8 E2 protein has increased half-life due to the phosphorylated S253. This phosphorylation also promotes partition of the viral genome to daughter cells due to more efficient binding of the phosphorylated E2 (S253) to cellular chromatin. However, the authors failed to demonstrate convincingly that this phosphorylation is mediated by PKA because of the presence of some residual amount of the phosphorylated E2 in the CV-1 cells treated with PKA inhibitor H89. In addition, this study failed to find the connection between the phosphorylated S253 and HPV8 replication, showing that S253 mutation to alanine (S253A) did not influence the ability of E2 protein to support viral replication in U2OS cells. Therefore, although the S253 phosphorylation might be crucial for regulation of E2 proteins of β HPVs, direct association of this phosphorylation with PKA and HPV replication efficiency has not been demonstrated. Taken together the results of both Sekhar and McBride and our study, it can be concluded that either another protein kinase may be responsible for the S253 phosphorylation or PKA-mediated regulation of HPV5 and HPV8 replication differs, since PKA inhibits the HPV5 replication. However, further studies are required to investigate the exact molecular mechanism of PKA-mediated suppression of the HPV5 replication.

In contrast to HPV5, PKA catalytic activity intensifies HPV18 replication suggesting that PKA may be a positive regulator of the HPV18 life cycle. We have shown that up-regulation of the HPV18 replication in response to PKAwt over-expression is associated with decreased amount of the *E8^E2* transcript. However, the replication of HPV18E8$^-$ mutant genome deficient for the *E8^E2* expression was also stimulated by the over-expressed PKA, but to less extent compared to wt HPV18. PKA-mediated up-regulation of HPV18E8$^-$ replication suggests the involvement of some yet unknown additional mechanism(s) along with the observed down-regulation of the *E8^E2* expression.

It can be hypothesized that PKA acts via activation of CREB-, ATF-1- or CREM-mediated pathway, which either inactivates or blocks the promoter p1193 required for the synthesis of HPV18 *E8^E2* transcript. This group of transcription factors (CREB family) participate in activation or inhibition of their target gene expression via binding to either palindromic full (TGACGTCA) or half CRE (TGACG/CGTCA) sites (Montminy, 1997). The activated PKA phosphorylates and promotes the binding of CREB family transcription factors to the CRE region, which regulates the expression of its downstream targets (Sakamoto and Frank, 2009). There are three CRE half-sites in the HPV18 genome (PaVE accession number: gi|60975): 761 (CGTCA), 1015 and 3440 (TGACG) nucleotides (numbering is given from the 1st 5` nucleotide of the CREB consensus site). The *E8^E2* transcript is initiated from p1193 promoter (ranging 1142-1319 nucleotides) and is spliced as -1357/3434 (Toots *et al*., 2014). One of the CRE half-sites is located near the p1193 promoter. Binding of the CREB family transcription factors in the proximity of this promoter may influence it activity and somehow interfere with the expression of E8^E2 transcriptional repressor.

In the HPV5 genome, there are four CRE half-sites (PaVE accession number: gi|333071): nucleotides 962, 1386, 3241 (TGACG) and 6588 (CGTCA) (numbering is given from the 1st 5` nucleotide of the CREB consensus site). One of these sites locates near the p840 promoter mapped to 708-915 nucleotides. It has been demonstrated that the HPV5 promoter p840 is involved in expression of the early genes encoding the E1 and E2 replication proteins (Sankovski *et al*., 2014). It can be hypothesized that inhibition of the HPV5 replication in response to the catalytically active PKA occurs because of the binding of the CREB family transcription factors to p840 promoter that in turn inhibits the expression of the regulatory proteins E1 and E2. In contrast to HPV5 and HPV18, no CRE consensus sequences have been found in the HPV11 genome, which can explain the observed inability of PKA to regulate the HPV11 replication. Also, PKA can influence HPV replication via direct phosphorylation and regulation of the viral E1 and E2 proteins or their interacting partners involved in regulation of HPV replication. However, to make these kinds of conclusions, further research is needed.

The obtained results are important for better understanding the life cycle of different types of HPVs. Furthermore, it can be speculated that the PKA effects are HPV type-dependent (if PKA-mediated effects are different in LR and HR types) or tropism-dependent (if PKA-mediated effects are different in mucosal and cutaneous HPVs); however, more experiments are needed to make this kind of conclusions. Most certainly, the long-term goal of this project is to get deeper insight the interactions between the host cell factors such as PKA, and different HPV types to develop novel strategies for effective treatment of HPV infections.

CONCLUSION

The present research was focused on the ability of PKACα subunit to influence the level of replication of HPV5, HPV11 and HPV18. The study resulted in the following conclusions:

- The mutant PKACα subunits carrying the point mutations in the amino acid residues T197 and K72 exhibit only minimal residual catalytic activity, and therefore may be used as a negative control for over-expression studies investigating the PKA catalytic activity-dependent processes.
- IBMX-mediated stimulation of the endogenous PKA catalytic activity or over-expression of catalytically active PKA results in strong inhibition of the HPV5 genome replication.
- The replication of HPV11 is not PKA-dependent.
- The over-expressed PKA up-regulates the HPV18 genome replication in a kinase activity-dependent manner.
- PKA phosphorylation activity influences HPV18 replication using a mixed-type activation mechanism. We show that PKA-mediated activation of the HPV18 replication occurs mainly due to down-regulation of the *E8^E2* transcript expression that encodes the strong transcriptional repressor negatively regulating the HPV18 replication.

Mõnede HPV tüüpide replikatsioon on moduleeritud cAMP-sõltuva proteiinkinaasi aktiivsuse poolt

Olga Sahharov

RESÜMEE

Inimese papilloomiviirused (HPV) on kaheahelalise DNA genoomiga viirused, mis nakatavad basaalseid keratinotsüüte nahas või limaskestades ja levivad mikrokahjustuste kaudu. HPV kodeerib tüüpiliselt kuute varajast ja kahte hilist valku. Varajased valgud vastutavad viiruse replikatsiooni regulatsiooni ja nakatunud raku rakutsükli muutuste eest. Hilised valgud moodustavad kapsiidi ja vastutavad rakust väljumise eest. HPV alatüüpe jagatakse kahte kategooriasse vastavalt viiruse onkogeensele potentsiaalile: „madalariski“ tüübid põhjustavad tüükaid, „kõrge riskiga“ HPV-d tekitavad kaela-, pea-, naha- ja anogenitaalpiirkonna vähki, millest kõige levinum on emakakaelavähk.

Üheks terapeutiliseks sihtmärgiks on HPV replikatsioonitsükli inhibeerimine, aga selle strateegia kasutamiseks on vaja lähemalt uurida molekulaarseid interaktsioone peremeesraku ja viiruse vahel. Proteiinkinaasid reguleerivad erinevaid bioloogilisi protsesse rakkudes, muutes märklaudvalkude aktiivsust. HPV E1 ja E2 regulaator-valgud sisaldavad mitut proteiinkinaaside konsensus saiti, sealhulgas ka proteiinkinaas A (PKA) oma. Antud magistritöö eesmärgiks oli teada saada, kuidas PKA katalüütiline subühik α (PKACα) mõjutab erinevate HPV alatüüpide replikatsiooni efektiivsust U2OS rakkudes. Valitud tüüpideks oli HPV5, HPV11 ja HPV18, mis kuuluvad erinevatesse klassidesse ja riskirühmadesse.

Uurimistöö raames loodi plasmiidid, mis kodeerivad FLAG-märgisega PKACα subühikut (PKAwt) ja tema kahte katalüütiliselt inaktiivset mutanti (PKA(T197A) ja PKA(K72R)). Töö esimene osa kirjeldab viiruse elutsüklit, HPV valkude funktsioone ja PKA rolli raku- ja viiruse elutsüklis. Eksperimentaalne osa kirjeldab plasmiidide loomist, PKA valkude ekspressiooni taseme ja bioloogilise aktiivsuse analüüsi ja nende üle-ekspressiooni mõju erinevate HPV tüüpide replikatsioonile. Leiti, et PKA aktiivsus ei mõjuta HPV11 replikatsiooni U2OS rakuliinis, kuid HPV5 ja HPV18 replikatsioonid sõltuvad PKA aktiivsusest. Endogeense PKA aktivaatori IBMX (3-isobutüül-1-metüül-ksanteen) või aktiivse PKA üle-ekspressiooni tagajärjel oli HPV5 replikatsioon inhibeeritud, kuid üle-ekspresseeritud PKA suurendas HPV18 replikatsiooni aktiivsust. Töö selgitab ka, et PKA fosforüleemine mõjutab HPV18 replikatsiooni segatüüpi mehhanismi kaudu, kus üheks osaks on E8^E2 transkriptsioonilise repressori ekspressiooni vähendamine. Antud töö on tehtud Tartu Ülikooli Tehnoloogiainstituudis molekulaarse viroloogia uurimisrühmas.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my supervisor Alla Piirsoo for support, patience and advises during thesis writing. Furthermore, I appreciate that she taught me new methods and techniques, and motivated me throughout the research. I also would like to thank Marko Piirsoo, labor assistant Regina Pipitš and all of my colleagues from the Institute of Technology, University of Tartu for their constant help and explanations.

REFERENCES

Adams, J.A., McGlone, M.L., Gibson, R., and Taylor, S.S. (1995). Phosphorylation modulates catalytic function and regulation in the cAMP-dependent protein kinase. Biochemistry *34*, 2447–2454.

Ammermann, I., Bruckner, M., Matthes, F., Iftner, T., and Stubenrauch, F. (2008). Inhibition of transcription and DNA replication by the papillomavirus E8-E2C protein is mediated by interaction with corepressor molecules. J. Virol. *82*, 5127–5136.

Anantharaman, D., Gheit, T., Waterboer, T., Abedi-Ardekani, B., Carreira, C., McKay-Chopin, S., Gaborieau, V., Marron, M., Lagiou, P., Ahrens, W., *et al*. (2013). Human Papillomavirus Infections and Upper Aero-Digestive Tract Cancers: The ARCAGE Study. JNCI J. Natl. Cancer Inst. *105*, 536–545.

Antonsson, A., Karanfilovska, S., Lindqvist, P.G., and Hansson, B.G. (2003). General acquisition of human papillomavirus infections of skin occurs in early infancy. J. Clin. Microbiol. *41*, 2509–2514.

Arbyn, M., de Sanjosé, S., Saraiya, M., Sideri, M., Palefsky, J., Lacey, C., Gillison, M., Bruni, L., Ronco, G., Wentzensen, N., *et al*. (2012). EUROGIN 2011 roadmap on prevention and treatment of HPV-related disease. Int. J. Cancer *131*, 1969–1982.

Ateenyi-Agaba, C., Franceschi, S., Wabwire-Mangen, F., Arslan, A., Othieno, E., Binta-Kahwa, J., van Doorn, L.-J., Kleter, B., Quint, W., and Weiderpass, E. (2010). Human papillomavirus infection and squamous cell carcinoma of the conjunctiva. Br. J. Cancer *102*, 262–267.

Bacallao, K., and Monje, P.V. (2013). Opposing Roles of pka and epac in the cAMP-Dependent Regulation of Schwann Cell Proliferation and Differentiation. PLOS ONE *8*, e82354.

Baker, C.T., and Calef, C. (1996). Maps of Papillomavirus mRNA Transcripts.

Becker, K.A., Florin, L., Sapp, C., and Sapp, M. (2003). Dissection of human papillomavirus type 33 L2 domains involved in nuclear domains (ND) 10 homing and reorganization. Virology *314*, 161–167.

Bedell, M.A., Hudson, J.B., Golub, T.R., Turyk, M.E., Hosken, M., Wilbanks, G.D., and Laimins, L.A. (1991). Amplification of human papillomavirus genomes in vitro is dependent on epithelial differentiation. J. Virol. *65*, 2254–2260.

Bergvall, M., Melendy, T., and Archambault, J. (2013). The E1 proteins. Virology *445*.

Bernard, H.-U., Burk, R.D., Chen, Z., van Doorslaer, K., zur Hausen, H., and de Villiers, E.-M. (2010). Classification of Papillomaviruses (PVs) Based on 189 PV Types and Proposal of Taxonomic Amendments. Virology *401*, 70–79.

Bilder, D., Li, M., and Perrimon, N. (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. Science *289*, 113–116.

Bodily, J.M., Hennigan, C., Wrobel, G.A., and Rodriguez, C.M. (2013). Regulation of the human papillomavirus type 16 late promoter by E7 and the cell cycle. Virology *443*, 11–19.

Bouvard, V., Matlashewski, G., Gu, Z.M., Storey, A., and Banks, L. (1994). The human papillomavirus type 16 E5 gene cooperates with the E7 gene to stimulate proliferation of primary cells and increases viral gene expression. Virology *203*, 73–80.

Bravo, I.G., and Alonso, A. (2004). Mucosal human papillomaviruses encode four different E5 proteins whose chemistry and phylogeny correlate with malignant or benign growth. J. Virol. *78*, 13613–13626.

Buck, C.B., Cheng, N., Thompson, C.D., Lowy, D.R., Steven, A.C., Schiller, J.T., and Trus, B.L. (2008). Arrangement of L2 within the Papillomavirus Capsid. J. Virol. *82*, 5190–5197.

Buck, C.B., Day, P.M., and Trus, B.L. (2013). The Papillomavirus Major Capsid Protein L1. Virology *445*, 169–174.

Burdyga, A., Surdo, N.C., Monterisi, S., Benedetto, G.D., Grisan, F., Penna, E., Pellegrini, L., Zaccolo, M., Bortolozzi, M., Swietach, P., *et al*. (2018). Phosphatases control PKA-dependent functional microdomains at the outer mitochondrial membrane. Proc. Natl. Acad. Sci. *115*, E6497–E6506.

Burley, M., Roberts, S., and Parish, J.L. (2020). Epigenetic regulation of human papillomavirus transcription in the productive virus life cycle. Semin. Immunopathol.

Byrne, D.P., Vonderach, M., Ferries, S., Brownridge, P.J., Eyers, C.E., and Eyers, P.A. (2016). cAMP-dependent protein kinase (PKA) complexes probed by complementary differential scanning fluorimetry and ion mobility–mass spectrometry. Biochem. J. *473*, 3159–3175.

Cadd, G., and McKnight, G.S. (1989). Distinct patterns of cAMP-dependent protein kinase gene expression in mouse brain. Neuron *3*, 71–79.

Cheadle, C., Nesterova, M., Watkins, T., Barnes, K.C., Hall, J.C., Rosen, A., Becker, K.G., and Cho-Chung, Y.S. (2008). Regulatory subunits of PKA define an axis of cellular proliferation/differentiation in ovarian cancer cells. BMC Med. Genomics *1*, 43.

Chellappan, S., Kraus, V.B., Kroger, B., Munger, K., Howley, P.M., Phelps, W.C., and Nevins, J.R. (1992). Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc. Natl. Acad. Sci. U. S. A. *89*, 4549–4553.

Chen, T.C., Hinton, D.R., Zidovetzki, R., and Hofman, F.M. (1998). Up-regulation of the cAMP/PKA pathway inhibits proliferation, induces differentiation, and leads to apoptosis in malignant gliomas. Lab. Investig. J. Tech. Methods Pathol. *78*, 165–174.

Cheng, S., Schmidt-Grimminger, D.C., Murant, T., Broker, T.R., and Chow, L.T. (1995). Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. Genes Dev. *9*, 2335–2349.

Cheng, X., Ma, Y., Moore, M., Hemmings, B.A., and Taylor, S.S. (1998). Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase. Proc. Natl. Acad. Sci. U. S. A. *95*, 9849–9854.

Cheng, Y., Zhang, Y., and McCammon, J.A. (2006). How does activation loop phosphorylation modulate catalytic activity in the cAMP-dependent protein kinase: A theoretical study. Protein Sci. Publ. Protein Soc. *15*, 672–683.

Chesson, H.W., Dunne, E.F., Hariri, S., and Markowitz, L.E. (2014). The estimated lifetime probability of acquiring human papillomavirus in the United States. Sex. Transm. Dis. *41*, 660–664.

Corbin, J.D., Sugden, P.H., Lincoln, T.M., and Keely, S.L. (1977). Compartmentalization of adenosine 3':5'-monophosphate and adenosine 3':5'-monophosphate-dependent protein kinase in heart tissue. J. Biol. Chem. *252*, 3854–3861.

Corbin, J.D., Sugden, P.H., West, L., Flockhart, D.A., Lincoln, T.M., and McCarthy, D. (1978). Studies on the properties and mode of action of the purified regulatory subunit of bovine heart adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. *253*, 3997–4003.

Cubie, H.A. (2013). Diseases associated with human papillomavirus infection. Virology *445*, 21–34.

Czech, M.P., Tencerova, M., Pedersen, D.J., and Aouadi, M. (2013). Insulin signalling mechanisms for triacylglycerol storage. Diabetologia *56*, 949–964.

Daling, J.R., Madeleine, M.M., Johnson, L.G., Schwartz, S.M., Shera, K.A., Wurscher, M.A., Carter, J.J., Porter, P.L., Galloway, D.A., and McDougall, J.K. (2004). Human papillomavirus, smoking, and sexual practices in the etiology of anal cancer. Cancer *101*, 270–280.

Davy, C., McIntosh, P., Jackson, D.J., Sorathia, R., Miell, M., Wang, Q., Khan, J., Soneji, Y., and Doorbar, J. (2009). A novel interaction between the human papillomavirus type 16 E2 and E1^E4 proteins leads to stabilization of E2. Virology *394*, 266–275.

Davy, C.E., Jackson, D.J., Wang, Q., Raj, K., Masterson, P.J., Fenner, N.F., Southern, S., Cuthill, S., Millar, J.B.A., and Doorbar, J. (2002). Identification of a G(2) arrest domain in the E1 wedge E4 protein of human papillomavirus type 16. J. Virol. *76*, 9806–9818.

Day, P.M., Roden, R.B., Lowy, D.R., and Schiller, J.T. (1998). The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. J. Virol. *72*, 142–150.

Dell'Acqua, M.L., and Scott, J.D. (1997). Protein Kinase A Anchoring. J. Biol. Chem. *272*, 12881–12884.

Deng, W., Lin, B.Y., Jin, G., Wheeler, C.G., Ma, T., Harper, J.W., Broker, T.R., and Chow, L.T. (2004). Cyclin/CDK Regulates the Nucleocytoplasmic Localization of the Human Papillomavirus E1 DNA Helicase. J. Virol. *78*, 13954–13965.

DiMaio, D., and Petti, L. (2013). The E5 Proteins. Virology *445*, 99–114.

Doorbar, J. (2006). Molecular biology of human papillomavirus infection and cervical cancer. Clin. Sci. *110*, 525–541.

Doorbar, J. (2013). The E4 protein; structure, function and patterns of expression. Virology *445*, 80–98.

Doorbar, J., Ely, S., Sterling, J., McLean, C., and Crawford, L. (1991). Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. Nature *352*, 824–827.

Dowhanick, J.J., McBride, A.A., and Howley, P.M. (1995). Suppression of cellular proliferation by the papillomavirus E2 protein. J. Virol. *69*, 7791–7799.

Dreer, M., van de Poel, S., and Stubenrauch, F. (2017). Control of viral replication and transcription by the papillomavirus E8^E2 protein. Virus Res. *231*, 96–102.

Duensing, S., Lee, L.Y., Duensing, A., Basile, J., Piboonniyom, S., Gonzalez, S., Crum, C.P., and Munger, K. (2000). The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. Proc. Natl. Acad. Sci. U. S. A. *97*, 10002–10007.

Dyson, N., Howley, P.M., Münger, K., and Harlow, E. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science *243*, 934–937.

El-Abaseri, T.B., Putta, S., and Hansen, L.A. (2006). Ultraviolet irradiation induces keratinocyte proliferation and epidermal hyperplasia through the activation of the epidermal growth factor receptor. Carcinogenesis *27*, 225–231.

Fisher, C. (2015). Recent Insights into the Control of Human Papillomavirus (HPV) Genome Stability, Loss, and Degradation. J. Clin. Med. *4*, 204–230.

Flores, E.R., and Lambert, P.F. (1997). Evidence for a switch in the mode of human papillomavirus type 16 DNA replication during the viral life cycle. J. Virol. *71*, 7167–7179.

Geimanen, J., Isok-Paas, H., Pipitch, R., Salk, K., Laos, T., Orav, M., Reinson, T., Ustav, M., Ustav, M., and Ustav, E. (2011). Development of a Cellular Assay System To Study the Genome Replication of High- and Low-Risk Mucosal and Cutaneous Human Papillomaviruses. J. Virol. *85*, 3315–3329.

Gillison, M.L., and Shah, K.V. (2003). Chapter 9: Role of Mucosal Human Papillomavirus in Nongenital Cancers. JNCI Monogr. *2003*, 57–65.

Grabowska, A.K., and Riemer, A.B. (2012). The Invisible Enemy – How Human Papillomaviruses Avoid Recognition and Clearance by the Host Immune System. Open Virol. J. *6*, 249–256.

Graham, S. (2010). Human papillomavirus: gene expression, regulation and prospects for novel diagnostic methods and antiviral therapies. Future Microbiol. *5*, 1493–1506.

Hanks, S.K., and Hunter, T. (1995). Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *9*, 576–596.

Herbst, L.H., Lenz, J., Van Doorslaer, K., Chen, Z., Stacy, B.A., Wellehan, J.F.X., Manire, C.A., and Burk, R.D. (2009). Genomic characterization of two novel reptilian papillomaviruses, Chelonia mydas papillomavirus 1 and Caretta caretta papillomavirus 1. Virology *383*, 131–135.

Herrero, R., González, P., and Markowitz, L.E. (2015). Present status of human papillomavirus vaccine development and implementation. Lancet Oncol. *16*, e206–e216.

Hirochika, H., Hirochika, R., Broker, T.R., and Chow, L.T. (1988). Functional mapping of the human papillomavirus type 11 transcriptional enhancer and its interaction with the trans-acting E2 proteins. Genes Dev. *2*, 54–67.

Holmgren, S.C., Patterson, N.A., Ozbun, M.A., and Lambert, P.F. (2005). The Minor Capsid Protein L2 Contributes to Two Steps in the Human Papillomavirus Type 31 Life Cycle. J. Virol. *79*, 3938–3948.

Howe, A.K. (2011). Cross-talk between calcium and protein kinase A in the regulation of cell migration. Curr. Opin. Cell Biol. *23*, 554–561.

Hummel, M., Hudson, J.B., and Laimins, L.A. (1992). Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. J. Virol. *66*, 6070–6080.

Hunter, T. (2000). Signaling—2000 and Beyond. Cell *100*, 113–127.

Iannacone, M.R., Gheit, T., Waterboer, T., Giuliano, A.R., Messina, J.L., Fenske, N.A., Cherpelis, B.S., Sondak, V.K., Roetzheim, R.G., Ferrer-Gil, S., *et al*. (2013). Case–Control Study of Cutaneous Human Papillomavirus Infection in Basal Cell Carcinoma of the Skin. J. Invest. Dermatol. *133*, 1512–1520.

Iannacone, M.R., Gheit, T., Pfister, H., Giuliano, A.R., Messina, J.L., Fenske, N.A., Cherpelis, B.S., Sondak, V.K., Roetzheim, R.G., Silling, S., *et al*. (2014). Case–control study of genus-beta human papillomaviruses in plucked eyebrow hairs and cutaneous squamous cell carcinoma. Int. J. Cancer *134*, 2231–2244.

Iftner, T., Haedicke-Jarboui, J., Wu, S.-Y., and Chiang, C.-M. (2017). Involvement of Brd4 in different steps of the papillomavirus life cycle. Virus Res. *231*, 76–82.

Ilves, I., Kivi, S., and Ustav, M. (1999). Long-Term Episomal Maintenance of Bovine Papillomavirus Type 1 Plasmids Is Determined by Attachment to Host Chromosomes, Which Is Mediated by the Viral E2 Protein and Its Binding Sites. J. Virol. *73*, 4404–4412.

Iyer, G.H., Moore, M.J., and Taylor, S.S. (2005). Consequences of Lysine 72 Mutation on the Phosphorylation and Activation State of cAMP-dependent Kinase. J. Biol. Chem. *280*, 8800–8807.

Johnson, D.A., Akamine, P., Radzio-Andzelm, E., Madhusudan, and Taylor, S.S. (2001). Dynamics of cAMP-Dependent Protein Kinase. Chem. Rev. *101*, 2243–2270.

Joyce, J.G., Tung, J.S., Przysiecki, C.T., Cook, J.C., Lehman, E.D., Sands, J.A., Jansen, K.U., and Keller, P.M. (1999). The L1 major capsid protein of human papillomavirus type 11 recombinant virus-like particles interacts with heparin and cell-surface glycosaminoglycans on human keratinocytes. J. Biol. Chem. *274*, 5810–5822.

Kamenetsky, M., Middelhaufe, S., Bank, E.M., Levin, L.R., Buck, J., and Steegborn, C. (2006). Molecular details of cAMP generation in mammalian cells: a tale of two systems. J. Mol. Biol. *362*, 623–639.

Kämper, N., Day, P.M., Nowak, T., Selinka, H.-C., Florin, L., Bolscher, J., Hilbig, L., Schiller, J.T., and Sapp, M. (2006). A membrane-destabilizing peptide in capsid protein L2 is required for egress of papillomavirus genomes from endosomes. J. Virol. *80*, 759–768.

Kay, M.A., He, C.-Y., and Chen, Z.-Y. (2010). A Simple And Rapid Minicircle DNA Vector Manufacturing System. Nat. Biotechnol. *28*, 1287–1289.

Kemp, B.E., Graves, D.J., Benjamini, E., and Krebs, E.G. (1977). Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. J. Biol. Chem. *252*, 4888–4894.

Kennelly, P.J., and Krebs, E.G. (1991). Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. J. Biol. Chem. *266*, 15555–15558.

Kim, M., Lee, Y., Park, J., Ryu, J.M., Yun, S.P., and Han, H. (2012). PKA and cAMP stimulate proliferation of mouse embryonic stem cells by elevating GLUT1 expression mediated by the NF-κB and CREB/CBP signaling pathways. Biochim. Biophys. Acta *1820*, 1636–1646.

Kines, R.C., Thompson, C.D., Lowy, D.R., Schiller, J.T., and Day, P.M. (2009). The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. Proc. Natl. Acad. Sci. U. S. A. *106*, 20458–20463.

Kirnbauer, R., Booy, F., Cheng, N., Lowy, D.R., and Schiller, J.T. (1992). Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. Proc. Natl. Acad. Sci. U. S. A. *89*, 12180–12184.

Kiyono, T., Hiraiwa, A., Fujita, M., Hayashi, Y., Akiyama, T., and Ishibashi, M. (1997). Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein. Proc. Natl. Acad. Sci. U. S. A. *94*, 11612–11616.

de Koning, M.N., Weissenborn, S.J., Abeni, D., Bavinck, J.N.B., Euvrard, S., Green, A.C., Harwood, C.A., Naldi, L., Neale, R., and Nindl, I. (2009). Prevalence and associated factors of betapapillomavirus infections in individuals without cutaneous squamous cell carcinoma. J. Gen. Virol. *90*, 1611–1621.

Kornev, A., Haste, N., Taylor, S., and Eyck, L. (2006). Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. Proc. Natl. Acad. Sci. U. S. A. *103*, 17783–17788.

Krebs, E.G., and Beavo, J.A. (1979). Phosphorylation-Dephosphorylation of Enzymes. Annu. Rev. Biochem. *48*, 923–959.

Kroupis, C., and Vourlidis, N. (2011). Human papilloma virus (HPV) molecular diagnostics. Clin. Chem. Lab. Med. *49*, 1783–1799.

Kurg, R., Uusen, P., Võsa, L., and Ustav, M. (2010). Human papillomavirus E2 protein with single activation domain initiates HPV18 genome replication, but is not sufficient for long-term maintenance of virus genome. Virology *408*, 159–166.

Lai, M.C., Teh, B.H., and Tarn, W.Y. (1999). A human papillomavirus E2 transcriptional activator. The interactions with cellular splicing factors and potential function in pre-mRNA processing. J. Biol. Chem. *274*, 11832–11841.

Leechanachai, P., Banks, L., Moreau, F., and Matlashewski, G. (1992). The E5 gene from human papillomavirus type 16 is an oncogene which enhances growth factor-mediated signal transduction to the nucleus. Oncogene *7*, 19–25.

Leemans, C.R., Braakhuis, B.J.M., and Brakenhoff, R.H. (2011). The molecular biology of head and neck cancer. Nat. Rev. Cancer *11*, 9–22.

Li, X., and Coffino, P. (1996). High-risk human papillomavirus E6 protein has two distinct binding sites within p53, of which only one determines degradation. J. Virol. *70*, 4509–4516.

Li, M., Beard, P., Estes, P.A., Lyon, M.K., and Garcea, R.L. (1998). Intercapsomeric Disulfide Bonds in Papillomavirus Assembly and Disassembly. J. Virol. *72*, 2160–2167.

Li, N., Franceschi, S., Howell-Jones, R., Snijders, P.J., and Clifford, G.M. (2011). Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: Variation by geographical region, histological type and year of publication. Int. J. Cancer *128*, 927–935.

Lipari, F., McGibbon, G.A., Wardrop, E., and Cordingley, M.G. (2001). Purification and biophysical characterization of a minimal functional domain and of an N-terminal Zn2+-binding fragment from the human papillomavirus type 16 E6 protein. Biochemistry *40*, 1196–1204.

Litchfield, D.W. (2003). Protein kinase CK2: structure, regulation and role in cellular decisions of life and death. Biochem. J. *369*, 1–15.

Liu, W.J., Gissmann, L., Sun, X.Y., Kanjanahaluethai, A., Müller, M., Doorbar, J., and Zhou, J. (1997). Sequence close to the N-terminus of L2 protein is displayed on the surface of bovine papillomavirus type 1 virions. Virology *227*, 474–483.

Logié, A., Dunois-Lardé, C., Rosty, C., Levrel, O., Blanche, M., Ribeiro, A., Gasc, J.-M., Jorcano, J., Werner, S., Sastre-Garau, X., *et al*. (2005). Activating mutations of the tyrosine kinase receptor FGFR3 are associated with benign skin tumors in mice and humans. Hum. Mol. Genet. *14*, 1153–1160.

López-Bueno, A., Mavian, C., Labella, A.M., Castro, D., Borrego, J.J., Alcami, A., and Alejo, A. (2016). Concurrence of Iridovirus, Polyomavirus, and a Unique Member of a New Group of Fish Papillomaviruses in Lymphocystis Disease-Affected Gilthead Sea Bream. J. Virol. *90*, 8768–8779.

Ma, T., Zou, N., Lin, B.Y., Chow, L.T., and Harper, J.W. (1999). Interaction between cyclin-dependent kinases and human papillomavirus replication-initiation protein E1 is required for efficient viral replication. Proc. Natl. Acad. Sci. *96*, 382–387.

Malumbres, M. (2014). Cyclin-dependent kinases. Genome Biol. *15*, 122.

McBride, A.A. (2008). Replication and Partitioning of Papillomavirus Genomes. Adv. Virus Res. *72*, 155–205.

McBride, A.A. (2013). The Papillomavirus E2 Proteins. Virology *445*, 57–79.

McCance, D.J., Kalache, A., Ashdown, K., Andrade, L., Menezes, F., Smith, P., and Doll, R. (1986). Human papillomavirus types 16 and 18 in carcinomas of the penis from Brazil. Int. J. Cancer *37*, 55–59.

Meharena, H.S., Chang, P., Keshwani, M.M., Oruganty, K., Nene, A.K., Kannan, N., Taylor, S.S., and Kornev, A.P. (2013). Deciphering the Structural Basis of Eukaryotic Protein Kinase Regulation. PLoS Biol. *11*.

Montminy, M. (1997). Transcriptional regulation by cyclic amp. Annu. Rev. Biochem. *66*, 807–822.

Moody, C.A., and Laimins, L.A. (2009). Human Papillomaviruses Activate the ATM DNA Damage Pathway for Viral Genome Amplification upon Differentiation. PLOS Pathog. *5*, e1000605.

Muller, M., and Demeret, C. (2012). The HPV E2-Host Protein-Protein Interactions: A Complex Hijacking of the Cellular Network. Open Virol. J. *6*, 173–189.

Murray, A.J. (2008). Pharmacological PKA Inhibition: All May Not Be What It Seems. Sci. Signal. *1*, re4–re4.

Orav, M., Henno, L., Isok-Paas, H., Geimanen, J., Ustav, M., and Ustav, E. (2013). Recombination-Dependent Oligomerization of Human Papillomavirus Genomes upon Transient DNA Replication. J. Virol. *87*, 12051–12068.

Orth, G. (2006). Genetics of epidermodysplasia verruciformis: Insights into host defense against papillomaviruses. Semin. Immunol. *18*, 362–374.

Ozbun, M.A., and Meyers, C. (1997). Characterization of late gene transcripts expressed during vegetative replication of human papillomavirus type 31b. J. Virol. *71*, 5161–5172.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B.E., Karandikar, M., Berman, K., and Cobb, M.H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr. Rev. *22*, 153–183.

Piirsoo, A., Piirsoo, M., Kala, M., Sankovski, E., Lototskaja, E., Levin, V., Salvi, M., and Ustav, M. (2019). Activity of CK2α protein kinase is required for efficient replication of some HPV types. PLOS Pathog. *15*, e1007788.

Plattner, F., and Bibb, J.A. (2012). Chapter 25 - Serine and Threonine Phosphorylation. In Basic Neurochemistry (Eighth Edition), S.T. Brady, G.J. Siegel, R.W. Albers, and D.L. Price, eds. (New York: Academic Press), pp. 467–492.

Pollard, T.D., Earnshaw, W.C., Lippincott-Schwartz, J., and Johnson, G.T. (2017). Protein Hardware for Signaling. In Cell Biology (Third Edition), (Elsevier), pp. 425–442.

Rajkumar, R. (2016). Human Papillomavirus: Research in a Global Perspective (BoD – Books on Demand).

Reinton, N., Haugen, T.B., Orstavik, S., Skålhegg, B.S., Hansson, V., Jahnsen, T., and Taskén, K. (1998). The gene encoding the C gamma catalytic subunit of cAMP-dependent protein kinase is a transcribed retroposon. Genomics *49*, 290–297.

Robinson-White, A., and Stratakis, C.A. (2002). Protein Kinase A Signaling. Ann. N. Y. Acad. Sci. *968*, 256–270.

Rosa, G.L., Fratini, M., Accardi, L., D'Oro, G., Libera, S.D., Muscillo, M., and Bonito, P.D. (2013). Mucosal and Cutaneous Human Papillomaviruses Detected in Raw Sewages. PLOS ONE *8*, e52391.

Sakamoto, K.M., and Frank, D.A. (2009). CREB in the Pathophysiology of Cancer: Implications for Targeting Transcription Factors for Cancer Therapy. Clin. Cancer Res. *15*, 2583–2587.

Sanders, C.M., and Stenlund, A. (1998). Recruitment and loading of the E1 initiator protein: an ATP-dependent process catalysed by a transcription factor. EMBO J. *17*, 7044–7055.

de Sanjosé, S., Diaz, M., Castellsagué, X., Clifford, G., Bruni, L., Muñoz, N., and Bosch, F.X. (2007). Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. Lancet Infect. Dis. *7*, 453–459.

de Sanjosé, S., Alemany, L., Ordi, J., Tous, S., Alejo, M., Bigby, S.M., Joura, E.A., Maldonado, P., Laco, J., Bravo, I.G., *et al*. (2013). Worldwide human papillomavirus genotype attribution in over 2000 cases of intraepithelial and invasive lesions of the vulva. Eur. J. Cancer *49*, 3450–3461.

Sankovski, E., Männik, A., Geimanen, J., Ustav, E., and Ustav, M. (2014). Mapping of Betapapillomavirus Human Papillomavirus 5 Transcription and Characterization of Viral-Genome Replication Function. J. Virol. *88*, 961–973.

Scheffner, M., Werness, B.A., Huibregtse, J.M., Levine, A.J., and Howley, P.M. (1990). The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell *63*, 1129–1136.

Schmitt, A., Rochat, A., Zeltner, R., Borenstein, L., Barrandon, Y., Wettstein, F.O., and Iftner, T. (1996). The primary target cells of the high-risk cottontail rabbit papillomavirus colocalize with hair follicle stem cells. J. Virol. *70*, 1912–1922.

Schuck, S., Ruse, C., and Stenlund, A. (2013). CK2 Phosphorylation Inactivates DNA Binding by the Papillomavirus E1 and E2 Proteins. J. Virol. *87*, 7668–7679.

Sedman, J., and Stenlund, A. (1995). Co-operative interaction between the initiator E1 and the transcriptional activator E2 is required for replicator specific DNA replication of bovine papillomavirus in vivo and in vitro. EMBO J. *14*, 6218–6228.

Sekhar, V., and McBride, A.A. (2012). Phosphorylation Regulates Binding of the Human Papillomavirus Type 8 E2 Protein to Host Chromosomes. J. Virol. *86*, 10047–10058.

Shabb, J.B. (2001). Physiological Substrates of cAMP-Dependent Protein Kinase. Chem. Rev. *101*, 2381–2412.

Small, J., Barton, V., Peterson, B., and Alberg, A.J. (2016). Chapter Six - Keratinocyte Carcinoma as a Marker of a High Cancer-Risk Phenotype. In Advances in Cancer Research, K.D. Tew, and P.B. Fisher, eds. (Academic Press), pp. 257–291.

Søberg, K., Jahnsen, T., Rognes, T., Skålhegg, B.S., and Laerdahl, J.K. (2013). Evolutionary paths of the cAMP-dependent protein kinase (PKA) catalytic subunits. PloS One *8*, e60935.

Steger, G., and Corbach, S. (1997). Dose-dependent regulation of the early promoter of human papillomavirus type 18 by the viral E2 protein. J. Virol. *71*, 50–58.

Stöppler, M.C., Straight, S.W., Tsao, G., Schlegel, R., and McCance, D.J. (1996). The E5 gene of HPV-16 enhances keratinocyte immortalization by full-length DNA. Virology *223*, 251–254.

Straub, E., Fertey, J., Dreer, M., Iftner, T., and Stubenrauch, F. (2015). Characterization of the Human Papillomavirus 16 E8 Promoter. J. Virol. *89*, 7304–7313.

Stubenrauch, F., Hummel, M., Iftner, T., and Laimins, L.A. (2000). The E8E2C protein, a negative regulator of viral transcription and replication, is required for extrachromosomal maintenance of human papillomavirus type 31 in keratinocytes. J. Virol. *74*, 1178–1186.

Sun-Kuie, T., Tew-Hongw, H., and Soo-Kim, L.-T. (1990). Is Genital Human Papillomavirus Infection Always Sexually Transmitted? Aust. N. Z. J. Obstet. Gynaecol. *30*, 240–242.

Terai, M., DeSalle, R., and Burk, R.D. (2002). Lack of canonical E6 and E7 open reading frames in bird papillomaviruses: Fringilla coelebs papillomavirus and Psittacus erithacus timneh papillomavirus. J. Virol. *76*, 10020–10023.

Titolo, S., Pelletier, A., Pulichino, A.-M., Brault, K., Wardrop, E., White, P.W., Cordingley, M.G., and Archambault, J. (2000). Identification of domains of the human papillomavirus type 11 E1 helicase involved in oligomerization and binding to the viral origin. J. Virol. *74*, 7349–7361.

Toots, M., Männik, A., Kivi, G., Ustav, M., Ustav, E., and Ustav, M. (2014). The Transcription Map of Human Papillomavirus Type 18 during Genome Replication in U2OS Cells. PLoS ONE *9*.

de Villiers, E.-M., Fauquet, C., Broker, T.R., Bernard, H.-U., and zur Hausen, H. (2004). Classification of papillomaviruses. Virology *324*, 17–27.

Walboomers, J.M.M., Jacobs, M.V., Manos, M.M., Bosch, F.X., Kummer, J.A., Shah, K.V., Snijders, P.J.F., Peto, J., Meijer, C.J.L.M., and Muñoz, N. (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. J. Pathol. *189*, 12–19.

Wall, E.A., Zavzavadjian, J.R., Chang, M.S., Randhawa, B., Zhu, X., Hsueh, R.C., Liu, J., Driver, A., Bao, X.R., Sternweis, P.C., *et al.* (2009). Suppression of LPS-Induced TNF-α Production in Macrophages by cAMP Is Mediated by PKA-AKAP95-p105. Sci. Signal. *2*, ra28–ra28.

Webster, K., Parish, J., Pandya, M., Stern, P.L., Clarke, A.R., and Gaston, K. (2000). The human papillomavirus (HPV) 16 E2 protein induces apoptosis in the absence of other HPV proteins and via a p53-dependent pathway. J. Biol. Chem. *275*, 87–94.

Wilson, V.G., West, M., Woytek, K., and Rangasamy, D. (2002). Papillomavirus E1 proteins: Form, function, and features. Virus Genes *24*, 275–290.

Xia, M., Huang, R., Guo, V., Southall, N., Cho, M.-H., Inglese, J., Austin, C.P., and Nirenberg, M. (2009). Identification of compounds that potentiate CREB signaling as possible enhancers of long-term memory. Proc. Natl. Acad. Sci. *106*, 2412–2417.

Xie, F., DeSmet, M., Kanginakudru, S., Jose, L., Culleton, S.P., Gilson, T., Li, C., and Androphy, E.J. (2017). Kinase Activity of Fibroblast Growth Factor Receptor 3 Regulates Activity of the Papillomavirus E2 Protein. J. Virol. *91*.

Yang, L., Mohr, I., Fouts, E., Lim, D.A., Nohaile, M., and Botchan, M. (1993). The E1 protein of bovine papilloma virus 1 is an ATP-dependent DNA helicase. Proc. Natl. Acad. Sci. U. S. A. *90*, 5086–5090.

Yu, J.-H., Lin, B.Y., Deng, W., Broker, T.R., and Chow, L.T. (2007). Mitogen-Activated Protein Kinases Activate the Nuclear Localization Sequence of Human Papillomavirus Type 11 E1 DNA Helicase To Promote Efficient Nuclear Import. J. Virol. *81*, 5066–5078.

Zanardi, T.A., Stanley, C.M., Saville, B.M., Spacek, S.M., and Lentz, M.R. (1997). Modulation of Bovine Papillomavirus DNA Replication by Phosphorylation of the Viral E1 Protein. Virology *228*, 1–10.

INTERNET REFERENCES

Papillomavirus Episteme database PaVE: Papilloma virus genome database. https://pave.niaid.nih.gov/#home (19.05.20)

Van Doorslaer, K., Chen, Z., Bernard, H.-U., Chan, P.K.S., and DeSalle, Rob, R. Papillomaviridae. (2018). https://talk.ictvonline.org/ictv-reports/ictv_online_report/dsdna-viruses/w/papillomaviridae (19.05.20)

SUPPLEMENTARY DATA

Appendix 1. Primers used in the study

№	Gene	Gene bank accession number	Primer	Sequence 5'-3'	Product length
1	PRKACA	NM_001304349.1 NM_002730.4 NM_207518.3	PKA A1 F HindIII	AAGCTTGGCAACGCCGC CGCCGCCAAGA	1064
2			PKA A1 R BamHI	GGATCCCTAAAACTCAG AAAACTCCTTG	
3	PRKACA	NM_001304349.1 NM_002730.4 NM_207518.3	PKA A1 T197A F	GAAGGGCCGCACTTGG**G** CCTTGTGCGGCACCCCT	
			PKA A1 T197A R	AGGGGTGCCGCACAAGG **C**CCAAGTGCGGCCCTTC	
4			PKA A1 K72R F	GAACCACTATGCCATGA **G**GATCCTCGACAAACAG	
			PKA A1 K72R R	CTGTTTGTCGAGGATC**C**T CATGGCATAGTGGTTC	
5			GAPDH F	CTCTCTGCTCCTCCTGTT CGAC	
			GAPDH R	TGAGCGATGTGGCTCGG CT	
6			HPV18 E2 F	GATAGTGGCTATGGCTG TTC	
			HPV18 E2 R	GCTGTTGTTGCCCTCTGT G	
7			HPV18 E1^E4 F	CATTTACCAGCCCGACG AG	
			HPV18 E1^E4 R	GACGTCTGGCCGTAGGT CTTTGC	

8			HPV18 E8^E2 MIX1 F	GATAGTGGCTATGGCTG TTC	
			HPV18 E8^E2 MIX 1 R	GACGTCTGGCCGTAGGT CTTTGC	
9			HPV18 E8^E2 MIX 2 F	CTGAAGTGGAAGCAACA CAG	
			HPV18 E8^E2 MIX 2 R	GACGTCTGGCCGTAGGT CTTTGC	
10	PRKACA	NM_001304349.1 NM_002730.4 NM_207518.3	PKA A F1 and R1	CTTATACATGGTCATGG AGTAC and CTGTAGATGAGATCCAG CGAG	149
11			PKA A F2 and R2	CTACCCGCCCTTCTTCGC AGAC and CAAAGCGCTTGGTGAGA TCTAC	142
12	PRKACB	NM_001242857.2 NM_001242858.2 NM_001242859.2 NM_001242860.2	PKA B F1 and R1	GGGTGAAATGTTTTCAC ATC and GATCTCTGTAGATGAGG TCTAG	124
13		NM_001242861.2 NM_001242862.2 NM_001300915.2 NM_001300916.2 NM_001300917.2 NM_002731.3 NM_182948.4 NM_207578.3	PKA B F2 and R2	GGCAGAACTTGGACATT ATGTG and GGTTGGTCTGCAAAGAA TGG	151

14	PRKACG	NM_002732.3	PKA G F1 and R1	CTGCAGGTGGACCTCAC CAAG and CGTCAAAGTTACTGGCA TCCC	168
15			PKA G F2 and R2	CTACAGCGCGTCGGAAG GTTTAG and GAAGTCCGTCACCTGCA GGTAG	161
16			pJET 1 F	CGACTCACTATAGGGAG AGCGGC	
			pJET 2 R	AAGAACATCGATTTTCC ATGGCAG	

Explanatory note: yellow indicates the restriction sites.

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