Universidade de Aveiro
2018

Departamento de Química

Ana Catarina Campos Ramalho

Human papillomavirus and human cytomegalovirus: evasion from the cellular antiviral response

Vírus do papiloma humano e o citomegalovírus humano: evasão da resposta celular antiviral

Universidade de Aveiro
2018

Departamento de Química

Ana Catarina Campos Ramalho

Human papillomavirus and human cytomegalovirus: evasion from the cellular antiviral response

Vírus do papiloma humano e o citomegalovírus humano: evasão da resposta celular antiviral

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Dr. Daniela Maria Oliveira Gandra Ribeiro, Investigadora do Departamento de Ciências Médicas da Universidade de Aveiro.

Thesis submitted at University of Aveiro to fulfil the requirements to obtain the Master's degree in Biotechnology, held under the scientific guidance of Dr Daniela Maria Oliveira Gandra Ribeiro, Researcher at the Department of Medical Sciences at University of Aveiro.

PTDC/BIACEL/31378/2017 (POCI-01-0145-FEDER-031378) através do Programa Operacional Competitividade e Internacionalização (COMPETE2020).

Cofinanciado pelo Fundo Comunitário Europeu FEDER e Fundação para a Ciência e Tecnologia (FCT).

o júri

presidente

Doutora Luísa Alexandra Seuanes Serafim Martins Leal
Professora Auxiliar do Departamento de Química da Universidade de Aveiro

Doutora Daniela Maria Oliveira Gandra Ribeiro
Investigadora do Departamento de Ciências Médicas da Universidade de Aveiro

Doutora Rute Conceição do Nascimento Veríssimo Afonso
Investigadora do Instituto Gulbenkian de Ciência

Agradecimentos

Com o fim desta etapa, quero agradecer a todos aqueles que me acompanharam ao longo do meu percurso.

À Dra. Daniela, por me ter recebido neste grupo, por me ter orientado neste projeto e por me ter transmitido um enorme gosto por esta área. Agradeço todo o conhecimento e conselhos oferecidos e por ter acreditado em mim e nas minhas capacidades.

Aos meus colegas de trabalho, agradeço em primeiro lugar pela amizade e pelo enorme apoio, sem vocês teria sido impossível.
À Isabel, por me teres acompanhado desde o início da minha tese e por teres sempre interesse no meu trabalho para me poder ajudar, apesar de "não perceberes nada" de vírus. Agradeço por tudo o que me ensinaste no laboratório e por toda a ajuda.
À Mariana, por estares sempre pronta a ajudar, pelo teu entusiamo contagiante pelo trabalho, por tudo o que me ensinaste e pelas gargalhadas.
À Beatriz, por seres a minha grande companheira, nunca me deixares sozinha até tarde, por estares sempre disposta a ajudar-me e pelas nossas conversas.
À Rita, por toda a ajuda, por me incentivares a pensar positivo e nunca desistir, por acreditares em mim, por tudo o que me ensinaste e por todas as discussões sobre o meu trabalho. Não consigo agradecer o suficiente pelo apoio que me deste na fase final da minha tese.
Ao Rui, pelo bom companheiro que foste desde que começámos este percurso há 1 ano e por me teres ajudado sempre que eu precisei.
À Carolina, pela companhia nos últimos meses e por me dares força.

Ao Pedro, por me ouvires quando as coisas corriam menos bem e me fazeres esquecer os problemas. Obrigada por acreditares sempre em mim.
À minha família, os meus pais, o meu irmão e os meus avós, por me apoiarem e por terem orgulho em mim, e por terem sempre interesse no meu trabalho.

palavras-chave

Vírus do Papiloma Humano, Citomegalovírus Humano, Infeção viral, Peroxissomas, Imunidade inata, MAVS, E5, vMIA

resumo

Após infeção viral, as células hospedeiras reconhecem o agente patogénico e ativam o sistema imunitário na tentativa de eliminá-lo. O vírus do papiloma humano (HPV) e o citomegalovírus humano (HCMV) codificam diversas proteínas, entre as quais a E5 e a vMIA, respetivamente, que alteram ou até inibem a resposta imune do hospedeiro. Neste trabalho, o nosso principal objetivo foi examinar em mais detalhe a influência destes na sinalização antiviral dependente dos peroxissomas.

A vMIA do HCMV é uma proteína com atividade anti-apoptótica que também foi observada a suprimir a sinalização antiviral mediada pela MAVS nos peroxissomas e mitocôndria, e a induzir a fragmentação de ambos os organelos. No presente trabalho, propusemo-nos a analisar os domínios da vMIA responsáveis pela inibição, de forma a complementar estudos anteriores que mostraram que os mutantes de deleção Δ2-23, Δ23-34, Δ115-130 e Δ131-147 inibem a sinalização de forma semelhante à vMIA completa. Os nossos resultados indicam que a vMIA Δ35-109 também suprime a via antiviral dependente dos peroxissomas, o que sugere que domínios distintos atuam de forma independente na via, ou que um dos segmentos mantidos em todos os mutantes (aa 110 a 114 e 148 a 163) são responsáveis por esta função da vMIA. Para além disso, otimizámos um protocolo para analisar a oligomerização da MAVS usando eletroforese semi-desnaturante com detergente em gel de agarose (SDD-AGE) e eletroforese em gel de poliacrilamida com dodecil sulfato de sódio (SDS-PAGE) em condições não redutoras. Estas análises iriam indicar se os mutantes da vMIA impedem a oligomerização da MAVS, que ocorre após a estimulação da via antiviral.

Em relação ao HPV, um estudo recente que sugeriu uma interação entre a proteína E5 e a MAVS levou-nos a direcionar a nossa investigação prática para a análise desta relação. Para esta análise, construímos um plasmídeo que codifica a E5 do HPV16 acoplada à proteína de fusão FLAG. Contudo, apesar dos nossos esforços não foi possível detetar a sua transfeção, o que impossibilitou a realização dos estudos propostos.

keywords

Human Papillomavirus, Human Cytomegalovirus, Viral infection, Peroxisomes, Innate Immunity, MAVS, E5, vMIA

abstract

Upon viral infection, the host cells recognize the pathogen and activate their immune system to attempt its elimination. The Human papillomavirus (HPV) and Human cytomegalovirus (HCMV) encode several proteins, among which E5 and vMIA, respectively, which affect or even inhibit the host immune response. In this work, our main goal was to further examine their influence on the peroxisome-dependent antiviral signalling.

HCMV vMIA is a protein with anti-apoptotic activity, which has also been shown to suppress the MAVS-mediated antiviral signalling at the peroxisomes and mitochondria and to induce fragmentation of both organelles. In the present work, we proposed to analyse the domains of vMIA responsible for the inhibition, complementing previous studies showing that the deletion mutants Δ2-23, Δ23-34, Δ115-130 and Δ131-147 inhibited signalling in a similar way as the wild-type. Our results indicate that vMIA Δ35-109 also suppresses the peroxisome-dependent antiviral pathway, which suggests that distinct domains act independently on the pathway or that either one of the segments maintained in all mutants (aa 110 to 114 and 148 to 163) are responsible for this role of vMIA. In addition, we have optimized a protocol for the analysis of MAVS oligomerization using semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). These analyses would indicate whether the vMIA mutants block MAVS oligomerization, which occurs upon stimulation of the antiviral pathway.

Regarding HPV, a recent study suggesting an interaction between the E5 protein and MAVS led us to direct our practical research towards the examination of their relationship. For our studies, we developed a plasmid encoding HPV16 E5 with a FLAG tag. However, regardless of our efforts it has not been possible to detect its transfection thus far, which has hindered the possibility of performing the studies proposed.

List of abreviations

AIM2	Absent In Melanoma 2
APOBEC3	Apolipoprotein B mRNA-Editing Catalytic Polypeptide 3
ASC	Apoptosis-Associated Speck-Like Protein Containing CARD
CARD	Caspase Activation And Recruitment Domains
CBP	CREB Binding Protein
cGAMP	Cyclic Guanosine Monophosphate-Adenosine Monophosphate
cGAS	Cyclic Guanosine Monophosphate-Adenosine Monophosphate Synthase
CIN	Cervical Intra-epithelial Neoplasia
COX-2	Cyclooxygenase-2
CpG	Cytosine-phosphate-Guanine
CR	Conserved Regions
CTD	C-Terminal Domain
DAI	DNA-Dependent Activator of Interferon-Regulatory Factors
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
dsDNA	double stranded DNA
dsRNA	double stranded RNA
E6AP	E3 Ubiquitin Ligase
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic Reticulum
HCMV	Human Cytomegalovirus
HDAC	Histone Deacetylase
HHV-5	Human Herpes Virus 5
HPV	Human Papillomavirus
HSIL	High-Grade Squamous Intra-Epithelial Lesion
IFI16	gamma-Interferon-Inducible Protein 16
IFNAR	Transmembrane Interferon-A/B Receptor
IL	Interleukin
IFN	Interferon
IKK	Inhibitor of NF-Kb Kinase
IRF	Interferon Regulatory Factor

ISG	Induced Stimulated Genes
ISRE	Interferon-Stimulated Response Element
JAK-STAT	Janus Kinase-Signal Transducers And Activators Of Transcription
LCR	Long Coding Region
LGP2	Laboratory of Genetics and Physiology 2
LSIL	Low-grade Squamous Intra-epithelial Lesion
MAM	Mitochondrion-Associated Membrane
MAPK	Mitogen Activated Protein Kinase
MAVS	Mitochondrial Antiviral Signalling
MDA5	Melanoma Differentiation Associated Gene 5
MHC I	Major Histocompatibility Complex
MEF	Mouse Embryonic Fibroblasts
MyD88	Myeloid Differentiation Primary Response Gene 88
mRNA	messenger RNA
NF-κB	Nuclear Factor Kappa B
NLR	NOD-Like Receptor
NOD	Nucleotide-Binding Oligomerization Domain
ORF	Open Reading Frame
PAMP	Pathogen-Associated Molecular Pattern
pRB	Retinoblastoma Protein
PRR	Pattern Recognition Receptor
P/CAF	P300/CBP-Associated Factor
RD	Repressor Domain
RIG-I	Retinoic Acid Inducible Protein 1
RLRs	RIG-I Like Receptors
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SCC	Squamous Cell Carcinoma
SCJ	Squamo-Columnar Junction
SIL	Squamous Intra-Epithelial Lesion
ssRNA	single-stranded RNA
STING	Stimulator of Interferon Genes
TGF-β	Transforming Growth Factor B

TIR	Toll/IL-1R Homology
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TRAF	Tumor Necrosis Factor Receptor-Associated Factor
TRIF	TIR-Domain-Containing Adapter Inducing Interferon-B
TRIM25	Tripartite Motif-containing 25
TYK2	Tyrosine Kinase 2
UCHL1	Ubiquitin Carboxyl-Terminal Hydrolase L1
VEGF	Vascular Endothelial Growth Factor
vMIA	Viral Mitochondrial Inhibitor of Apoptosis

Index

List of Figures

List of Tables

List of Equations

I. Introduction

1.1 Antiviral Innate Immune Response

The innate immune system is an early defense mechanism triggered upon detection of pathogens, such as viruses. Once a viral infection occurs, the recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) leads to the activation of the innate immune response, which stimulates the adaptive immune response (Mogensen, 2009; Takeuchi and Akira, 2010). Viral sensing by PRRs triggers the initiation of a cascade of downstream signalling pathways, namely the interferon regulatory factor (IRF) pathway, the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling pathway (Hoesel and Schmid, 2013; Villarino et al., 2015). Consequently, pro-inflammatory cytokines (such as type I interferons (IFNs)) and interferon stimulated genes (ISGs) are produced for the activation of the innate and adaptive immunity (Crosse et al., 2018).

In general, cellular PRRs are classified according to their intracellular location as membrane-associated or cytosolic, and they can detect either viral RNA or DNA. Membrane-bound PRRs comprise Toll-like receptors (TLRs), from which TLR1/2/4/5/6 are mainly expressed on the cell surface while TLR3/7/8/9 are found in intracellular compartments and are the ones responsible for the recognition of viral nucleic acids (Kawasaki and Kawai, 2014). TLR3 binds dsRNA, TLR7 and TLR8 recognize ssRNA and finally TLR9 recognizes unmethylated CpG motifs in DNA (Chaturvedi and Pierce, 2009). Cytosolic RNA sensors correspond to retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), including RIG-I, the melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), as well as the nucleotide-binding oligomerization domain (NOD)-like receptors (Seth et al., 2006). Viral DNA sensors include cyclic GMP-AMP (cGAMP) synthase (cGAS), DNA-dependent activator of IFN-regulatory factors (DAI), absent in melanoma 2 (AIM2) and γ-IFN-inducible protein 16 (IFI16) (Reinholz et al., 2013, p. 2). Importantly, dsRNA transcribed by polymerase III from a DNA virus may be recognized by RIG-I (Ablasser et al., 2009; Chiu et al., 2009).

The PRRs use different pathways to convey their signals, ultimately culminating in the production of IFNs and ISGs which will target the pathogen. TLRs signal via the adapter molecule myeloid differentiation primary response gene 88 (MyD88) or via TIR-domain-containing adapter-inducing interferon-β (TRIF) (Kawasaki and Kawai, 2014). RIG-I and MDA5 induce the NF-κB signalling and IRF-mediated IFN production via the mitochondrial antiviral-signalling protein (MAVS) (Seth et al., 2006). On the other hand, most cytosolic DNA sensors convey their signal via

the stimulator of interferon genes (STING), while AIM2 acts by assembling an inflammasome with the apoptosis-associated speck-like protein containing CARD (ASC) and caspase 1 (Paludan and Bowie, 2013). As a result, the IRF1-9 transcription factors are activated and migrate to the nucleus, causing the expression of IFNs and ISGs. Subsequently, IFNs are secreted and bind to receptors on adjacent cells in order to propagate the antiviral response by initiating the JAK-STAT pathway (Tummers and Burg, 2015). In parallel, the NF-κB signalling pathway is activated as well, driving the production of pro-inflammatory cytokines and other factors (Abbas et al., 2017).

The most common and best characterized IFNs belong to type I, which includes IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ω. Their synthesis is mainly stimulated by IRF1, 3, 5 and 7 upon viral genome recognition. Type II corresponds to IFN-γ, which is mainly produced by natural killer (NK) cells as well as CD4 and CD8 T cells (Hong and Laimins, 2017). Finally, type III comprises IFN-λ1, IFN-λ2 and IFN-λ3, which act mainly in tissues highly susceptible to viral infection, such as mucosal tissues. Type I and type III IFNs act in a similar way, by stimulating the expression of genes in both infected and non-infected cells, thus inducing a strong antiviral state (Wack et al., 2015).

1.1.1 RIG-I-like Receptors and MAVS Signalling Pathway

RIG-I, MDA5 and LGP2 are important sensors, expressed in most tissues, for the recognition of a wide range of viruses. The RLRs belong to the DExD/H-box family of helicases, containing two helicase domains, Hel1 and Hel2, and a C-terminal domain (CTD), responsible for RNA binding and conformational changes involving the hydrolysis of ATP (Jiang et al., 2011; Yoneyama et al., 2005). In addition, RIG-I and MDA5 contain two N-terminal caspase activation and recruitment domains (CARDs), which interact with downstream molecules containing CARD (Jiang et al., 2011).

Upon binding to viral RNA, RIG-I is activated due to conformational changes which expose its CARD domains, allowing downstream signalling through CARD–CARD-mediated interactions (Jiang et al., 2011). In the absence of stimuli, RIG-I is maintained in a closed inactive conformation by the repressor domain (RD). In contrast, the CARD domains in MDA5 are not sequestered by RD but are permanently exposed instead (Berke and Modis, 2012).

The activation of RIG-I rapidly propagates the antiviral signalling cascade via MAVS. Four different groups discovered MAVS simultaneously, which accounts for the three other names attributed to the protein: IPS-1, VISA, and Cardif. It functions as a signalling adaptor protein, since it links the identification of viral RNA to the initiation of antiviral signalling (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). The protein comprises a CARD domain, a proline-rich region and a C-terminal transmembrane domain (Seth et al., 2005). Furthermore, two

tumor necrosis factor receptor-associated factor (TRAF)-interacting motifs (TIM) are present in the proline-rich region, while another one is located near the transmembrane domain (Xu et al., 2005). Regarding its location, MAVS is a tail-anchored membrane protein found at the outer membrane of mitochondria, peroxisomes and at the mitochondrion-associated membrane (MAM) (Seth et al., 2005; Dixit et al., 2010; Horner et al., 2011).

Mitochondria and peroxisomes are exceptionally dynamic and multifunctional organelles responsible for important metabolic functions (Camões et al., 2009). The close relationship between both organelles has been extensively explored, mainly after the discovery of their cooperation in the oxidation of fatty acids and in the metabolism of reactive oxygen species (ROS), as well as of some overlapping components from the division machinery (Bonekamp et al., 2009; Wanders, 2004). Importantly, they have also been shown to participate coordinately in antiviral signaling (Dixit et al., 2010). Mitochondria are organelles bounded by a double-membrane and containing their own genome, which are proposed to have evolved from bacteria by endosymbiosis (Cooper, 2000). Despite their typical spherical structure, mitochondria are continuously remodeling their shape through cycles of fusion and fission, forming dynamic tubular networks (Okamoto and Shaw, 2005) In contrast to mitochondria, no DNA or protein translation machinery is found in peroxisomes. They are dynamic organelles that respond to modifications in the cellular environment by adapting their morphology, number and protein composition, which is achieved by controlled events of biogenesis and pexophagy. Peroxisome biogenesis occurs by asymmetric growth and division of pre-existing organelles or *de novo* formation from the endoplasmic reticulum (Hoepfner et al., 2005; Lazarow and Fujiki, 1985). Regarding their size, mitochondria range from 0.5 to 0.7 µm and peroxisomes between 0.1 to 1 µm in their spherical or rod-like shape, although both can form elongated structures (Schrader, 2006).

After binding viral RNA, activated RIG-I recruits and activates the adaptor molecule MAVS via CARD-CARD interactions, mediated by K63-linked ubiquitin chains synthesized by the Riplet and tripartite motif-containing 25 (TRIM25) ubiquitin ligases. (Gack et al., 2007, p. 2; Oshiumi et al., 2013; Zeng et al., 2010) The CARD domains of MAVS then form large prion-like aggregates composed of several MAVS molecules, which determines a gain of function for the molecule (Hou et al., 2011). Signalling is propagated by interaction with TRAF molecules, leading to IRF and NF-κB activation (Loo and Gale, 2011).

Differential MAVS localization to the mitochondria and peroxisomes determines the activation of distinct and complementary signalling pathways. Peroxisomal MAVS promotes a rapid but transient response to viral infection, while the activation of mitochondrial MAVS leads to a later

and sustained antiviral response (Dixit et al., 2010). Furthermore, although still controversial, it has been suggested that the mitochondrial MAVS induces the expression of type I IFNs while peroxisomal MAVS promotes type III IFNs, independently of type I IFN production. (Bender et al., 2015; Odendall et al., 2014) The RIG-I/MAVS pathway via the peroxisomes and the mitochondria is represented in Figure 1.

Although STING and RIG-I-MAVS pathways are involved in DNA and RNA sensing, respectively, their signalling is interconnected (Zhong et al., 2008). Ishikawa *et al.* proposed that STING, RIG-I and MAVS form a complex which is stabilized upon viral infection, and that STING is an important mediator of RIG-I downstream signalling (Ishikawa and Barber, 2008). However, contradictory results arise from other reports, where the absence of cGAS or STING does not affect IFN-β induction by an RNA virus (Li et al., 2013).

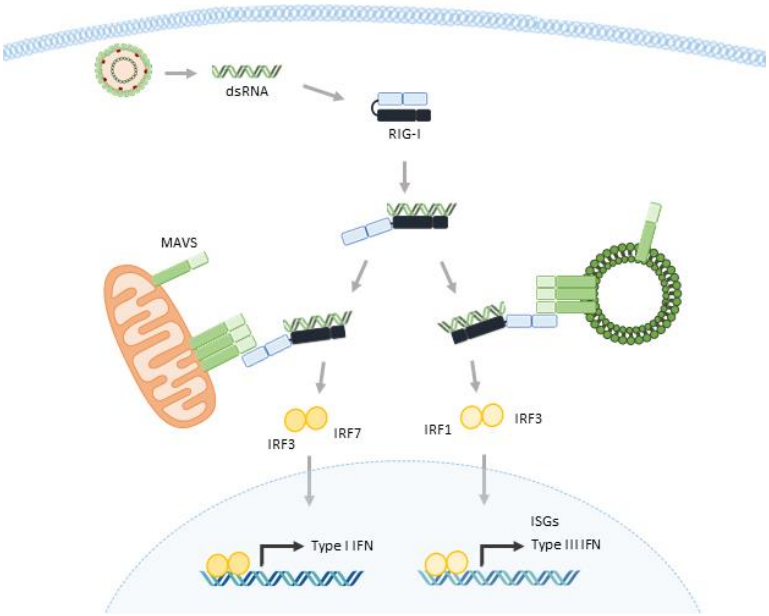

Figure 1. RIG-I/MAVS signalling pathway via the mitochondria and peroxisomes.

1.2. Human Cytomegalovirus

1.2.1 Characteristics and Epidemiology of the virus

The human cytomegalovirus (HCMV), or human herpes virus 5 (HHV-5) as it has recently been referred to in the literature, belongs to the Herpesviridae family, sub-family Betaherpesvirinae. HCMV is a common and globally distributed pathogen, exhibiting great geographic variability, with higher seroprevalence in Third World countries (Cannon et al., 2010). Similar to other herpes viruses, HCMV enters latency, establishing a lifelong infection of the host. Hematopoietic stem cells and cells of the myeloid lineage are the most common cellular reservoirs for HCMV latency (Hahn et al., 1998; Goodrum et al., 2002). In immunocompetent individuals, primary infection is usually asymptomatic, although symptoms resembling that of influenza or a mild mononucleosis may be manifested (Lancini et al., 2014). However, HCMV infection or reactivation of the latent virus in immunocompromised patients and congenital infection may lead to life-threatening clinical manifestations (Kenneson and Cannon, 2007; Limaye et al., 2008). After infection, viraemia occurs and the virus is excreted in body fluids. Thus, HCMV may be transmitted via saliva, sexual contact and also via the placenta, during birth or through breast milk. In addition, blood transfusion or stem cell/organ transplants may also transmit the virus (de Jong et al., 1998).

1.2.2 Genome and Structure

The genome of HCMV corresponds to linear non-segmented double-stranded DNA with a size of around 240 kb, one of the longest genomes of all human viruses. HCMV exhibits a complex coding capacity and it is estimated to contain over 165 open reading frames (ORFs) (Stern-Ginossar et al., 2012). Some of the encoded proteins are carried by viral particles, whereas others are expressed in the cell at different stages of infection. The virion has a diameter of around 150–200 nm and it is composed of a lipid bilayer envelope surrounding an icosahedral capsid constituted by 162 capsomers which encloses the genome. The envelope is derived from membranes of the endoplasmic reticulum (ER)-Golgi compartments of the host, and it presents at least 20 viral glycoproteins important for cell attachment and entry (Varnum et al., 2004). Between the envelope and the capsid is the tegument, an unstructured layer containing viral RNA and proteins (Robert F. Kalejta, 2008). The glycoproteins of the envelope mediate viral fusion with the host membrane, allowing the release of tegument proteins and the genome-containing capsid into the cytoplasm.

1.2.3 Life Cycle

Following primary infection, HCMV can lead to a productive life cycle in some tissues, with active viral gene expression and release of infectious particles. Simultaneously, latent infection is established in certain cell types, where low levels of gene transcription are verified, and no viral particles are synthesized, until an eventual reactivation. As described ahead, the lytic cycle of HCMV, depicted in Figure 2, induces cytopathic effects that include cytoplasmic and nuclear enlargement, culminating in cell death.

Viral glycoproteins at the envelope mediate cell entry by interaction with host receptors, causing fusion or endocytosis of the virion (Isaacson et al., 2008). gB and gH/gL are two glycoproteins essential for internalization (Wille et al., 2013). Upon viral entry, the fully formed and active tegument proteins released in the cytoplasm act rapidly to regulate the initiation of viral gene expression, as well as to inhibit the initial steps of immune response (R. F. Kalejta, 2008). The capsid is delivered to the cytoplasm as well and transported to the nuclear envelope for import of the viral genome into the nucleus. Proteins attached to the capsid have been suggested to associate with the cellular microtubule network to mediate the transport of the capsid to the nucleus (Ogawa-Goto et al., 2003).

In the nucleus, viral genes are expressed using the cellular transcriptional machinery. HCMV genes are divided in three classes: immediate early, early or late, depending on the moment and conditions of their expression.

The assembly of the capsid occurs in the nucleus, whereas the final virion assembly and maturation takes place in large cytoplasmic inclusions - the virion assembly complex (Sanchez et al., 2000). Here, the capsid associates with tegument proteins and acquires the envelope. Finally, two to three days post-infection the virions are released by exocytosis.

1.2.4 Evasion from the innate immune response

In most cases of HCMV infection, the human organism is able to mount a rapid, strong immune response against the virus, rendering the infection asymptomatic. Nevertheless, the manipulation and evasion strategies developed by HCMV allow a first stage of infection with efficient dissemination through several host cell types and high rate of virion release, as well as the subsequent maintenance of the virus in a latent state (Jackson et al., 2011). The interplay between HCMV and the immune system has been widely studied for years, although further investigations to elucidate all details of their interaction are required.

HCMV rapidly induces the innate immune response within the infected cell, as the viral DNA is recognized by PRRs, initiating a cascade of pro-inflammatory cytokines, IFNs and ISGs production. Several host sensors have been implicated in HCMV recognition and a wide range of immune strategies are clearly activated against HCMV shortly after infection (reviewed in (Marques et al., 2018)) However, the virus is equipped with several proteins which counteract the host response, allowing its life cycle to proceed (Marques et al., 2018).

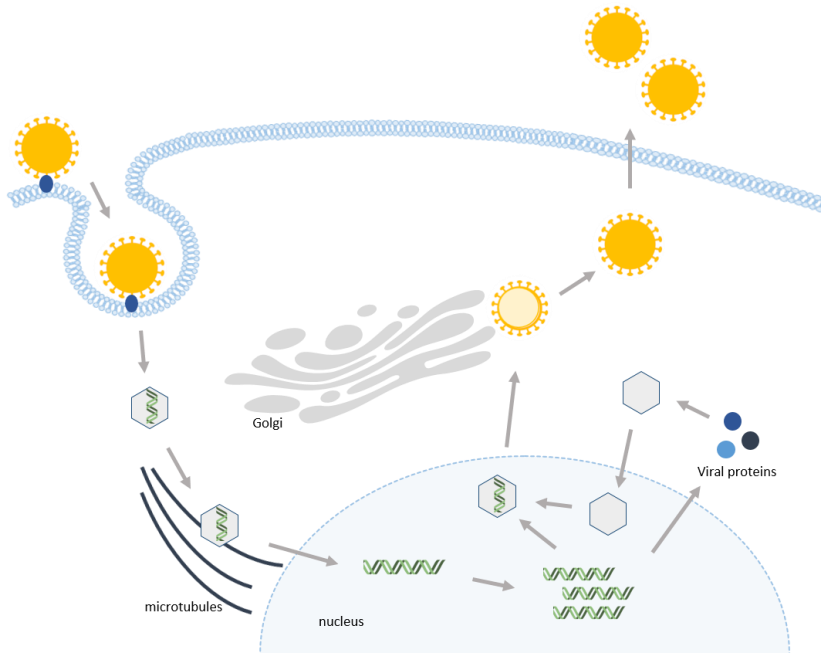

Figure 2. HCMV life cycle. The virus enters the cells by interaction with cell surface receptors. The viral capsid is released in the cytoplasm and associates with microtubules to migrate to the nucleus, where it delivers the genome. In the nucleus, gene expression and amplification of the genome take place. Encapsidation of the DNA occurs at the nucleus, and the envelope is added before virion release.

1.2.5 vMIA: an antiapoptotic protein with antiviral activity

HCMV must also prevent the apoptotic pathways which are naturally induced in the host cell upon viral infection, since cell viability must be maintained for its replication. HCMV encodes multiple proteins with anti-apoptotic activity in the UL36 to UL38 loci. The immediate early protein pUL37x1 termed vMIA (viral mitochondria-localized inhibitor of apoptosis), encoded by the UL37 exon 1 gene, has been shown by Goldmacher *et al.* (Goldmacher et al., 1999) to suppress apoptosis by cell death receptors. Two domains have been demonstrated to be necessary and sufficient for the anti-apoptotic activity of vMIA: the regions between aa 5-34 and aa 118-147 (Hayajneh et al., 2001). The same authors showed that the segment between aa 2-30 contains the mitochondrial

localization signal. Intrinsic apoptosis requires Bax and/or Bak for mitochondrial outer membrane permeabilization. vMIA has been demonstrated to bind and sequester Bax at the mitochondria, hampering its pro-apoptotic activity (Arnoult et al., 2004). vMIA has also been shown to alter mitochondrial fission and/or fusion events, disrupting the tubular networks formed by the organelle, which is suggested to contribute to inhibition of apoptosis (McCormick et al., 2003). The vMIA-mediated release of Ca^{2+} from the endoplasmic reticulum (ER) induces mitochondrial fission as well (Sharon-Friling et al., 2006, p.).

Besides its anti-apoptotic activity, vMIA exhibits an important role in the inhibition of innate immune signalling. The viral protein inhibits MAVS-mediated signalling at mitochondria and peroxisomes, possibly via different mechanisms (Castanier et al., 2010; Magalhães et al., 2016). Castanier *et al.* (Castanier et al., 2010) suggested that the RLR-induced mitochondrial elongation enhances MAVS signalling and that vMIA blocks this pathway by inducing the fragmentation of the organelle, consequently dampening the association between MAVS and STING (Castanier et al., 2010). At the peroxisomes, vMIA causes fragmentation and inhibits the MAVS-dependent immune signalling as well, although peroxisomal fragmentation is not crucial for the inhibition of the pathway. It has also been demonstrated that vMIA interacts directly with peroxisomal MAVS. However, the precise mode of RIG-I-MAVS signalling inhibition by vMIA is unknown.

1.3. Human Papillomavirus

The following chapter is the result of a detailed bibliographic study on the human papillomavirus, focusing not only on its constitution, genome/protein organization and life cycle but also discussing on the latest results related to its oncogenesis and evasion from the innate immune response. For clarity, this part of the work was included in the Introduction chapter.

1.3.1 Epidemiology

To this day, over 180 human papillomavirus (HPV) types have been identified and divided into five genera (alpha, beta, gamma, mu, and nu) belonging to the *Papillomaviridae* family (Groves and Coleman, 2015). HPV can infect epithelial cells of the skin or mucosal tissues. Depending on their tropism, the HPV types are categorized either as cutaneous or mucosal (Doorbar et al., 2015), and the mucosal HPV types can be further divided into two categories: low-risk HPV which cause benign warts, and high-risk HPV with oncogenic potential.

Human papillomaviruses are best known as the causative agents of cervical cancer, being responsible for virtually all cases of the disease (Schiffman et al., 2016). Nevertheless, most HPV infections are asymptomatic and are cleared by the immune system within 12 months (Rodríguez et al., 2008). In fact, infection by HPV is quite ubiquitous, since it is the most common sexually transmitted infection worldwide. (Dunne et al., 2007) In some cases, women do not clear infection, causing the persistence of HPV, which is the major risk factor for malignant progression (Bodily and Laimins, 2011). Immunocompromised individuals, for instance those with human immunodeficiency virus infection or organ transplant recipients who are receiving immunosuppressive therapy, are more susceptible to the development of HPV-associated carcinomas (Denny et al., 2012; Freiberger et al., 2015). Besides cervical cancer, high-risk HPV types are associated with the development of several other carcinomas, namely anal, vulvovaginal and penile cancers, and also head and neck cancers (Abramowitz et al., 2011; De Vuyst et al., 2009; Marur et al., 2010). The incidence of these cancers is increasing, and for anal as well as head and neck cancers most cases are associated with infection by HPV16 (Abramowitz et al., 2011; Kreimer et al., 2005). However, cervical cancer remains the most common HPV-related cancer, since over 500,000 cases of invasive cervical cancer have been registered, from a total of 600,000 invasive cancer cases caused by HPV in 2012 (“Globocan 2012 - Home,” n.d.). Furthermore, 250,000 deaths resulted from cervical cancer cases.

1.3.2 Virus structure and genome and protein organization

The genomes of different HPV types share their main structural and organizational features, although slight variations are verified. The viral genetic material corresponds to circular double-stranded DNA with a size of approximately 8 kb, comprising a set of open reading frames (ORFs). The genome is divided in three regions: early, late and long control region (LCR). The genes E1, E2, E4, E5, E6 and E7 encoding for six individual proteins belong to the early region. The late region is constituted by the genes L1 and L2, which encode for the major and minor capsid proteins, respectively (Danos et al., 1982). The LCR corresponds to a non-coding segment located between the L1 and E6 genes which contains the origin of replication as well as promoters and transcription factor binding sites. E1, E2, L1 and L2 have well-conserved sequences, while the remaining genes display a greater variability, which is reflected in the differences observed in infections with each HPV type (García-Vallvé et al., 2005).

The HPV gene products include core proteins (E1, E2, L1 and L2), which are essential for the viral life cycle, and accessory proteins (E4, E5, E6 and E7) that are not strictly required, although they greatly favor the progression of HPV infection. E1, a DNA helicase, and E2, a regulatory protein, are responsible for genome amplification (Chiang et al., 1992). The E2 protein is a DNA binding protein with sequence specificity to motifs in the viral genome. It performs numerous functions, not only as a transcriptional activator and auxiliary replication factor, but also in immune evasion. The major capsid protein L1 and the minor capsid protein L2 assemble in an icosahedral structure around the viral genome (Hagensee et al., 1993). Besides their structural function, both capsid proteins play a crucial role in cell binding and entry, as well as endosome escape (Joyce et al., 1999; Kämper et al., 2006).

The E4 protein displays numerous functions in the late stages of the HPV life cycle, including the disruption of the keratin cytoskeleton and causing abnormalities in the cornified envelope, thus facilitating virion release (Doorbar et al., 1991; Brown and Bryan, 2000). Furthermore, E4 causes the redistribution of mitochondria, leading to apoptosis (Raj et al., 2004), and it also interferes with the cell cycle by inducing G2 arrest, thus preventing cell proliferation while promoting the onset of vegetative viral genome amplification (Knight et al., 2004).

Finally, E5, E6 and E7 are the HPV oncogenic proteins. The E6 and E7 proteins from low- and high-risk HPV types display different functions, which justifies the ability of high-risk HPV to drive neoplasia, in contrast with low-risk HPV types. Similarly, there are differences between the high-risk and low-risk E5 proteins as well, and they are found in alpha HPV types only (Bravo and Alonso, 2004). E5 is a short (only 83 amino acids long in HPV16) and hydrophobic molecule, which accounts

for its membrane localization and affinity for membrane-bound proteins (Krawczyk et al., 2010). E6 and E7 are small proteins with well-characterized functions, comprising approximately 150 and 100 amino acids, respectively. The E6 protein locates predominantly in the nucleus, but it can also be found in the cytoplasm, depending on the cell environment (Masson et al., 2003). The E7 protein, on the other hand, exists mainly in the cytoplasm while it is also detected in nuclear pools (Sato et al., 1989; Smotkin and Wettstein, 1987).

HPV particles are non-enveloped icosahedral structures assembled from 360 molecules of the L1 protein, arranged into 72 capsomeres composed of 5 L1 molecules each (Chen et al., 2000). The number of L2 molecules present in the viral capsid is variable, but the virion can incorporate up to 72, one per pentamer (Buck et al., 2008). Inside this shielding structure, the viral genome is present in a chromatin-like structure, associated with host histones (Favre et al., 1977). The L2 protein is buried below the capsid at the hollow center of L1 pentamers in a mature virion (Hagensee et al., 1993; Liu et al., 1997). During the infectious stage, the capsid undergoes conformational changes which expose the L2 protein (Day et al., 2008).

1.3.3 Virus life cycle

The differentiation-dependent life cycle of HPV, shown in Figure 3, takes advantage of the continuous tissue renewal characteristic of the stratified squamous epithelia (Doorbar, 2005). During the different stages of the viral life cycle, the viral gene products are variable due to the control of gene expression by multiple promoters and mRNA splicing (Schwartz, 2013). Most HPV infections are unapparent and asymptomatic, since they do not disrupt the infected cells and only elicit a minor immune response. This means that the virus is able to go unnoticed through its life cycle, from the moment of infection until the production and release of thousands of virions, which takes about 2-3 weeks (Doorbar, 2005).

Infection

HPV infects basal cells of the epithelium, which it reaches through microabrasions in the tissue (Egawa, 2003). Binding of the L1 capsid protein to heparan sulfate proteoglycans present in the cell surface has been proposed to be necessary for viral internalization (Joyce et al., 1999; Combita et al., 2001; Giroglou et al., 2001). Upon recognition of L1 at the cell surface, the capsid undergoes structural modifications which allow the cleavage of L2 by furin (Richards et al., 2006), leading to the endocytosis of the virion (Bousarghin et al., 2003; Schelhaas et al., 2012). Then, HPV travels along the endosomal pathway from the plasma membrane to the trans-Golgi network while the capsid is uncoated. The L1 protein dissociates from the viral genome in this process, while L2

remains associated with the DNA to guide HPV trafficking and mediate viral egressing from endosomes (Bienkowska-Haba et al., 2012; DiGiuseppe et al., 2014; Kämper et al., 2006). When the nuclear envelope is disrupted during mitosis, HPV-containing transport vesicles are released from the trans-Golgi network and traffic along microtubules to deliver HPV to the nucleus (Aydin et al., 2014; DiGiuseppe et al., 2016; Pyeon et al., 2009). Once HPV is established in the nucleus, genome amplification and gene expression begin.

Replication

Three phases of viral replication in the viral life cycle have been identified: initial amplification, maintenance replication and vegetative amplification. Importantly, HPV recruits the DNA damage response, exploiting it to facilitate productive replication (Gautam and Moody, 2016). Upon entry in the nucleus, L2-viral DNA complexes accumulate at nuclear domain 10 (ND10) bodies, where the initial amplification of DNA occurs (Day et al., 2004). Cellular factors promote the transcription of E1 and E2, the proteins necessary for replication of the viral genome (McKinney et al., 2016, p. 4). Given the low sequence specificity of E1 for the viral origin of replication, E2 has been shown to recruit the E1 helicase (Sanders and Stenlund, 1998). The E1 protein then forms two enzymatically active hexamers, each surrounding one DNA strand, ready for DNA unwinding (Schuck and Stenlund, 2005). The remaining proteins involved in viral replication belong to the host (McBride, 2008). After the first stage of replication, a low copy number of around 100 copies per cell is reached.

Afterwards, during maintenance replication, the low copy number is maintained as the genome is partitioned to daughter cells in proliferating cells. This is accomplished through the establishment of stable episomes at specific regions of the nucleus. The E2 protein is responsible for tethering the viral genome to host chromatin, thus guarantying its successful partition upon cell division (McBride, 2008). In the differentiated cells of infected epithelium, the HPV genome undergoes a second amplification to a high copy number (Reinson et al., 2015), a crucial step before virion assembly and release from the epithelium surface to maximize the number of virions produced.

Virion assembly and release

The process of virion assembly comprises the formation of the capsid and genome packaging in the nucleus. Day *et al*. (Day et al., 1998) proposed that L2 is the first to localize to specific regions within the nucleus at the assembly stage, followed by the recruitment of L1 as well as E2-genome complexes. The concentration of virion components within these regions confers an

appropriate environment for virion assembly. In these differentiated cells, high levels of E4 are found as well, since it plays an important role at this stage, facilitating virus release through disruption of the keratin cytoskeleton (Doorbar et al., 1991). Virion assembly and release from the epithelial surface upon desquamation is represented in Figure 3.

1.3.4 Carcinogenic progression

Carcinogenesis associated with HPV infection is a process that takes several years and involves four major steps: infection of the cervical epithelium, viral persistence, progression of persistent infection to neoplasia and progression to invasive cancer. Most cases of cervical cancer correspond to squamous cell carcinomas (SCC), although adenocarcinomas are also observed. Recently, new evidence has shown that cuboidal cells at the squamo-columnar junction (SCJ) may be implicated in lesion progression, following HPV infection (Herfs et al., 2012; Mirkovic et al., 2015). The precursor non-invasive lesions which may evolve to cancer are classified according to the three-tier cervical intra-epithelial neoplasia (CIN) or two-tier squamous intra-epithelial lesion (SIL) systems (Baldwin et al., 2003). Low-grade squamous intra-epithelial lesions (LSILs) correspond to CIN1, which describe productive HPV infections with a relatively low risk of malignant progression. In high-grade squamous intra-epithelial lesions (HSILs), corresponding to CIN2/3, a greater risk of progression to carcinoma is verified. HSILs are observed in persistent HPV infections in which viral gene expression is deregulated and no virions are released at the surface of the epithelium (Baldwin et al., 2003). The life cycle of HPV and the progression to invasive cancer are depicted in Figure 3.

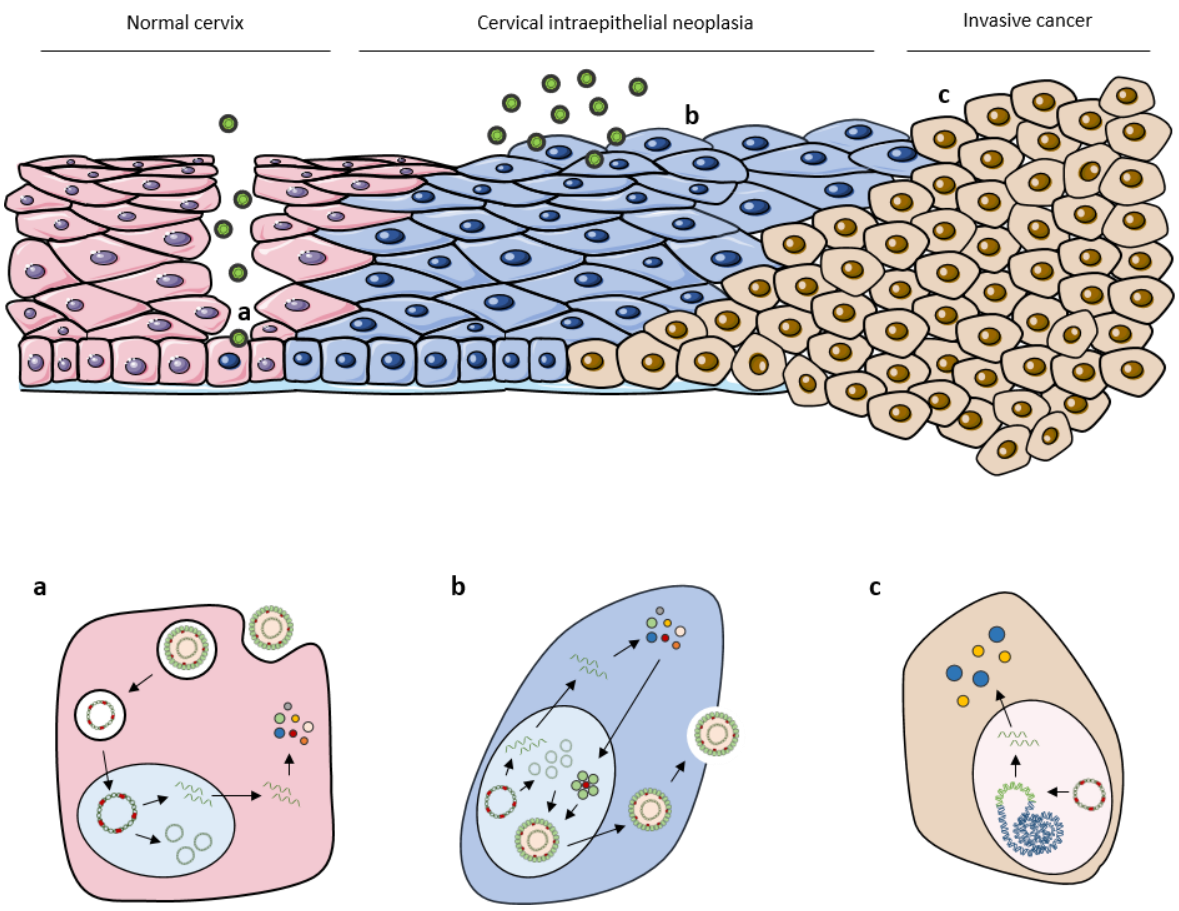

Figure 3. HPV life cycle and progression to cancer. HPV infects basal cells of the epithelium (a) that differentiate towards the surface of the tissue. At the surface, virions are released upon desquamation (b). Viral persistence in basal cells may lead to integration of the genome, which favours carcinogenesis (c).

During a productive life cycle, both low- and high-risk HPV types accompany the normal growth of differentiating keratinocytes, yielding virions at the epithelial surface. Carcinogenesis never occurs in productive infections, since the infected cells are progressively eliminated from the epithelium undergoing constant renewal. Conversely, persistence of HPV in a latent state within basal keratinocytes constitutes a risk factor for carcinogenic development (Schiffman et al., 2005; Gravitt, 2012). Persistence of papillomavirus consists in genome maintenance at the basal layer with low expression levels. It has been hypothesized that in some cases the immune-mediated regression may lead to the establishment of latency, which is kept by constant immune surveillance, while the suppression of T-cell immunity may result in the reactivation of latent HPV due to the increase in viral DNA copy number as well as viral activity (Ozsaran et al., 1999; Maglennon et al., 2011, 2014). Hence, latency explains the reappearance of disease at sites of previous infection,

which has been observed in older women (Maglennon et al., 2011). In addition to the persistence of HPV, the risk factor of the HPV type as well as the susceptibility of the host are aspects of utmost importance for carcinogenesis (Moody and Laimins, 2010).

The HPV genome is found integrated in the DNA of the host cell in most cervical cancers (Cullen et al., 1991). The integration process has been suggested to be inadvertent, occurring randomly throughout the genome, although integrants are often found within common fragile sites with genomic instability and propensity to double strand breaks (DSBs) (Dall et al., 2008; Yu et al., 2005). Hu *et al*. (Hu et al., 2015) has proposed that the integration mechanism consists in the fusion between viral and human DNA through microhomology-mediated DNA repair pathways. The HPV genome can be integrated as a single copy (type I integrants) or as multiple consecutive copies (type II). In type I integrants, the E2 gene is consistently disrupted, although variable portions of the genome are deleted. Thus, upon integration the E2 gene is deleted or truncated (Choo et al., 1987; Schwarz et al., 1985). The loss of E2 allows the overexpression of E6 and E7, since no transcriptional repression is verified (Pett and Coleman, 2007). The polyadenylation site is disrupted as well, hence the transcription of the early genes becomes dependent on host polyadenylation signals, leading to the synthesis of virus-host fusion transcripts with increased stability (Jeon and Lambert, 1995). Type II integrants, less frequent, correspond to concatemers of full-length HPV arranged head-to-tail. Nevertheless, epigenetic silencing of the full-length copies by DNA methylation prevents the transcription of E2 as well, while the viral expression of E6 and E7 in regions lacking the E2 gene is allowed (Van Tine et al., 2004). Hence, integration contributes to deregulation of the viral oncogenes E6 and E7 in both cases. After integration, the expression of E5 ceases in most cases as well (zur Hausen, 2002).

1.3.5 Oncogenic activity of the HPV proteins

E6 contains two zinc finger domains separated by a short linker region, which allow the interaction with cellular proteins displaying the LXXLL motif (Barbosa et al., 1989). These binding domains allow high-risk E6 to bind a wide array of cellular proteins. The most notable interaction mediated by the zinc finger domains corresponds to the association with the E3 ubiquitin ligase (E6AP), which triggers the polyubiquitination and proteasome-mediated degradation of p53 (Scheffner et al., 1993, 1990). Upon cellular damage in normal cells, expression of p53 is increased and it is activated via post-translational modifications. Depending on the damage, p53 might trigger the activation of DNA repair pathways, cell cycle arrest and/or apoptosis (Banin et al., 1998). Thus, the E6-mediated degradation of p53 allows the replication of damaged DNA, and the proliferation

of cells with deleterious mutations. The interaction with the core region of p53 is essential for its degradation by E6. Since low-risk HPV do not interact with this region, they do not degrade p53 (Li and Coffino, 1996).

The E7 protein contains two conserved regions (CR1 and CR2) at the N-terminal and a zinc-binding motif at the C-terminal, similar to the ones exhibited by E6 (Barbosa et al., 1989). The CR2 domain contains a conserved LXCXE motif responsible for the interaction of E7 with the retinoblastoma protein (pRB) proteins (Münger et al., 1989). The pRB family of proteins, also known as the pocket proteins, includes pRB, p107 and p130. Their main function is the regulation of G1/S entry and progression by controlling the activity of E2F transcription factors. E7 preferentially associates with pRB at the pRB/E2F repressor complex, causing its degradation and consequently the release of E2F (Boyer et al., 1996; Münger et al., 1989). The repressor complex is crucial for the regulation of the G1 phase, while the dissociated E2F promotes S phase entry by activating the transcription of the necessary genes, and it is involved in the regulation of apoptosis, cellular differentiation and genomic stability as well. Importantly, low- and high-risk HPV E7 proteins display different pRB-binding efficiencies, which accounts for the oncogenicity of high-risk HPV types (Gage et al., 1990).

The E5 protein has been proposed to play a supportive role in carcinogenesis, since it enhances the transforming activity of E6 and E7, inducing cell proliferation and immortalization (Bouvard et al., 1994; Stöppler et al., 1996). E5 has been implicated in the regulation of the actin cytoskeleton, as well as in migration and invasion of human cervical cancer cells (Liao et al., 2013b). In addition, it has been demonstrated that E5 upregulates IFN-β through the induction of IRF1 expression (Muto et al., 2011). This leads to the establishment of an antiviral state, which in turn may contribute to the loss of viral episomes in infected cervical keratinocytes, driving the progression to invasive cancer. One of the best characterized roles of E5 corresponds to the modulation of the epidermal growth factor receptor (EGFR), resulting in a multitude of downstream effects that culminate in abnormal cell proliferation. Early studies identified the ability of HPV16 E5 to enhance EGFR signalling, and the resultant transforming and mitogenic activity (Leechanachai et al., 1992; Pim et al., 1992; Straight et al., 1993).

The HPV oncoproteins have been shown to abrogate many other cellular processes, ultimately causing genomic instability, which in turn drives malignant progression and explains the long latency periods verified before the emergence of cancer. Aneuploidies, chromosomal rearrangements and mutations in the DNA are found in most HPV-associated carcinomas (zur Hausen, 1999). Although in most cases the genomic instability promoted by HPV leads to nonviable

cells, the accumulation of slight chromosomal changes over the years might originate cells with propensity to carcinogenesis. Nevertheless, it should be noted that malignant progression is an unintended side effect of the HPV life cycle, since activation of the DNA damage response and impairment of cell cycle checkpoints are events required for viral replication.

Host defense mechanisms against HPV

In most infected individuals, the host defense mechanisms are capable of eliminating HPV infection in 6-18 months (Plummer et al., 2007). Hence, only around 10% of the cases lead to the establishment of persistent infections, which pose a risk for cancer development (Schiffman et al., 2007).

Keratinocytes constitutively express low levels of cytokines and chemokines, however, the activation of PRRs or the stimulation with inflammatory cytokines induces their secretion (Barker et al., 1991). Several studies have reported the recognition of the HPV genome by host sensors. A role for AIM2 has been suggested in a study where the AIM2 inflammasome is triggered in HPV16-infected keratinocytes, leading to the production of interleukin-1β (IL-1β) (Reinholz et al., 2013). Furthermore, another study reported that IFI16 inhibits HPV transcription and replication (Cigno et al., 2015). Another work reported an association between the clearance of initial HPV infection in young women and higher expression levels of TLR3, TLR7, TLR8 and TLR9 (Daud et al., 2011). Furthermore, several cytokines, namely IL-1α, IL-4, IL-13, transforming growth factor β (TGF-β), TNF-α, IFN-α and IFN-β, were identified as inhibitors of HPV16 LCR activity, suppressing early gene transcription (Kyo et al., 1994; Lembo et al., 2006). Type I IFNs, TNF-α and TGF-β restrain the growth of keratinocytes, either normal or HPV-containing, although in oncogenic cells the suppression tends to cease (Kowli et al., 2013, p.; Scott et al., 2001). In a recent study, it was shown that TGF-β promotes the transcription of IFN-κ in HPV16-infected cells for the production of ISGs (Woodby et al., 2018). The IFN-inducible antiviral family of proteins apolipoprotein B mRNA-editing catalytic polypeptide 3 (APOBEC3) have been demonstrated to hypermutate the HPV genome (Vartanian et al., 2008). Furthermore, it was reported that the member APOBEC3A reduces HPV infectivity (Warren et al., 2015, p. 3). Another important class of molecules for the arrest of HPV infection are human α-defensins, since they block virion escape from endocytic vesicles (Buck et al., 2006). The α-defensin 5 (HD5) has been reported to be particularly effective, by inhibiting the furin cleavage of L2 (Wiens and Smith, 2015). Furthermore, defensins have been proposed to recruit immune cells, thus contributing to the activation of adaptive immunity (Hubert et al., 2007). Recently, the Myb-related transcription factor, partner of profilin has been shown to have antiviral activity against HPV by repression of the LCR function (Wüstenhagen et al., 2018). These findings confirm that several

immune effectors mount a response to HPV infection, which eventually leads to clearance. However, HPV proteins have strong counteraction strategies to escape and suppress the host immune response, as described in the following chapter.

1.3.6 Immune evasion

HPV has developed numerous strategies to evade and manipulate the immune surveillance, by interfering with the function of host proteins or by inhibiting their expression. The reduced expression of late proteins and the fact that HPV hides within mucosal cells also contribute to the evasion of the immune system. The agents responsible for the deregulation of the immune response are primarily the oncoproteins E6 and E7, although E5 and E2 interfere with some cell components as well. HPV targets viral genome sensors and IRFs and suppresses both the JAK-STAT and the NF-κB pathways at several points, resulting in an impaired immune response. In addition, E5 seems to contribute to immune evasion by modulating the cell-surface expression of cellular proteins.

Deregulation of Pattern-Recognition Receptors by HPV

Initially, a study reported that the expression levels of TLR3, TLR9, IFN-inducible double-stranded RNA-dependent protein kinase (PKR), RIG-I, and MDA5 were not altered in keratinocytes containing HPV16 or 18 episomes (Karim et al., 2011). However, several studies show contradictory results as they demonstrate that most TLRs, including TLR3 and TLR9, as well as RIG-I, MDA5 and the adaptor molecule MyD88 are downregulated in these cells by E6 and E7 (Hasan et al., 2007; Reiser et al., 2011; Hasan et al., 2013; Morale et al., 2018). In addition, a recent study by Chiang *et al*. (Chiang et al., 2018) identified a novel immune escape mechanism of HPV by E6-mediated suppression of RIG-I activity. Several downstream components in their signalling pathway are targeted by HPV as well, as described ahead.

Suppression of the NF-κB signalling pathway

HPV has evolved mechanisms to abrogate the antiviral immune response promoted by NF-κB signalling, as described ahead. E7 associates with the inhibitor of NF-κB kinase (IKK) complex, hence the release of NF-κB is hampered (Spitkovsky et al., 2002). Furthermore, HPV16 E6 reduces the transcriptional activity of p65, which is justified by the interaction of E6 with the CREB binding protein (CBP) and p300: the coactivators CBP/p300 and p300/CBP-associated factor (P/CAF) must associate with p65 to enable the transcription of certain genes, but the association of E6 with CBP/p300 prevents it from binding p65 (Patel et al., 1999). Interestingly, the E6 protein from HPV6

(low-risk) binds the coactivators as well, although a lower interaction level was observed (Patel et al., 1999). E7 was also reported to decrease the DNA binding activity of NF-κB and to cause reduced nuclear translocation and acetylation of the p65 subunit of NFκB (Perea et al., 2000; Richards et al., 2015). Furthermore, E7 was shown to bind to P/CAF, impairing its interaction with p65 (Huang and McCance, 2002), and to target the transcriptional coactivator p300. However, E2-dependent transcription has been demonstrated to require CBP/p300. Thus, E7 interferes with the regulation of E2 transcriptional activity by associating with p300 (Bernat et al., 2003). It has also been reported that HPV16 E6 and E7 proteins cause an overexpression and modulate the subcellular localization of p105 and p100 NF-κB precursors (Havard et al., 2005).

HPV16 has also been demonstrated to cause the overexpression of ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) (Karim et al., 2013). Binding of UCHL1 to TRAF6 leads to the degradation of NEMO, which in turn results in the suppression of p65 phosphorylation, blocking the canonical NF-κB signalling (Karim et al., 2013). UCHL1 also targets IκBα by attenuating its ubiquitination, which prevents the release of NF-κB (Takami et al., 2007).

Suppression of the IRF signalling pathway

Several downstream elements in the IRF signalling cascade have been shown to be targeted by HPV, deregulating the expression of IFNs and ISGs. In the work of Karim *et al*. (Karim et al., 2011) it was shown that multiple ISGs, as well as the adaptor protein TRIF, were downregulated by HPV. HPV16 and -18 E2 proteins reduce the transcription of STING, suggesting for the first time the involvement of the adaptor molecule in the immune response to HPV (Sunthamala et al., 2014). Importantly, E7 has also been reported to bind to STING, inhibiting its function (Lau et al., 2015). On the other hand, IRF1 is targeted by E7, preventing the correct binding to the IFN-β promoter region in a mechanism involving histone deacetylases (HDACs), which leads to reduced IFN-β production (Park et al., 2000). The E6 protein from HPV16 binds to IRF3, hampering its transcriptional activity (Ronco et al., 1998, p. 3). The upregulation of UCHL1 caused by HPV16 has consequences in the IRF signalling as well. One of the roles of this molecule is to by inhibit TNF receptor-associated factor (TRAF3) K63 poly-ubiquitination, thus its overexpression suppresses the IRF pathway (Karim et al., 2013).

Deregulation of the JAK-STAT signalling pathway

The JAK-STAT pathway is activated upon recognition of extracellular IFNs or other cytokines secreted by adjacent cells or the same cell. Given its ability to threaten the survival of the virus, HPV interferes with the JAK-STAT pathway at several points. HPV18 E6 protein interacts with tyrosine

kinase 2 (TYK2), suppressing its transmembrane interferon-α/β receptor (IFNAR) binding activity, thus the JAK-STAT pathway via IFN-α/β is hampered (Li et al., 1999). Furthermore, in keratinocyte cell lines harboring HPV16, -18 and -31 the downregulation of IFN-κ transcription by E6 causes a lower transcriptional level of certain ISGs, including STAT1 (Reiser et al., 2011). The expression of IFN-κ is reduced by E2 as well (Sunthamala et al., 2014). It was also shown that E6 and E7 directly decrease the levels of STAT1 and its ability to bind the interferon-stimulated response element ISRE (Nees et al., 2001). The reduced level of STAT1 has been described to be necessary for differentiation-dependent HPV amplification and episomal maintenance (Hong et al., 2011). In contrast, STAT3 is activated by HPV18 E6. The increased activity of STAT3 leads to cell cycle progression and cell survival, suggesting its importance in the life cycle of HPV18 (Morgan et al., 2018). Likewise, STAT5 is activated by E7, inducing genome amplification in differentiating cells through the exploitation of the DNA damage response (Hong and Laimins, 2013). It has also been reported that the oncoprotein E7 interacts directly with IRF9 in the cytosol, preventing its association with STAT1/2 and consequently blocking the IFN-α-mediated signal transduction (Barnard and McMillan, 1999). Furthermore, E6 and E7 target PKR, an antiviral ISG, by multiple mechanisms (Hebner et al., 2006).

1.3.7 The E5 oncoprotein

The E5 protein is a short and hydrophobic molecule involved in malignant progression, which interacts with cellular proteins to modulate their activity. Although several aspects concerning its contribution for oncogenesis remain to be elucidated, the growing knowledge on E5 suggests that this oncoprotein is important in some HPV types for viral infection and cell transformation. Hence, further studies should be conducted for a better understanding of the E5-mediated cell deregulation.

A differential expression of E5 throughout the different phases of the viral life cycle has been verified. During the early phases, low levels of E5 protein are likely to be observed, since the HPV E5 ORF is the fourth on polycistronic transcripts. On the other hand, E5 is expressed as the second ORF of late transcripts in differentiating keratinocytes, thus, E5 is likely to be expressed primarily in differentiating suprabasal cells and in the late stages of the HPV life cycle (Longworth and Laimins, 2004).

Most residues in E5 are hydrophobic, which justifies its membrane localization and affinity for membrane-bound proteins. HPV16 E5 is located mainly at the endoplasmic reticulum, but its presence in the Golgi apparatus membranes, plasma membrane, nuclear envelope, early

endosomes and lysosomes has also been reported (Conrad et al., 1993; Oetke et al., 2000; Disbrow et al., 2005; Suprynowicz et al., 2008; Hu and Ceresa, 2009; Krawczyk et al., 2010). E5 displays three well-defined hydrophobic regions. It has been reported that the deletion of the first hydrophobic region, at the N-terminal, resulted in abrogation of its presence at the ER, early endosomes and lysosomes, while deletion at the C-terminus caused no significant changes on the location of E5 (Lewis et al., 2008). Regarding the orientation of E5 in the membranes, conflicting results have arisen. In a work reporting the localization of a fraction of HPV16 E5 to the plasma membrane, the N-terminal was exposed to the cytoplasm, whereas the C-terminal was extracellular (Hu and Ceresa, 2009). In another study, however, it was reported that the C-terminal of E5 faced the cytoplasm and the N-terminal was exposed to the lumen of the ER (Krawczyk et al., 2010).

Oncogenic activity of E5

The E5 protein has been proposed to play a supportive role in carcinogenesis, since it enhances the transforming activity of E6 and E7. E5 has been shown to cooperate with both oncoproteins to induce cell proliferation and immortalization (Bouvard et al., 1994; Stöppler et al., 1996). In addition, E5 has been implicated in the regulation of the actin cytoskeleton, as well as in migration and invasion of human cervical cancer cells (Liao et al., 2013b). In the course of time, several new E5 targets have been unveiled, as well as the mechanisms underlying their interactions. Hence, the E5 oncoprotein contributes to evasion of the immune response and cell transformation in multiple ways, as shown in Figure 4.

One of the best characterized roles of E5 corresponds to the modulation of the epidermal growth factor receptor (EGFR), resulting in a multitude of downstream effects that culminate in abnormal cell proliferation. Early studies identified the ability of HPV16 E5 to enhance EGFR signalling, and the resultant transforming and mitogenic activity (Crusius et al., 1998; Leechanachai et al., 1992; Pim et al., 1992; Straight et al., 1993). In fact, EGFR has been reported to be necessary for hyperplasia induced by E5 in transgenic mice (Genther Williams et al., 2005). However, the molecular mechanisms driving the increase in EGFR levels and signalling are not fully established. There are conflicting results regarding the association of HPV16 E5 with EGFR, indicating that other cellular molecules mediate this deregulation (Conrad et al., 1994; Hwang et al., 1995). It has been proposed that EGFR levels are increased at the plasma membrane due to an increased recycling of the receptor from endosomes to the cell surface. E5 is proposed to induce this event by inhibiting the acidification of endosomes, which in turn prevented the natural lysosomal degradation of internalized EGFR and allowed its return to the plasma membrane. It was hypothesized that the cause was the disruption of V-ATPase function by direct association of HPV16 E5 with the 16-kDa

subunit (16K) of V-ATPase (Disbrow et al., 2005; Straight et al., 1995). Also, the C-terminal of E5 has been reported to be essential for endosomal alkalization and overactivation of EGFR (Disbrow et al., 2005; Rodríguez et al., 2000). Nevertheless, other studies showed that binding of E5 to 16K is not sufficient for impairment of V-ATPase function and EGFR overactivation. Hence, another mechanism must be responsible for this phenotype (Adam et al., 2000; Rodríguez et al., 2000). It has been reported that HPV16 E5 suppresses EGFR endocytic trafficking by inhibiting vesicle fusion independently of endosome acidification (Suprynowicz et al., 2010; Thomsen et al., 2000). Thus, EGFR signalling is proposed to be enhanced through this pathway. Suprynowicz *et al*. (Suprynowicz et al., 2010) also suggested that the V-ATPase function was not directly inhibited by E5, since they found that the oncoprotein associated with only a small fraction of endogenous 16K in keratinocytes. Another mechanism of E5-mediated upregulation of EGFR signal transduction corresponds to the inhibition of the interaction between c-Cbl and the receptor (Zhang et al., 2005). Since the role of the ubiquitin ligase c-Cbl is the ubiquinitation and degradation of EGFR, the disruption of their association by E5 leads to the promotion of EGFR signalling. In all cases, EGFR signalling is increased due to the heightened receptor activation in response to EGF, which in turn is caused by the higher level of EGFR at the cell surface. Although the precise steps driving the activation of EGFR and the EGF-dependent proliferation remain unresolved, the evidence described here points to an important role of E5 in expanding HPV16-infected keratinocytes.

E5 displays the ability to stimulate signalling pathways downstream of EGFR as well. The oncoprotein has been shown to activate mitogen activated protein kinase (MAPK) p38 both in the presence and absence of EGF (Crusius et al., 1997; Gu and Matlashewski, 1995). E5 also upregulates the activation of ERK1/2, another MAPK. (Crusius et al., 2000, p. 200) Thus, E5 enhances the cell response through the MAPK pathway. In addition, E5 promotes cell cycle progression by upregulating c-jun and c-fos oncogenes via the NF1 binding element (Chen et al., 1996a, 1996b). It has also been reported that E5 induced the expression of cyclooxygenase-2 (COX-2) and the vascular endothelial growth factor (VEGF) through the EGFR signalling pathway (Kim et al., 2009, 2006). COX-2 has been implicated in carcinogenesis, while VEGF is pivotal for angiogenesis during early cervical carcinogenesis. Furthermore, the downregulation of the tumour suppressor genes p21 and p27 by transcriptional or post-translational mechanisms has been attributed to E5 as well. The downregulation of these cyclin dependent protein kinase inhibitors allows cell cycle progression (Tsao et al., 1996; Pedroza-Saavedra et al., 2010).

The E5 oncoprotein promotes ligand signalling in keratinocytes outside the EGF pathway as well. Venuti *et al*. (Venuti et al., 1998) showed that HPV16 E5 enhances the signalling of the G

protein-coupled endothelin receptor (ET_A), which is activated by endothelin-1, the specific ligand of ET_A. Hence, E5 improves the mitogenic activity of endothelin-1, driving cell growth. Importantly, endothelin-1 has been previously suggested to play a preponderant role in epithelial proliferation (Simeone et al., 2004).

In contrast to the previous E5 functions, the oncoprotein has been shown to down-modulate the keratinocyte growth factor receptor/fibroblast growth factor receptor 2b (KGF-R/FGF-R2b) at both transcript and protein levels. The perturbation of the receptor is suggested to decrease the differentiation of suprabasal keratinocytes, which may be important for virus replication (Belleudi et al., 2011). Recently, Ranieri *et al*. (Ranieri et al., 2015) proposed that E5 induced switching from fibroblast growth factor receptor 2b to the mesenchymal fibroblast growth factor receptor 2c isoform, causing the initiation of the epithelial–mesenchymal transition, which in turn leads to cancer progression (Al Moustafa, 2015).

Other activities of E5 may render the transformed cells more insensitive to extracellular growth control signals. Particularly, the decrease in phosphorylation and expression of connexin 43 by E5 drives the downregulation of cell-cell communication through gap junctions (Oelze et al., 1995; Tomakidi et al., 2000). It has been hypothesized that 16K may be implicated in the impairment of connexin 43, since both are present in gap junctions and 16K is known to be targeted by E5 (DiMaio and Petti, 2013).

Another relevant effect of E5 is the modulation of the membrane lipid composition and dynamics (Bravo et al., 2005). Suprynowicz *et al*. (Suprynowicz et al., 2008) reported the upregulation of caveolin-1 and ganglioside GM1, two components of the lipid raft, at the plasma membrane, caused by E5. This may constitute an immune evasion strategy for HPV, since gangliosides have been shown to strongly inhibit cytotoxic T lymphocytes and block the formation of immune synapses. In addition, the overexpression of GM1 enhanced the cell proliferation in response to EGF (Nishio et al., 2005).

E5 also binds to the zinc transporter ZnT-1, as well as to the EVER1 and EVER2 transmembrane channel-like proteins. Their association abrogates the ability of these proteins to inhibit c-jun and zinc-regulated transcription factors (Lazarczyk et al., 2008). Recently, a new binding partner of E5 has been identified, YIP1 family member 4, which is a poorly characterized membrane protein. However, no cellular modifications resulting from the interaction have been uncovered. (Müller et al., 2015) Furthermore, HPV16/18 E5 has been proposed to be responsible for the reduced levels of the spindle checkpoint proteins Bub1 and Mad2, crucial for cell

proliferation and differentiation. Thus, E5 seems to deregulate cell proliferation via this mechanism as well (Liao et al., 2013a).

HPV16 E5 has also been proposed to drive the transformation of normal cervical cells into carcinoma by downregulating miRNA-196a. A target protein of miRNA-196a is HoxB8, which has been implicated in a few cancers. In this study, it has been shown to be upregulated due to the E5-mediated decrease of miRNA-196a levels (Liu et al., 2015).

In most cancers, the HPV genome is found integrated into the host DNA, in which case the E2 and E5 genes are frequently lost (zur Hausen, 2002). Nevertheless, HPV16 genome has been observed as integrated DNA, episomal DNA, or both in cervical malignant lesions, unlike other HPV types, including HPV18. Chang *et al*. (Chang et al., 2001) reported the expression of E5 in 60% of HPV16-infected cancers. In addition, HPV16 E5 has been detected in precancerous lesions, indicating that it may play a role in malignant progression (Kell et al., 1994; Paolini et al., 2017; Sahab et al., 2012). Also, it has been shown that HPV16 E5 contributes to the promotion and progression of skin carcinogenesis in transgenic mice (Maufort et al., 2007). Finally, as previously mentioned, the upregulation of IFN-β by E5 leads to the establishment of an antiviral state, which could drive the loss of viral episomes (Muto et al., 2011).

E5 and apoptosis

In addition to causing cell proliferation, E5 also leads to the inhibition of apoptosis through a number of mechanisms. To begin with, HPV16 E5 protects cells from apoptosis by enhancing the PI3K-Akt and ERK1/2 MAPK signalling pathways (Zhang et al., 2002). In addition, E5 prevents FasL- or TRAIL-mediated apoptosis in cultures of human keratinocytes (Kabsch et al., 2004; Kabsch and Alonso, 2002). The hydrogen peroxide-induced apoptosis of cervical cancer cells is suppressed by E5 as well, by ubiquitination and proteasome-mediated degradation of Bax protein, in a pathway involving COX-2, prostaglandin E2 and protein kinase A (Oh et al., 2010). Finally, E5 has been shown to colocalize with the Bcl-2 antiapoptotic protein on intracellular membranes (Auvinen et al., 2004, p. 200). Hence, the E5 protein inhibits apoptosis by multiple mechanisms, possibly contributing to the carcinogenesis of cervical epithelial cells.

E5 and chromosomal instability

A group has reported two mechanisms promoted by the oncoprotein to induce morphological and chromosomal changes in the cell. They have shown that E5 mediates cell-cell fusion with the formation of binucleated HaCaT cells, for which they suggested the interaction of E5 proteins in the plasma membranes of adjacent cells (Hu et al., 2009; Hu and Ceresa, 2009). In a

later study, they reported that E5 promotes an increase in nuclear size, as well as polyploidy by endoreplication (Hu et al., 2010). These discoveries are very relevant because such abnormalities are characteristic of precancerous cervical lesions (Gao and Zheng, 2010). Therefore, E5 may have a major role in the early stages of cancer progression.

E5 and immune evasion

The ability of HPV to evade the immune system is crucial for the establishment of a productive infection, and eventually for the persistence of the virus, an essential step in cervical carcinogenesis. E6 and E7 oncoproteins play an important role towards that end, but E5 also seems to contribute to immune evasion by modulating the cell-surface expression of cellular proteins. E5 interacts with the heavy chain of the class I major histocompatibility complex (MHC I), which is denominated HLA I in humans (Ashrafi et al., 2005; G. H. Ashrafi et al., 2006). Their association is mediated by the first hydrophobic domain of E5, and leads to the retention of HLA I in the golgi apparatus, thus the migration of HLA I to the cell surface is hampered (G. Hossein Ashrafi et al., 2006; Cortese et al., 2010). The retention of HLA I in the golgi apparatus has been proposed to result from another mechanism as well, the golgi alkalinization by E5, which may in turn be caused by the interaction of E5 with the 16K subunit.

E5 has also been reported to associate with Bap31, a chaperone that may be involved in the quality control of MHC I molecules, and A4, a transmembrane ion channel protein of the endoplasmic reticulum (Kotnik Halavaty et al., 2014; Regan and Laimins, 2008). The effect of the interaction of E5 with both proteins is not clearly understood, however, it is associated with increased HPV genome amplification and enhancement of cell proliferation upon differentiation. Also, it has been hypothesized that the interaction of E5 with Bap31 may be involved in the retention of HLA I, if E5 dissociates Bap31 from HLA I (Schapiro et al., 2000). Another target of E5 is calnexin, a molecular chaperone involved in HLA I maturation and surface transport. Their association is essential for retention of HLA I molecules in the endoplasmic reticulum (Gruener et al., 2007). The downregulation of cell-surface HLA I by E5 might seriously affect the adaptive immune response, given that the presentation of antigenic viral peptides to effector T cells is compromised. The expression of surface HLA class II is downregulated by E5 as well, since the oncoprotein can prevent the endosomal breakdown of the invariant chain, a chaperone which must be degraded to allow the proper maturation of HLA class II (Zhang et al., 2003).

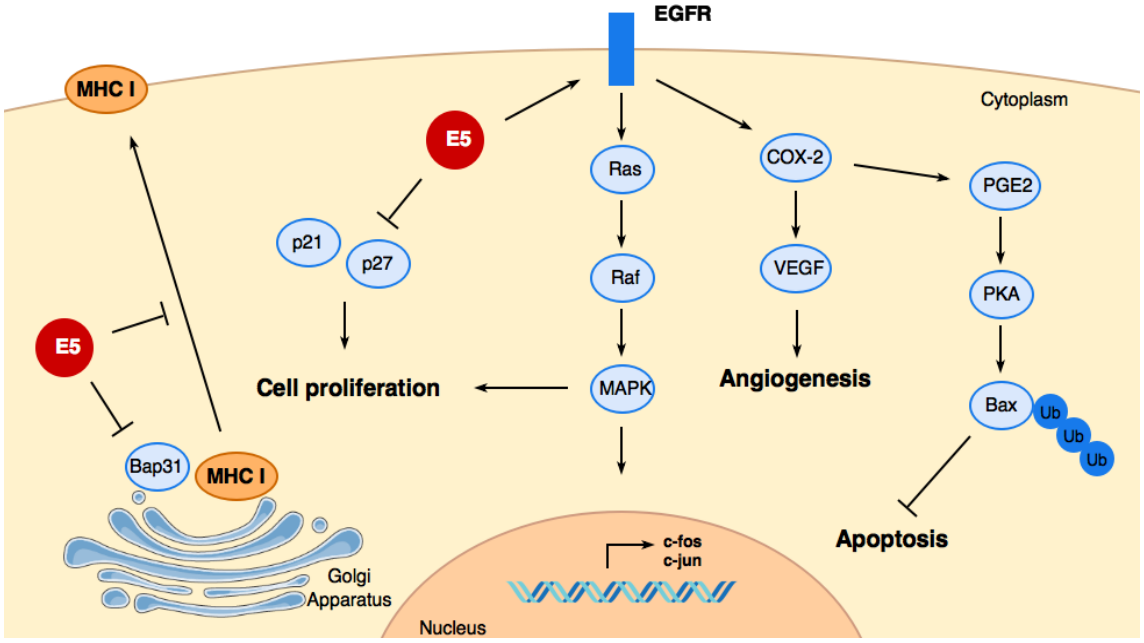

Figure 4. Cellular proteins targeted by the E5 oncoprotein. E5 enhances EGFR signalling, leading to cell proliferation and angiogenesis, while suppressing apoptosis. Furthermore, E5 downregulates the tumour suppressor genes p21 and p27, and it suppresses the migration of the major histocompatibility complex to the cell surface.

E5 and the RIG-I-MAVS pathway

As mentioned before, the RLRs initiate an antiviral innate immune response upon RNA virus sensing. In addition, dsRNA transcribed by polymerase III from DNA viruses can also trigger RIG-I (Chiu et al., 2009). Hence, there is the possibility that this RNA sensor activates antiviral immunity in response to HPV. A recent study by Chiang *et al.* (Chiang et al., 2018) identified a novel immune escape mechanism of HPV causing the abrogation of the RIG-I-MAVS signalling pathway. They reported the formation of a complex constituted by E6, TRIM25 and USP15. USP15 corresponds to an upstream regulator of TRIM25, responsible for its stabilization. By targeting these two enzymes, the E6 oncoprotein antagonizes the activation of RIG-I. Finally, they suggest that RIG-I is important for detecting HPV16 infection, since they showed that the innate immune response mounted is partially mediated by the RIG-I-MAVS pathway (Chiang et al., 2018). This was the second study to show the interaction of HPV oncoproteins with host molecules of the RIG-I-MAVS pathway. In a previous work, it was suggested that the E5 oncoprotein from HPV16, HPV18, as well as the low-risk HPV6 and HPV11 interacts with MAVS (Rozenblatt-Rosen et al., 2012). This was concluded based on an experiment consisting of tandem affinity purification followed by mass spectrometry (TAP-MS). Thus, it would be interesting to ascertain their association in cell culture and whether E5

targets the mitochondrial or the peroxisomal MAVS, as well as to investigate the effects of this interaction.

II. Aims

In the scope of this dissertation, two main goals were determined:

1. Further unravel the mechanism of Human cytomegalovirus vMIA's inhibition of the peroxisome-dependent antiviral signalling

The inhibition of the peroxisomal MAVS antiviral signalling by the viral mitochondria-localized inhibitor of apoptosis (vMIA) protein of Human cytomegalovirus (HCMV) has been extensively studied in our group. Recently, there has been an effort towards discovering the precise molecular mechanisms of this inhibition, as well as the specific domains of vMIA involved. In the scope of this work, we proposed to create a deletion mutant of vMIA (Δ35-109) to complement the analyses performed with previously studied deletions mutants, which have shown no differences compared to the wild-type protein, concerning the inhibition of the peroxisomal antiviral innate immunity.

2. Human papillomavirus and the antiviral innate immunity

In this dissertation we proposed to perform a thorough examination of the literature on the possible roles of the Human papillomavirus (HPV) on the innate immune signalling and the progression of HPV infection to cancer. Given the major research focus of the group on antiviral innate immune signalling via the peroxisomes, our aim was also to specifically gather the current knowledge on the role of HPV proteins on immune evasion, in order to find a starting point for our practical research. Consequently, we discovered that E5, a small oncoprotein encoded by HPV, was suggested to interact with MAVS, a protein involved in innate immunity found at the mitochondrial and peroxisomal membranes Thus, the main goal of our practical work became the evaluation of the localization of E5 to the mitochondria and the peroxisomes and further analysis of its role on the antiviral immunity mediated by MAVS.

III. Materials and Methods

3.1. Methods

The list of chemicals and reagents can be found in Appendix A, while the solutions, buffers and gels are in Appendix B.

3.1.1 Cloning

Flag-E5 (HPV16)

Amplification of E5

A construct containing E5 with a HA-tag was kindly provided by Dr. Saveria Campo (University of Glasgow, UK). In order to obtain a FLAG-tagged construct, E5 was cloned into pCMV-Tag2B, a mammalian expression vector with an N-terminal FLAG epitope. Therefore, a polymerase chain reaction (PCR) was performed in the My Cycler Thermal Cycler (Bio-Rad) to amplify E5 using the following primers (Eurofins) and under the conditions exhibited in Table 1 and Figure 5:

Forward primer: 5'- CCGCTCGAGTTATGTAATTAAAAAGCGTGCAT – 3'

Reverse primer: 5'- CGCGGATCCATGACAAATCTTGATACAGC – 3'

The primers were designed with restriction sites for BamHI and XhoI at the 3' region to allow the insertion in pCMV-Tag2B. The amplification was performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific).

Table 1. Components of the PCR for the amplification of E5.

Components	V (µL)
H_2O (Milli-Q)	35
5x Phusion HF Buffer	10
dNTPs	1
Fw primer	1,5
Rv primer	1,5
DNA	0,5
Phusion HF	0,5
Total	50 µL

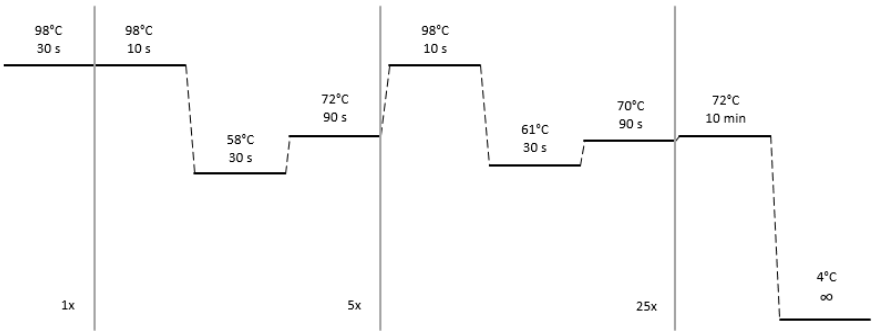

Figure 5. PCR conditions for the amplification of E5.

Gel Electrophoresis and DNA isolation

After the PCR, the solution was submitted to electrophoresis for separation of the DNA products in a 1% agarose gel, prepared in 1x TAE with Midori Green DNA stain. Previously to loading, the PCR product was mixed with a 6x Orange DNA Loading Dye (Thermo Scientific) to allow DNA visualization after migration and the GeneRuler DNA Ladder Mix (Thermo Scientific) was used as a marker. The migration was performed at 100 V for 45 minutes with 1x TAE buffer. The Gel Doc equipment (Bio-Rad) was used to obtain pictures of the gels, which were analysed in the Image Lab Software (Bio-Rad).

The band with the expected size was excised from the gel under UV light and purified with the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel). The purified DNA sample was quantified using the DS-11 FX series spectrophotometer (DeNovix). This equipment measures the absorbance at 230, 260 and 280 nm, providing information on the concentration of nucleic acids based on the absorbance at 260 nm. The A260/A230 and A260/A280 ratios are provided as a measure of nucleic acid purity. The A260/A230 ratio should be between 2.0 and 2.2, while the expected A260/A280 ratio for DNA is around 1.8 and 2.0 for RNA. Lower values indicate contamination of the sample.

DNA digestion and dephosphorylation

Both the E5 PCR product and the plasmid pCMV-Tag2B were digested with BamHI and XhoI (NEB) for 6h at 37°C, using the 10x NEBuffer 3.1 (NEB). Further details of the reaction are exhibited below (Table 2).

The plasmid was subjected to a dephosphorylation with an Alkaline Phosphatase, Calf Intestinal (CIP) from NEB for 40 minutes at 37°C, immediately after the end of the digestion, to prevent its re-circularization. For the dephosphorylation, 0,5 µL of the enzyme were added to the

restriction digest mix, since it is active in NEBuffer 3.1. After this step, both restriction mixes were submitted to gel electrophoresis and the desired bands isolated with the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel) and quantified with the DeNovix spectrophotometer, as described above.

Table 2. Components of the restriction digestion.

Components	E5	Plasmid
H_2O (Milli-Q)	41,85	41,96
10x NEBuffer 3.1	5	5
BamHI	1	1
XhoI	1	1
DNA (2 µg)	1,15	1,04
Total	50 µL	

DNA ligation

The digested constructs were ligated overnight at 16°C using T4 DNA Ligase (NEB), as described below. 1:3 and 1:6 molar ratios of vector to insert were prepared, using 60 ng of the plasmid in both cases. The following formula (Equation 1) was used to calculate the amount of E5 required (for the 1:3 ratio).

Equation 1. Formula for the mass of E5 required.

$$mass\ insert\ (ng) = \frac{mass\ vector\ (ng) \times length\ insert\ (kb)}{length\ vector\ (kb)} \times 3$$

The ligation mix was directly used in the transformation of competent bacteria.

Table 3. Components for the ligation.

Components	1:3	1:6
H_2O (Milli-Q)	12,1	10,2
10x T4 Ligase buffer	2	2
T4 DNA Ligase	1	1
Plasmid	2,99	2,99
Insert (E5)	1,90	3,81
Total	20 µL	

Bacterial transformation

For the transformation of competent *Escherichia coli* XL-1 Blue, 5 to 15 µL of ligated DNA were added to 30 µL of a bacterial suspension, gently mixed and incubated on ice for 30 minutes. After the incubation, the bacteria were submitted to a heat shock, which corresponded to 90 seconds at 42°C. Then, the bacteria were briefly chilled on ice before the addition of 750 µL of LB medium (Fisher Scientific). The bacteria were grown for 1 hour at 37°C, shaking at 750 rpm. The suspension was centrifuged for 3 minutes at 3000 rpm and the supernatant was discarded. The pellet was resuspended in the small volume of supernatant remaining in the tube. The suspension was added to LB agar plates (LB medium with agar (Formedium)) supplemented with kanamycin and spread with the aid of glass beads. The plates were incubated overnight at 37°C. The protocol was conducted under a sterile environment and the appropriate controls were performed.

Colony selection and plasmid isolation

Incubation of the LB agar plates allowed the growth of individual colonies of bacteria carrying the plasmid. Several colonies were randomly picked, inoculated in ~3 mL of LB medium + kanamycin and grown overnight shaking at 180 rpm at 37°C. The liquid culture provided the necessary density of bacteria for plasmid isolation, which was achieved with a miniprep protocol. All steps were performed on ice. The first step was to centrifuge 1-1,5 mL of the culture at 12.000 g for 2 minutes. The pellet, constituted by bacterial cells, was allowed to dry for 10 minutes at room temperature before being resuspended in 100 µL of cold solution 1 and vigorously vortexed. 200 µL of solution 2 were added to the solution, and the tube was inverted 5 times to mix the solution. The next step was the addition of 150 µL of cold solution 3. The tube was then inverted 5 times, kept on ice for 3-5 minutes, and centrifuged at 17.000 g for 10 minutes at 4°C. The supernatant was transferred to a new tube, where 2 volumes of 100% ethanol were subsequently added to precipitate the DNA. The mixture was vortexed and incubated for 2 minutes at room temperature. Another centrifugation followed, at 17.000 g for 5 minutes at 4°C. The supernatant was discarded, and 1 mL of 70% ethanol was added to the tube to wash the pellet. The solution was vortexed for a few seconds, leaving the pellet intact. A new centrifugation was performed at 17.000 g for 5 minutes at 4°C and the supernatant was completely removed. The plasmid DNA was resuspended in 30 µL of water containing 20 µg/mL RNase.

To confirm the success of the cloning procedure, the samples were digested with the same restriction endonucleases as before (BamHI and XhoI). After visualization on gel, one of the test colonies was selected, since its digestion yielded two fragments with the expected sizes. It was

purified with the NucleoSpin Plasmid kit (Macherey-Nagel) following the protocol recommended by the manufacturer and quantified in the DeNovix spectrophotometer.

Sequencing

Finally, to assess the occurrence of mutations, the plasmid DNA was sent for sequencing at Stab Vida according to their specifications. The sequences were analysed with the FinchTV and Serial Cloner Softwares.

After confirmation of the sequence, the plasmid was obtained in high quantity with the NucleoBond® Xtra Midi kit (Macherey-Nagel) for transfection experiments. 3 mL of the culture were grown for 6 hours and transferred to an Erlenmeyer containing 200 mL of LB medium + kanamycin, which was in turn incubated overnight for the attainment of a high number of cells for plasmid purification with the kit.

vMIAΔ35-109-myc (HCMV)

Amplification of pcDNA3-vMIA

An alternative cloning methodology was followed for the development of vMIAΔ35-109, which used the pcDNA3-vMIA-myc as template. The amplification step was performed with the following primers (Eurofins), complementary to the vMIA sequence:

Forward primer: 5'- CGGACGCCCGAGGTGAAC – 3'

Reverse primer: 5'- GAGAGGATCTTCAAGGCGTTTTCG – 3'

Using these primers, it was possible to amplify the whole vector, excluding the Δ35-109 region. The PCR mix and reaction are displayed below. The amplification was performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) in the the My Cycler Thermal Cycler (Bio-Rad).

Table 4. PCR components for the amplification of pcDNA3-vMIA.

Components	V (µL)
H_2O (Milli-Q)	33,1
5x Phusion HF buffer	10
dNTPs	1
Fw primer	2,5
Rv primer	2,5
DNA (10 ng)	0,427
Phusion HF	0,5
Total	50 µL

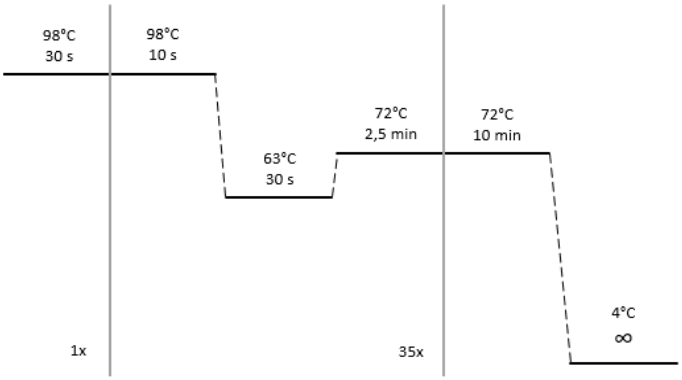

Figure 6. PCR conditions for the amplification of pcDNA3-vMIA.

The PCR yielded a linear fragment of DNA with blunt ends, which was isolated by gel electrophoresis and purified with the NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel).

Before ligation, it was necessary to phosphorylate the PCR product to allow the ligation of the blunt ends on the amplified DNA. For this, the necessary components (Table 5) were mixed and incubated for 20min at 37°C before being inactivated at 75°C for 10min. PEG 4000 was added to the reaction because it increases the ligation efficiency of blunt-end DNA ligation.

Table 5. Components of the phosphorylation reaction.

Components	DNA (20 ng)	DNA (40 ng)
H_2O (Milli-Q)	33.26	32,52
10x T4 Ligase Buffer	5	5
50% PEG 4000	5	5
ATP (10mM)	5	5
T4 PNK	1	1
DNA	0.739	1.478
Total	50 µL	

Ligation of vMIA Δ35-109

For the ligation, 1µL was added to the previous mix (Table 5), and it was incubated at 16°C overnight.

Bacterial transformation with vMIA Δ35-109

The transformation protocol previously described was followed, adjusting the volume of ligated DNA to 15 and 35 µL.

After isolation of the plasmid from a few colonies with the miniprep protocol, the presence of the vMIA Δ35-109 sequence was assessed by visualization on gel after a restriction digestion with BamHI and XbaI. A colony was selected based on the size of the fragments obtained for purification with the NucleoSpin Plasmid kit (Macherey-Nagel). The plasmid was quantified with the DeNovix equipment and sent for sequencing at Stab Vida.

3.1.2 Cell Culture

Cell maintenance

Human embryonic kidney cells expressing the SV40 large T antigen (HEK293T), Mouse Embryonic Fibroblasts with MAVS solely at peroxisomes (Mefs MAVS-Pex) were cultured in 100 mm × 20 mm culture dishes with complete DMEM: Dulbecco's Modified Eagle Medium, High Glucose w/ L-Glutamine w/o Sodium Pyruvate (Gibco) supplemented with 10% Fetal Bovine Serum (FBS), qualified, E.U.-approved, South America origin (Gibco) and 1% (100U/mL) penicillin and streptomycin (Gibco). They were kept in a humidified atmosphere at 37°C containing 5% CO_2. Cells

were split twice a week, when they reached confluency. For that purpose, cells were washed with Dulbecco's Phosphate Buffered Saline (PBS) w/o Calcium w/o Magnesium (Gibco) and incubated for 1-2 minutes with 2 mL of Trypsin-EDTA (Gibco) at 37°C and 5% CO_2 until they detached from the plate surface. After harvesting the cells with 8 mL of complete DMEM, they were centrifuged at 1000 g for 3 minutes at room temperature and then resuspended in 10 mL of medium and seeded in a 1:20 dilution (~105 cells/mL).

Cell storage, freezing and thawing

For the preparation of cell stocks, confluent cells were resuspended in freezing medium (DMEM supplemented with 10% FBS and 10% DMSO) in cryovials. Before cryopreservation in liquid nitrogen, cells were frozen at -80°C.

Thawing of cell stocks was performed through resuspension with pre-warmed complete DMEM. Cells were seeded in a 100 mm culture dishes and after a few hours (~5h), when cells were adhered, the medium was replaced with fresh culture medium to remove cell debris and DMSO.

3.1.3 Transient Mammalian Transfection Methods

Lipofectamine 3000

The Lipofectamine™ 3000 Transfection Reagent (Invitrogen) was used for the transfection of Mefs MAVS-PEX cells with pcDNA3-vMIAΔ35-109-myc, pCMV-2B-FLAG-E5 and GFP-RIG-I-CARD. The protocol was followed according to the manufacturer's instructions. For 6-well plates, the quantity of DNA used was 3.5 µg. The P3000 reagent was slowly added to 90 µL of Opti-MEM (Opti-MEM Reduced-Serum Medium (Gibco)) containing the DNA in a 1:1 ratio. The mixture was added to another tube containing lipofectamine 3000 (1:1 ratio) in 90 µL of Opti-MEM and incubated for 5 minutes at room temperature. The medium was changed before the dropwise addition of the mixture to the cells. The dishes were incubated at 37°C with 5% CO_2.

Polyethylenimine (PEI)

The PEI Reagent (Sigma-Aldrich) was used for the transfection of HEK293T cells with pcDNA3-vMIA-myc, pcDNA3-vMIAΔ35-109-myc, pcDNA3-vMIAΔ131-147-myc, pcDNA3-vMIAΔ115-130-myc, pcDNA3-vMIAΔ23-34-myc and pcDNA3-vMIAΔ2-23-myc. For 10 ø cm plates, 8 µg of DNA were diluted in a tube containing 500 µL of serum-free DMEM without antibiotics, where 64 µL of PEI (1 µg/µL) were added. The mixture was vortexed for 10 seconds and incubated for 15 minutes

at room temperature. The medium was changed to serum-free DMEM and the mixture was added dropwise to the cells.

3.1.4 Viral infection

HEK293T were infected with Vesicular stomatitis virus (VSV) with a MOI of 3, diluted in serum and antibiotic free media. After removing the growth media, the viral dilution was added to the plates. Cells were incubated for 5 minutes at room temperature under agitation, and then for 1 hr in a humidified atmosphere at 37°C containing 5% CO_2. The same virus dilution volume of growth media containing 20% of FBS was then added to the cells. Cells were collected 8hrs post-infection.

3.1.5 Immunofluorescence

Cells grown in glass coverslips were fixed with 4% PFA for 20 minutes, permeabilized with the 0,2% Triton X-100 solution for 10 minutes and blocked for 10 minutes with 1% BSA in PBS. Then followed the incubation with the primary and secondary antibodies, for 1h each, and with Hoechst dye for 2 minutes. The plates were covered in wet paper to prevent the coverslips from drying and the latter two were incubated in the dark. Between each step the coverslips were washed 3 times with PBS, and the protocol was performed at room temperature. The coverslips were mounted in glass slides with mounting medium after being washed with dH_2O.

After allowing the coverslips to dry for 24 hours, they were observed in the AxioImager Z1 Zeiss Microscope using the AxioVision Software while the confocal images were obtained in the Zeiss confocal LSM 510M microscope with the Zeiss Black Edition Software. The 488 nm Argon-ion laser, 561 nm DPSS laser were used.

3.1.6 Protein extraction and quantification

Lysis protocol for MAVS oligomerization studies

Using 1 mL of cold PBS, cells were scraped from the plates and collected in tubes. They were centrifuged at 16200x g and 4°C for 1 min and the supernatant was discarded. The pellet was resuspended in 150 µL of lysis buffer (for MAVS oligomerization assays). To promote disruption of the cell membranes, samples were sonicated 3 times each with 15 pulses of 0.8s at 40%, while immersed in ice to prevent heating. A centrifugation at 800x g for 10 min at 4°C allowed the sedimentation of cellular debris. The supernatant was transferred to a new tube, where 150 µL of

lysis buffer supplemented with 0.1% SDS was immediately added. Before proceeding to quantification, the samples were incubated for 15 minutes. The samples were kept on ice throughout the whole procedure.

Bradford quantification method

For the Bradford protein assay, 3 µL of sample were mixed with 97 µL of 0.1 M NaOH in tubes and 1 mL of Bradford solution was subsequently added. The samples were incubated for 15 minutes at room temperature in the dark before optical density measurement at a wavelength of 595 nm. Duplicates were prepared in all experiments. At the same time, a set of standards ranging from 0 to 21 µg were prepared with bovine serum albumin (BSA) from a stock solution at 1 µg/µL. Each standard tube was filled to a final volume of 100 µL with the solution of 0.1 M NaOH before the addition of Bradford reagent. The absorbance measured for the standards was used for drawing a standard curve to calculate the sample concentrations.

3.1.7 Gel electrophoresis

After protein quantification, the samples were prepared for gel electrophoresis, using 50 µg of protein.

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were diluted in 6x laemmli (350 mM Tris pH 6,80, 10 mM SDS, 60 mM DTT, 0.06% bromophenol blue) with or without DTT for reducing or non-reducing SDS-PAGE, respectively, heated at 95°C for 5 minutes for protein denaturation and loaded on the gel with GRS Protein Marker MultiColour Tris-Glycine 4-20% (Grisp), a ready-to-use pre-stained protein marker. The resolving gel contained 10% polyacrylamide while the stacking gel had a 3% concentration. Migration was performed at 120 V using running buffer for SDS-PAGE.

Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE)

Before loading the gel, samples were diluted in 4x loading dye (20% glycerol, 8% SDS and bromophenol blue in 2x TAE buffer) and incubated at room temperature for 5 minutes. The migration was performed at 100 V for 40 min at 4°C using a running buffer composed of 1x TAE + 0,1% SDS.

3.1.8 Immunoblotting

After protein migration, gels were transferred onto nitrocellulose or PVDF membranes on a wet transfer system Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad). The transfer was performed at 400 mA for 2h at 4°C using transfer buffer without SDS or at 90V, overnight at room temperature. At the end, the membranes were carefully removed from the apparatus and washed with TBS-T in 3 rounds of 5 minutes. They were blocked with milk (5% in TBS-T) for 1h at room temperature.

Incubation with primary antibodies was performed at 4°C, overnight or at room temperature for 1h30 or 3h under agitation. Secondary antibodies were incubated at room temperature for 1 hour. 3 washing steps of 5 minutes were performed after each incubation. Both chemiluminescent and fluorescent detection methods were used.

Staining of the membrane with fluorescent tagged antibodies allows protein observation in the Odyssey system with the LI-COR software (Biosciences, US). For chemiluminescence detection, HRP coupled secondary antibodies were used for observation in the ChemiDoc equipment (BioRad). Clarity western ECL substrate (BioRad) was used to activate HRP and produce luminescent signal. For acquisition and image processing Image Studio Lite (LI-COR) or Image Lab Software (BioRad) were used. Protein quantification was performed in Image Lab Software (Bio-Rad), where total protein obtained from Ponceau S was used to normalize samples.

3.1.9 Reverse transcriptase - quantitative Polymerase Chain Reaction (RT-qPCR)

RNA extraction

Cells in 6-well plates were washed with PBS, lysed at room temperature with 500 µL of NZYol (nzytech), and then harvested by pipetting up and down. At this point, the samples could be stored at -80°C. Frozen samples should be thawed and incubated for 5 minutes at room temperature before use. After the addition of 100 µL of chloroform, the samples were shaken vigorously for 15 seconds, incubated for 5 minutes at room temperature, and centrifuged for 15 minutes at 12000 g. The upper aqueous phase containing RNA was extracted and incubated on ice with 250 µL of isopropanol for 10 minutes. Another centrifugation for 15 minutes at 12000 g was performed, after which the supernatant was discarded. The pellet was washed twice with 500 µL of 75% ethanol and the sample was centrifuged at maximum speed for 5 minutes at 4°C after each washing step. The ethanol was removed, and the pellet dried for 10 minutes at room temperature.

20 µL of RNAse free water was used to resuspend the pellet at 55°C. The RNA concentration was measured with the Nanodrop (DeNovix) equipment.

cDNA synthesis

Complementary DNA (cDNA) was obtained by reverse transcription of the RNA. 1µg of RNA extracted from the cells was mixed with a master mix of 280 pmol oligo-dT primers, 166 µM dNTPs, 1x M-MuLV Reverse transcriptase buffer (NEB), 100 U M-MuLV Reverse transcriptase (NEB), 20 U RNAse inhibitor and RNAse free water. The mixture was incubated for 10 minutes at room temperature before cDNA was synthesized by reverse transcription for 90 minutes at 42°C. The enzyme was inactivated for 20 minutes at 65°C. At this point cDNA could be stored at -30°C.

Real-time Quantitative Polymerase Chain Reaction

For the quantification of mouse IRF1, the primers used were Fw 5' GGTCAGGACTTGGATATGGAA 3' and Rv 5' AGTGGTGCTATCTGGTATAATGT, while for mouse GAPDH the primers were Fw 5' GCC TTC CGT GTT CCT ACC 3' and Rv 5' CCT GCT TCA CCA CCT TCT T 3'. The RT-qPCR mix was prepared by mixing 2 µL of cDNA from a 1:10 dilution, 10 µL of 2× iTaq SYBR Green Master Mix (BioRad) and the primers were added to a final concentration of 250 nM, to a final volume of 20 µL. Fluorescence was measured using the 7500 Real time PCR system (Applied Biosystems). The PCR was initiated with 95°C for 3 min, followed by 40 cycles of denaturation steps at 95°C for 12 seconds and annealing/elongation steps at 60°C for 30 seconds. The 2−ΔΔCT method was used for data analysis.

IV. Results and Discussion

4.1 Human Cytomegalovirus' vMIA: unravelling the mechanism of inhibition of the peroxisome-dependent antiviral response

vMIA has been shown to inhibit the peroxisome and mitochondria-dependent antiviral response(Castanier et al., 2010; Magalhães et al., 2016). However, the actual mechanism or the specific domains of the protein involved remain unknown. A previous study has been performed in our group where several vMIA mutants lacking distinct segments of the protein were analysed in order to understand which domain of vMIA is responsible for the inhibition of the peroxisomal MAVS-mediated immune response (Figure 7). The production of the ISG IRF1 mRNA was quantified by RT-qPCR upon overexpression of the mutants Δ2-23, Δ23-34, Δ115-130 and Δ131-147 in mouse embryonic fibroblasts containing MAVS solely at the peroxisomal membrane (Mefs MAVS-PEX cells)(Magalhães et al., 2016). The cellular immune response was activated by transfection with RIG-I-CARD, a constitutively active version of RIG-I composed of the two CARD domains of RIG-I (Magalhães et al., 2016). RIG-I-CARD mimics a viral infection since the CARD domains can directly associate with MAVS, even in the absence of a viral infection, as demonstrated by Magalhães *et al.*(Magalhães et al., 2016).

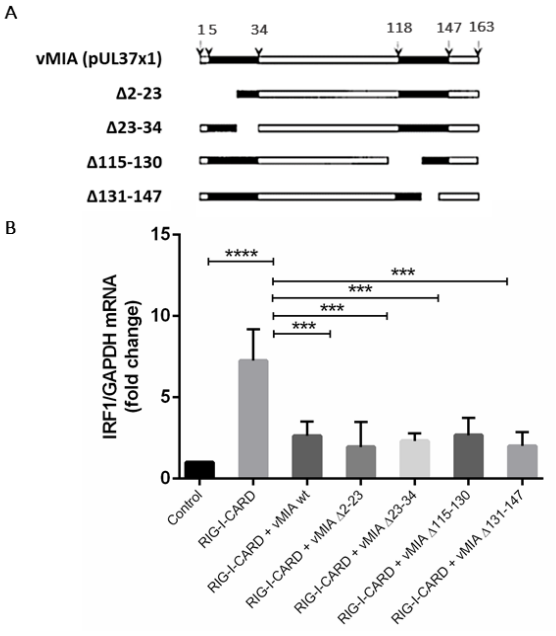

Figure 7. Deletion mutants of vMIA and their influence on the peroxisome-dependent antiviral signalling. A) Representation of vMIA and the respective previously studied deletion mutants. B) RT-qPCR analysis of IRF1 mRNA production in Mefs MAVS-Pex cells transfected with deletion mutants of vMIA and stimulated with GFP-RIG-I-CARD, using GAPDH as a normalizer gene (Marques *et al.*, unpublished results) Adapted from *(Hayajneh et al., 2001)*.

The results obtained demonstrated that the four mutants exhibit a similar behavior to the wild-type vMIA: while the overexpression of RIG-I-CARD alone promotes the transcription of IRF1, the presence of each mutant form of vMIA suppresses that activation, indicating that these mutants keep the ability to inhibit the peroxisomal antiviral response.

Construction of a vMIA mutant – vMIA Δ35-109

In this work, a new deletion mutant, vMIA Δ35-109 (Figure 8), was produced in order to evaluate if the lack of this segment would lead to an altered regulation of the immune response, in contrast with the four mutants previously tested.

The construct with vMIA Δ35-109 was obtained from a pcDNA3 plasmid containing vMIA-myc(Magalhães et al., 2016). The plasmid was amplified by PCR with specific primers designed for the regions of the gene adjacent to the segment to remove. Consequently, a fragment of linear DNA with blunt ends lacking the region corresponding to codons 35 to 109 was obtained. In order to re-circularize the vector, the phosphorylation of 5' ends with a polynucleotide kinase was required, given that DNA polymerase leaves unphosphorylated ends which DNA ligase is not able to ligate. The procedure is depicted in figure 9 and the plasmid obtained is represented in figure 10. The protein expressed by the construct has 127 aa and 14,84 kDa (including the three sequential myc tags at the C-terminal, derived from the original plasmid).

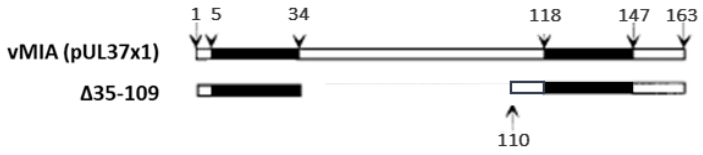

Figure 8. Representation of wild-type vMIA and the deletion mutant Δ35-109. Adapted from *(Hayajneh et al., 2001)*.

Figure 9. Cloning of vMIA Δ35-109. 1) Primers were designed for the amplification of the regions of interest of vMIA and the whole plasmid. 2) Phosphorylation of 5’ ends. 3) Re-circularization of the plasmid by ligation.

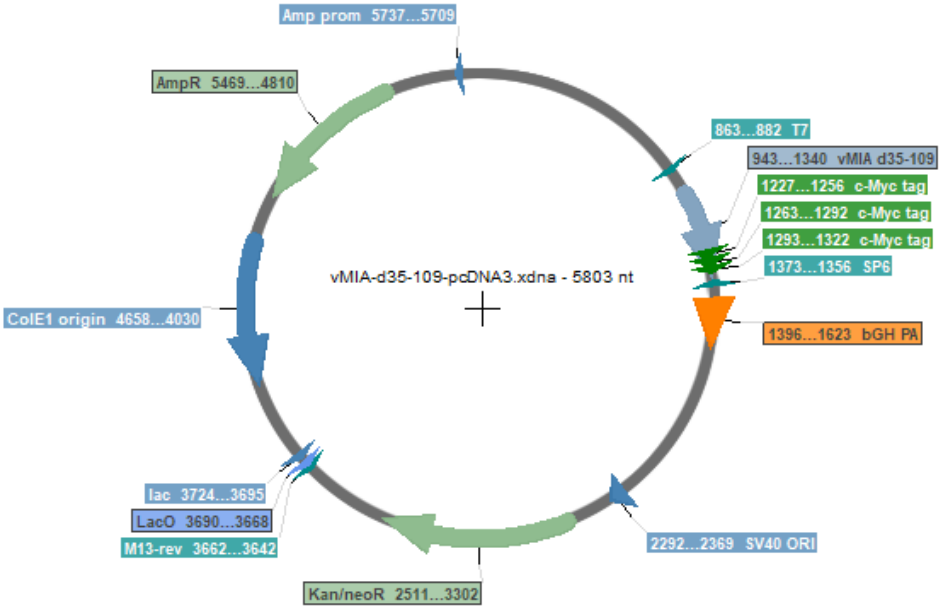

Figure 10. Map of the pcDNA3-vMIA Δ35-109 plasmid.

Upon confirmation by sequencing, the construct was transfected in 293T cells and protein expression was confirmed by Western blot, as shown in figure 11 in parallel with wild-type vMIA

and the other mutants. Given its molecular weight of 14,84 kDa, vMIA Δ35-109 is found in the gel at the expected size.

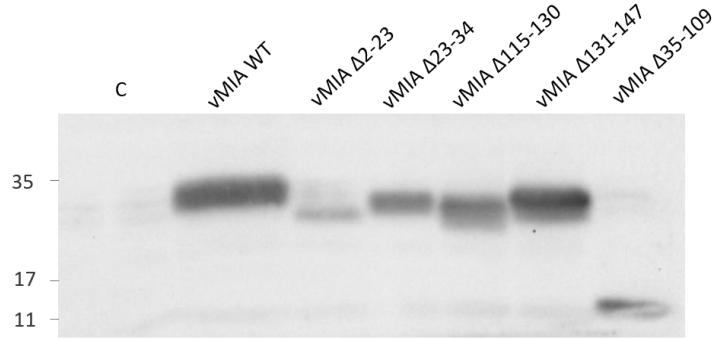

Figure 11. Immunoblot of wild-type vMIA and the mutants. SDS-PAGE of total protein after transfection of 293T cells incubated with an anti-Myc Rb antibody shows vMIA-myc and the myc-tagged deletion mutants.

The presence of vMIA Δ35-109 at the peroxisomes was observed by immunofluorescence in MAVS-PEX cells, as shown in Figure 12. The success of the transfection confirms the viability of the construct. Furthermore, the colocalization of myc and PMP70 staining indicates that the protein retains its ability to migrate to peroxisomes.

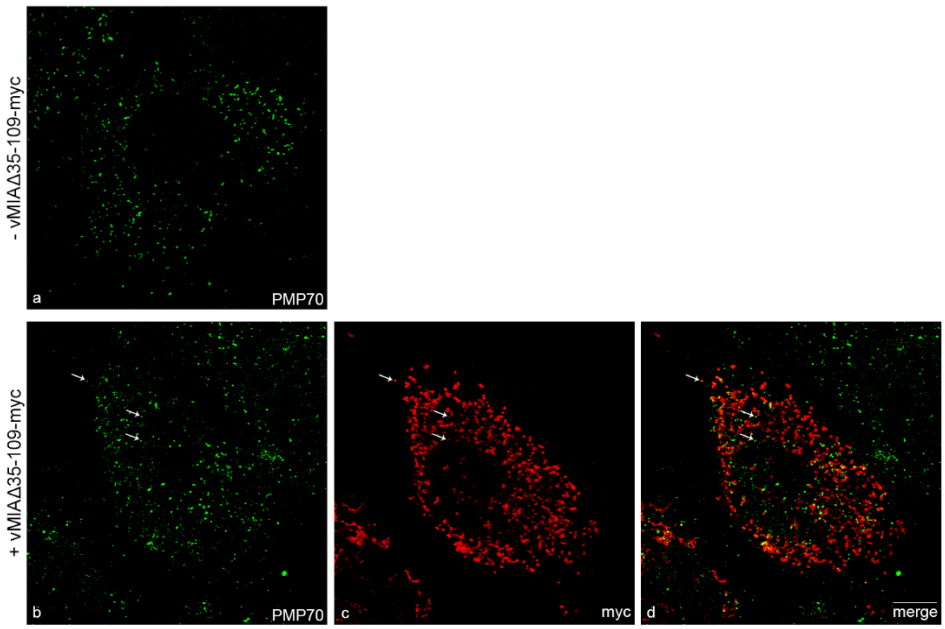

Figure 12. Localization of vMIA Δ35-109 in MAVS-PEX cells. Images obtained by confocal microscopy of immunofluorescence staining of peroxisomes with PMP70 and vMIA Δ35-109-myc with myc. a) untransfected cells. b-d) cells transfected with vMIA Δ35-109-myc. The arrows indicate colocalization of vMIA Δ35-109 with the peroxisomes.

vMIA Δ35-109 inhibits the antiviral innate immune response

Similar to what had been performed for the previous deletion mutants, we have analysed by RT-qPCR the production of IRF1 mRNA upon transfection with GFP-RIG-I-CARD of MAVS-PEX cells expressing the vMIA Δ35-109. The results are shown in Figure 13 and, surprisingly, demonstrate that vMIA Δ35-109 suppresses the IRF1 mRNA production induced by RIG-I-CARD. Combining this information with the results previously obtained with the other four deletion mutants, which also retain the ability to suppress the peroxisomal MAVS signalling, two possibilities remain for the mode of action of vMIA on this pathway. In the first place, as two short fragments of the protein, ranging from aa 110 to 114 and aa 148 to 163, are present in all mutants, it is possible that this sequence is responsible for abrogation of the pathway. On the other hand, two (or more) distinct domains of vMIA may influence the pathway, suppressing it independently.

In the future, it would be interesting to study additional deletion mutants of vMIA, specifically the Δ110-114 and Δ148-163 or others lacking distinct subsets of the protein in order to further restrict the exact domain responsible for the inhibition.

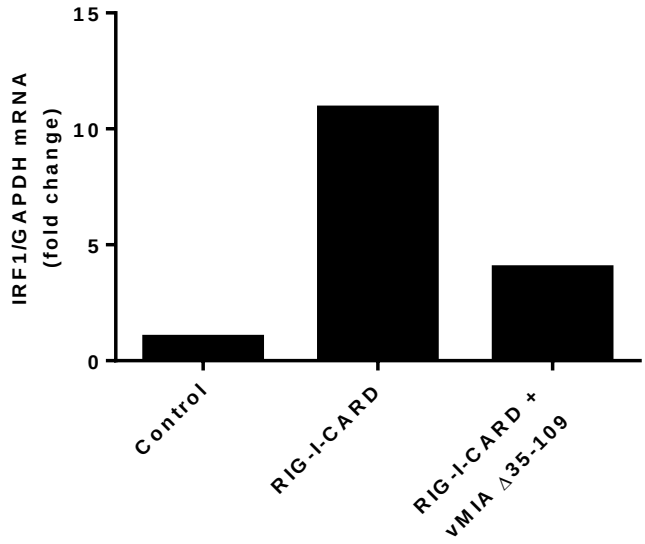

Figure 13. vMIA Δ35-109 inhibits the peroxisome-dependent antiviral signalling. Mefs MAVS PEX cells were transfected with vMIA Δ35-109-myc and GFP-RIG-I-CARD. The expression of IRF1 mRNA was analysed by RT-qPCR, using GAPDH as a normalizer gene. Image obtained with GraphPad Prism 6 (GraphPad).

Analyzing the influence of vMIA on MAVS oligomerization

Some recent results from our group indicate that vMIA may interfere directly with MAVS oligomerization at peroxisomes, which is necessary for the activation of downstream signalling (unpublished data). One of the objectives of this thesis-assignment was to develop and optimize a protocol to confidently analyse peroxisomal and mitochondrial MAVS oligomerization. To that end, we have used semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDD-AGE has been previously used for the study of MAVS aggregation, given the usefulness of agarose gels for the resolution of high molecular weight aggregates(Xu et al., 2014; Zamorano Cuervo et al., 2018). Non-reducing SDS-PAGE is indicated for the identification of MAVS oligomers of lower molecular weight. In non-reducing electrophoresis, the samples are heat-denatured as well but no reducing agents such as DTT or β-mercaptoethanol are added, whereas typical reducing SDS-PAGE requires the addition of either one of these agents.

With the aim of complementing the data obtained with the vMIA Δ2-23, Δ23-34, Δ115-130 and Δ131-147 mutants and well as obtaining novel data for the newly constructed vMIA Δ35-109 mutant, a preliminary study was performed with whole cell extracts from cells previously transfected with wild-type vMIA and the mutants. Protein was extracted after 8 hours of infection with vesicular stomatitis virus (VSV). The expected results would be the absence of oligomerization or aggregation in the control (since MAVS aggregation is solely verified upon RIG-I activation) as well as in the samples containing vMIA and the respective mutants. In the samples solely infected with VSV, an appreciably higher aggregation of MAVS should be observed when comparing to the control(Hou et al., 2011). An assay where all the deletion mutants were transfected is present in Figure 14, however, the results contradict the expected pattern for the control and the condition with VSV infection. Therefore, the protocol was further optimized by the use of a loading buffer for SDD-AGE with higher percentage of SDS, the use of PVDF membranes, the performance of wet transfer at 200 mA overnight and the incubation of the membranes for chemiluminescence. With the proposed improvements, it was possible to observe clearer immunoblots in both SDD-AGE and non-reducing SDS-PAGE, as exhibited in Figure 15. Unfortunately, at the time of completion of this thesis, it was not yet possible to apply the optimized protocol to the cells containing vMIA or its deletion mutants. This will surely be done in the near future and nicely complement the results obtained by RT-qPCR. In the future, it would also be interesting to evaluate the aggregation state of MAVS at the peroxisomes and the mitochondria individually, upon organelle separation by differential centrifugation. This optimized technique will also contribute to further studies on the

specific role of vMIA and other viral proteins on the RIG-I/MAVS signalling that are currently being performed in our laboratory.

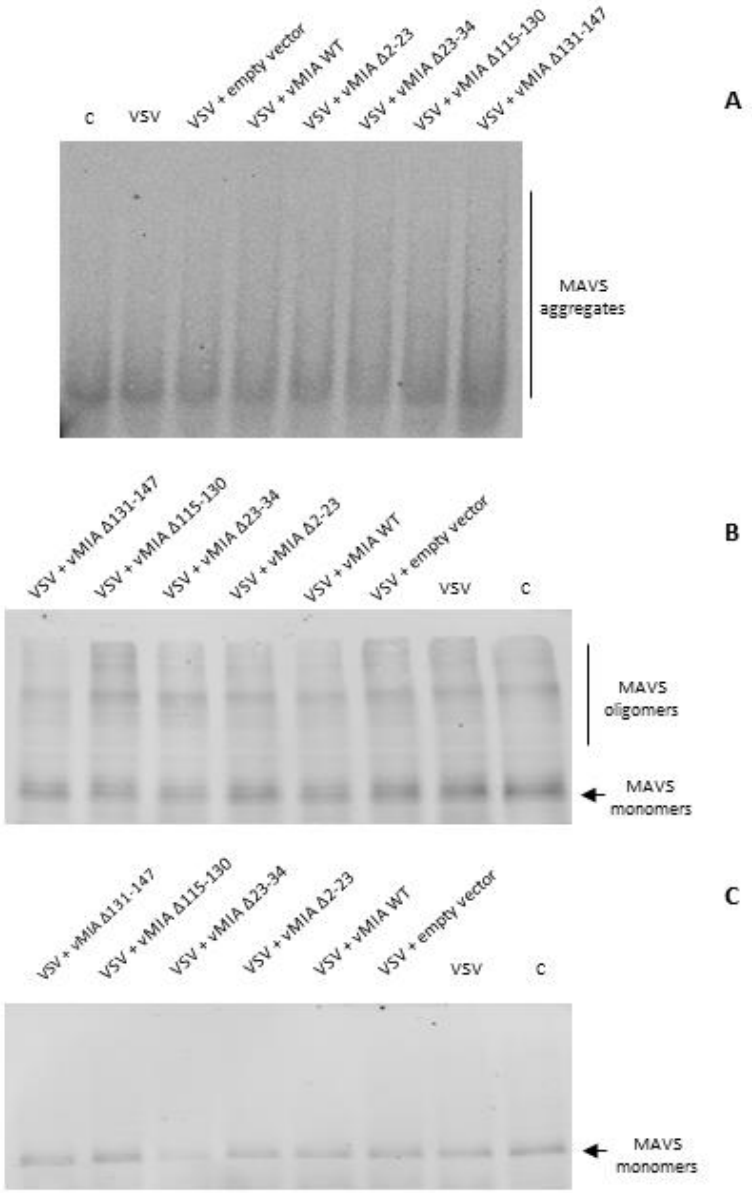

Figure 14. Immunoblots from the MAVS Oligomerization assay. A) SDD-AGE. B) Non-reducing SDS-PAGE. C) Reducing SDS-PAGE. The three membranes were incubated with anti-MAVS Ms antibody and observed in Odyssey.

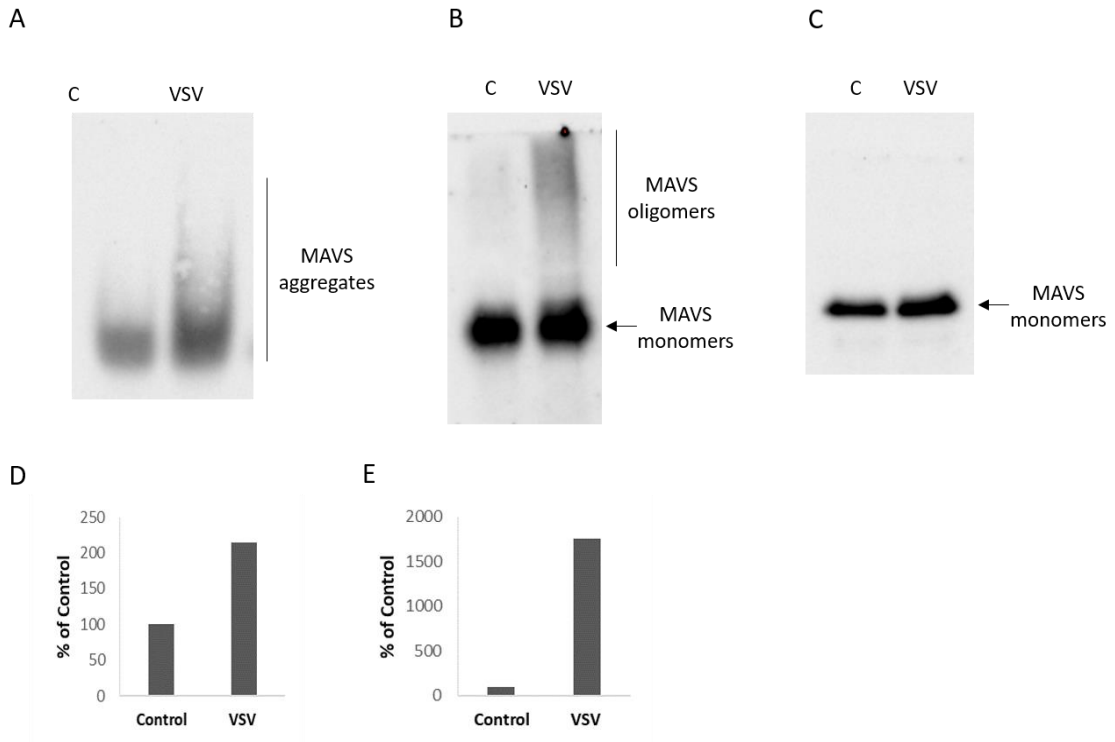

Figure 15. Immunoblots from the MAVS Oligomerization assay. A) SDD-AGE. B) Non-reducing SDS-PAGE. C) Reducing SDS-PAGE. A) SDD-AGE immunoblot. B) Non-reducing SDS-PAGE immunoblot. C) Reducing SDS-PAGE immunoblot. The three membranes were incubated with anti-MAVS Ms antibody and observed in ChemiDoc. D) Quantification of intensity of the bands from the SDD-AGE gel. E) Quantification of intensity of the bands from the non-reducing SDS-PAGE gel using total protein for normalization.

4.2 Study of a possible role of the Human Papillomavirus E5 protein on the peroxisome-dependent antiviral signalling

As explained in section 1.3, the HPV E5 protein plays a role in the modulation of a few cellular components, contributing to the progression of the infection by HPV. E5 contributes to immune evasion by suppressing the migration of MHC I to the cell surface. Furthermore, a study has suggested the interaction of E5 with MAVS(Rozenblatt-Rosen et al., 2012). Hence, we have decided to analyse whether this protein would exert any influence on (and possibly inhibit) the peroxisome-dependent antiviral signalling. To that end, we planned to overexpress a FLAG-tagged version of E5 on cells that expressed MAVS solely at peroxisomes and analyse the effect on the antiviral signalling by quantifying the production of specific ISGs mRNAs, as in section 4.1.

Construction of a FLAG-E5 plasmid

In order to construct a FLAG-tagged version of E5, we took advantage of a plasmid containing the HPV16 E5 gene that was generously sent by Dr. Saveria Campo (University of Glasgow)(G. Hossein Ashrafi et al., 2006) and pCMV-Tag2B, a mammalian expression vector with an N-terminal FLAG epitope. Firstly, a polymerase chain reaction (PCR) was performed in order to amplify the E5, inserting the restriction site for XhoI downstream from the gene, and maintaining the BamHI restriction site, which was already upstream from E5 in the received construct. The restriction enzymes were chosen considering the restriction sites found in the vector pCMV-Tag2B and in the construct containing the gene. A representation of the primers and their annealing to the gene is shown in Figure 16, whereas a picture of the agarose gel electrophoresis of the PCR product is present in Figure 17. The gel shows that the amplification was successful, due to the observation of a large band corresponding to the expected fragment size (270 bp) and the absence of non-specific bands. The vector pCMV-Tag2B and the PCR product were further digested with BamHI and XhoI, yielding the fragments with the expected size seen in Figure 17.

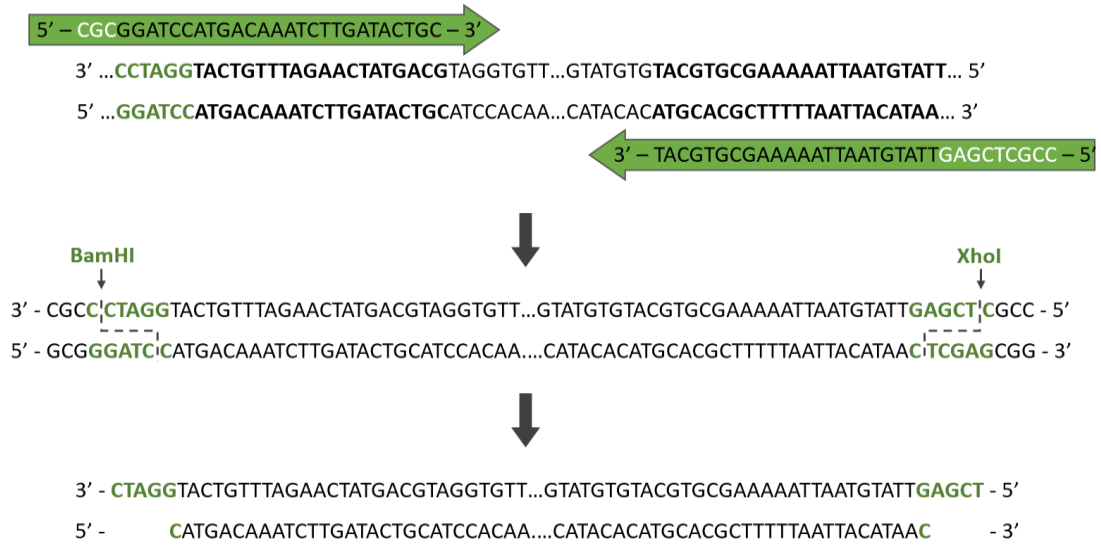

Figure 16. Cloning of the E5 gene into the pCMV-Tag2B plasmid. PCR amplification and restriction digestion.

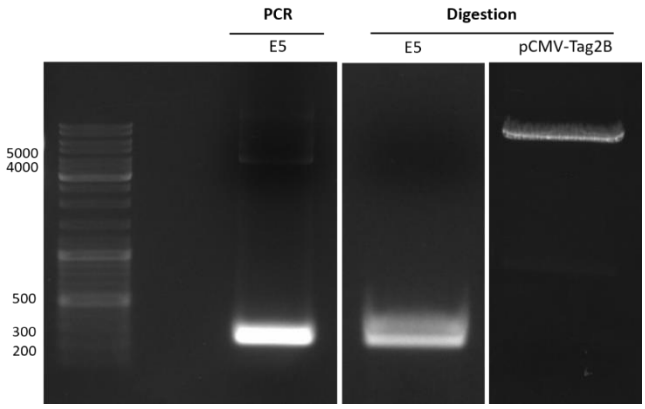

Figure 17. Agarose gel electrophoresis of the PCR product and the restriction products. The bands correspond to the expected size of the amplified E5 gene (270 bps).

Finally, the ligation of the two fragments was performed. After colony screening by restriction reactions, a positive colony was picked and sent for sequencing, which confirmed the success of the cloning procedure. The final construct is depicted in Figure 18.

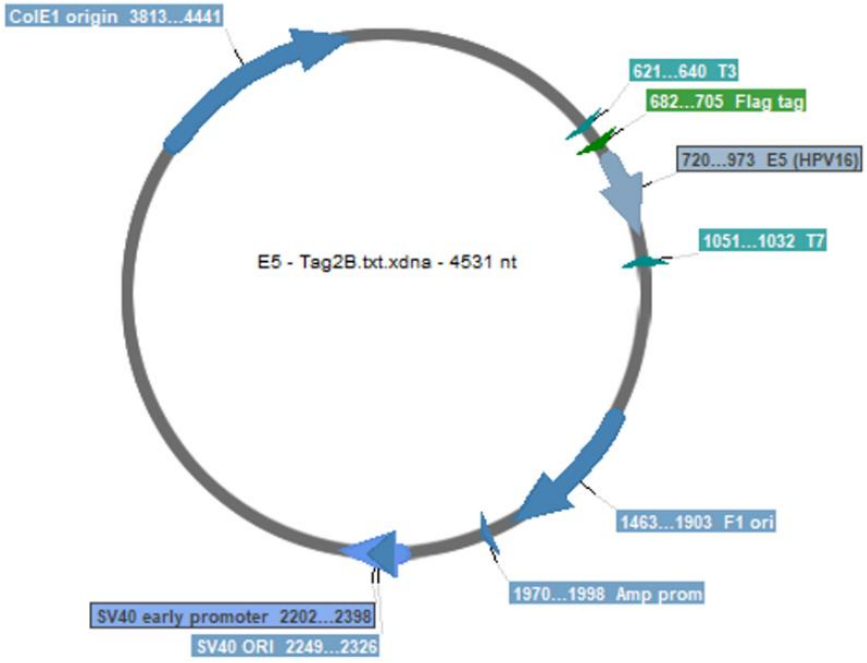

Figure 18. Map of the pCMV-Tag2B plasmid containing the E5 gene.

FLAG-E5 transfection assays

The FLAG-E5 protein expressed from the construct has 97 amino acids and a molecular weight of 10,4 kDa. In order to analyse the role of the protein on the peroxisomes and the MAVS

signalling pathway, transfection assays were performed in 293T cells and Mefs MAVS-Pex with distinct transfection reagents. In 293T cells, polyethylenimine was used whereas in Mefs MAVS-Pex the reagent used was Lipofectamine™ 3000. Although these experiments were repeated multiple times, with different DNA concentrations and amounts of transfection reagents, the presence of FLAG-E5 was not identified in any of these cells by fluorescence microscopy. E5 expression was also analysed by SDS-PAGE followed by Western blotting with similarly negative results. These results were quite surprising as the same transfection conditions have been repeatedly used in the group and the plasmid pCMV-Tag2B contains a Kozak consensus sequence immediately upstream of FLAG (which would suggest high translation levels of the protein). Thus, assuming the CMV promotor sequence is intact in the plasmid, protein expression should not be compromised. One possible explanation for the obtained results is a potential toxicity of E5 towards the cells, or its rapid degradation after expression. Further experiments towards optimization should be performed, or alternatively the gene should be cloned into another plasmid.

V. Concluding Remarks

5.1 Human Cytomegalovirus' vMIA: unravelling the mechanism of inhibition of the peroxisome-dependent antiviral response

The vMIA protein of HCMV localizes to mitochondria and peroxisomes, where it induces organelle fragmentation and inhibits the antiviral innate immunity mediated by MAVS. Unravelling the mechanisms behind this inhibition has been one of the aims of our group. In the present work, we proposed to analyse the influence of vMIA Δ35-109 on the peroxisomal RIG-I/MAVS pathway, considering that the deletion mutants Δ2-23, Δ23-34, Δ115-130 and Δ131-147 tested previously retained the ability to inhibit the peroxisome-dependent antiviral response. Surprisingly, we observed that vMIA Δ35-109 is also able to inhibit signalling via MAVS.

Further experiments were planned for the study of vMIA and its deletion mutants regarding the oligomerization of MAVS, which occurs upon activation of RIG-I by viral RNA. We have optimized a set of techniques in the laboratory: semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which have been increasingly used for the detection of protein aggregates and oligomers of low molecular weight, respectively. However, due to time constraints, it was not possible to apply the techniques optimized by us at the time of completion of this dissertation. In the near future, we intend to perform these assays to complement the results obtained with RT-qPCR, and to further explore the differential MAVS oligomerization state in the presence of vMIA at the mitochondria and the peroxisomes by organelle separation.

5.2 Study of a possible role of the Human Papillomavirus E5 protein on the peroxisome-dependent antiviral signalling

A wide bibliographic study was performed concerning the role of HPV proteins on immune evasion upon infection and on the implication of HPV in the progression to cancer. Regarding the practical investigation, a study reporting an association between E5 and MAVS led us to focus on this protein and its involvement in the suppression of the RIG-I/MAVS pathway. To that end, we prepared a construct containing the E5 gene with a FLAG tag and performed numerous transfection

assays. Unfortunately, after several transfection assays performed in 293T and Mefs MAVS-Pex cells with distinct transfection reagents, E5 expression was never detected. Thus, it was impossible to perform further assays for the analysis of the possible roles of E5 on innate immunity. E5 may be toxic to the cells or rapidly degraded, therefore, different conditions should be tested in order to attempt the optimization of the transfections.

References

Abbas, A.K., Lichtman, A.H.H., Pillai, S., 2017. Cellular and Molecular Immunology E-Book. Elsevier Health Sciences.

Ablasser, A., Bauernfeind, F., Hartmann, G., Latz, E., Fitzgerald, K.A., Hornung, V., 2009. RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. Nat. Immunol. 10, 1065–1072. https://doi.org/10.1038/ni.1779

Abramowitz, L., Jacquard, A.-C., Jaroud, F., Haesebaert, J., Siproudhis, L., Pradat, P., Aynaud, O., Leocmach, Y., Soubeyrand, B., Dachez, R., Riethmuller, D., Mougin, C., Pretet, J.-L., Denis, F., 2011. Human papillomavirus genotype distribution in anal cancer in France: the EDiTH V study. Int. J. Cancer 129, 433–439. https://doi.org/10.1002/ijc.25671

Adam, J.L., Briggs, M.W., McCance, D.J., 2000. A mutagenic analysis of the E5 protein of human papillomavirus type 16 reveals that E5 binding to the vacuolar H+-ATPase is not sufficient for biological activity, using mammalian and yeast expression systems. Virology 272, 315–325. https://doi.org/10.1006/viro.2000.0376

Al Moustafa, A.-E., 2015. E5 and E6/E7 of high-risk HPVs cooperate to enhance cancer progression through EMT initiation. Cell Adh Migr 9, 392–393. https://doi.org/10.1080/19336918.2015.1042197

Arnoult, D., Bartle, L.M., Skaletskaya, A., Poncet, D., Zamzami, N., Park, P.U., Sharpe, J., Youle, R.J., Goldmacher, V.S., 2004. Cytomegalovirus cell death suppressor vMIA blocks Bax- but not Bak-mediated apoptosis by binding and sequestering Bax at mitochondria. Proc. Natl. Acad. Sci. U.S.A. 101, 7988–7993. https://doi.org/10.1073/pnas.0401897101

Ashrafi, G. H., Brown, D.R., Fife, K.H., Campo, M.S., 2006. Down-regulation of MHC class I is a property common to papillomavirus E5 proteins. Virus Res. 120, 208–211. https://doi.org/10.1016/j.virusres.2006.02.005

Ashrafi, G. Hossein, Haghshenas, M., Marchetti, B., Campo, M.S., 2006. E5 protein of human papillomavirus 16 downregulates HLA class I and interacts with the heavy chain via its first hydrophobic domain. Int. J. Cancer 119, 2105–2112. https://doi.org/10.1002/ijc.22089

Ashrafi, G.H., Haghshenas, M.R., Marchetti, B., O'Brien, P.M., Campo, M.S., 2005. E5 protein of human papillomavirus type 16 selectively downregulates surface HLA class I. Int. J. Cancer 113, 276–283. https://doi.org/10.1002/ijc.20558

Auvinen, E., Alonso, A., Auvinen, P., 2004. Human papillomavirus type 16 E5 protein colocalizes with the antiapoptotic Bcl-2 protein. Arch. Virol. 149, 1745–1759.

Aydin, I., Weber, S., Snijder, B., Samperio Ventayol, P., Kühbacher, A., Becker, M., Day, P.M., Schiller, J.T., Kann, M., Pelkmans, L., Helenius, A., Schelhaas, M., 2014. Large scale RNAi reveals the requirement of nuclear envelope breakdown for nuclear import of human papillomaviruses. PLoS Pathog. 10, e1004162. https://doi.org/10.1371/journal.ppat.1004162

Baldwin, P., Laskey, R., Coleman, N., 2003. Translational approaches to improving cervical screening. Nat. Rev. Cancer 3, 217–226. https://doi.org/10.1038/nrc1010

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y., Ziv, Y., 1998. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science 281, 1674–1677.

Barbosa, M.S., Lowy, D.R., Schiller, J.T., 1989. Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. J. Virol. 63, 1404–1407.

Barker, J.N.W.N., Griffiths, C.E.M., Nickoloff, B.J., Mitra, R.S., Dixit, V.M., Nickoloff, B.J., 1991. Keratinocytes as initiators of inflammation. The Lancet, Originally published as Volume 1, Issue 8735 337, 211–214. https://doi.org/10.1016/0140-6736(91)92168-2

Barnard, P., McMillan, N.A., 1999. The human papillomavirus E7 oncoprotein abrogates signaling mediated by interferon-alpha. Virology 259, 305–313. https://doi.org/10.1006/viro.1999.9771

Belleudi, F., Leone, L., Purpura, V., Cannella, F., Scrofani, C., Torrisi, M.R., 2011. HPV16 E5 affects the KGFR/FGFR2b-mediated epithelial growth through alteration of the receptor expression, signaling and endocytic traffic. Oncogene 30, 4963–4976. https://doi.org/10.1038/onc.2011.203

Bender, S., Reuter, A., Eberle, F., Einhorn, E., Binder, M., Bartenschlager, R., 2015. Activation of Type I and III Interferon Response by Mitochondrial and Peroxisomal MAVS and Inhibition by Hepatitis C Virus. PLOS Pathogens 11, e1005264. https://doi.org/10.1371/journal.ppat.1005264

Berke, I.C., Modis, Y., 2012. MDA5 cooperatively forms dimers and ATP-sensitive filaments upon binding double-stranded RNA. EMBO J. 31, 1714–1726. https://doi.org/10.1038/emboj.2012.19

Bernat, A., Avvakumov, N., Mymryk, J.S., Banks, L., 2003. Interaction between the HPV E7 oncoprotein and the transcriptional coactivator p300. Oncogene 22, 7871–7881. https://doi.org/10.1038/sj.onc.1206896

Bienkowska-Haba, M., Williams, C., Kim, S.M., Garcea, R.L., Sapp, M., 2012. Cyclophilins facilitate dissociation of the human papillomavirus type 16 capsid protein L1 from the L2/DNA complex following virus entry. J. Virol. 86, 9875–9887. https://doi.org/10.1128/JVI.00980-12

Bodily, J., Laimins, L.A., 2011. Persistence of human papillomavirus infection: keys to malignant progression. Trends Microbiol. 19, 33–39. https://doi.org/10.1016/j.tim.2010.10.002

Bonekamp, N.A., Völkl, A., Fahimi, H.D., Schrader, M., 2009. Reactive oxygen species and peroxisomes: struggling for balance. Biofactors 35, 346–355. https://doi.org/10.1002/biof.48

Bousarghin, L., Touzé, A., Sizaret, P.-Y., Coursaget, P., 2003. Human Papillomavirus Types 16, 31, and 58 Use Different Endocytosis Pathways To Enter Cells. J. Virol. 77, 3846–3850. https://doi.org/10.1128/JVI.77.6.3846-3850.2003

Bouvard, V., Matlashewski, G., Gu, Z.M., Storey, A., 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. https://doi.org/10.1006/viro.1994.1456

Boyer, S.N., Wazer, D.E., Band, V., 1996. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Res. 56, 4620–4624.

Bravo, I.G., Alonso, Á., 2004. Mucosal Human Papillomaviruses Encode Four Different E5 Proteins Whose Chemistry and Phylogeny Correlate with Malignant or Benign Growth. J Virol 78, 13613–13626. https://doi.org/10.1128/JVI.78.24.13613-13626.2004

Bravo, I.G., Crusius, K., Alonso, A., 2005. The E5 protein of the human papillomavirus type 16 modulates composition and dynamics of membrane lipids in keratinocytes. Arch. Virol. 150, 231–246. https://doi.org/10.1007/s00705-004-0420-x

Brown, D.R., Bryan, J.T., 2000. Abnormalities of cornified cell envelopes isolated from human papillomavirus type 11-infected genital epithelium. Virology 271, 65–70. https://doi.org/10.1006/viro.2000.0317

Buck, C.B., Cheng, N., Thompson, C.D., Lowy, D.R., Steven, A.C., Schiller, J.T., Trus, B.L., 2008. Arrangement of L2 within the Papillomavirus Capsid. J. Virol. 82, 5190–5197. https://doi.org/10.1128/JVI.02726-07

Buck, C.B., Day, P.M., Thompson, C.D., Lubkowski, J., Lu, W., Lowy, D.R., Schiller, J.T., 2006. Human α-defensins block papillomavirus infection. PNAS 103, 1516–1521. https://doi.org/10.1073/pnas.0508033103

Camões, F., Bonekamp, N.A., Delille, H.K., Schrader, M., 2009. Organelle dynamics and dysfunction: A closer link between peroxisomes and mitochondria. J. Inherit. Metab. Dis. 32, 163–180. https://doi.org/10.1007/s10545-008-1018-3

Cannon, M.J., Schmid, D.S., Hyde, T.B., 2010. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. Rev. Med. Virol. 20, 202–213. https://doi.org/10.1002/rmv.655

Castanier, C., Garcin, D., Vazquez, A., Arnoult, D., 2010. Mitochondrial dynamics regulate the RIG-I-like receptor antiviral pathway. EMBO Rep. 11, 133–138. https://doi.org/10.1038/embor.2009.258

Chang, J.L., Tsao, Y.P., Liu, D.W., Huang, S.J., Lee, W.H., Chen, S.L., 2001. The expression of HPV-16 E5 protein in squamous neoplastic changes in the uterine cervix. J. Biomed. Sci. 8, 206–213. https://doi.org/54034

Chaturvedi, A., Pierce, S.K., 2009. How location governs toll-like receptor signaling. Traffic 10, 621–628. https://doi.org/10.1111/j.1600-0854.2009.00899.x

Chen, S.L., Huang, C.H., Tsai, T.C., Lu, K.Y., Tsao, Y.P., 1996a. The regulation mechanism of c-jun and junB by human papillomavirus type 16 E5 oncoprotein. Arch. Virol. 141, 791–800.

Chen, S.L., Lin, Y.K., Li, L.Y., Tsao, Y.P., Lo, H.Y., Wang, W.B., Tsai, T.C., 1996b. E5 proteins of human papillomavirus types 11 and 16 transactivate the c-fos promoter through the NF1 binding element. J. Virol. 70, 8558–8563.

Chen, X.S., Garcea, R.L., Goldberg, I., Casini, G., Harrison, S.C., 2000. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. Mol. Cell 5, 557–567.

Chiang, C., Pauli, E.-K., Biryukov, J., Feister, K.F., Meng, M., White, E.A., Münger, K., Howley, P.M., Meyers, C., Gack, M.U., 2018. The Human Papillomavirus E6 Oncoprotein Targets USP15 and TRIM25 To Suppress RIG-I-Mediated Innate Immune Signaling. J. Virol. 92. https://doi.org/10.1128/JVI.01737-17

Chiang, C.M., Ustav, M., Stenlund, A., Ho, T.F., Broker, T.R., Chow, L.T., 1992. Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. Proc. Natl. Acad. Sci. U.S.A. 89, 5799–5803.

Chiu, Y.-H., Macmillan, J.B., Chen, Z.J., 2009. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. Cell 138, 576–591. https://doi.org/10.1016/j.cell.2009.06.015

Choo, K.B., Pan, C.C., Han, S.H., 1987. Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. Virology 161, 259–261.

Cigno, I.L., Andrea, M.D., Borgogna, C., Albertini, S., Landini, M.M., Peretti, A., Johnson, K.E., Chandran, B., Landolfo, S., Gariglio, M., 2015. The Nuclear DNA Sensor IFI16 Acts as a Restriction Factor for Human Papillomavirus Replication through Epigenetic Modifications of the Viral Promoters. J. Virol. 89, 7506–7520. https://doi.org/10.1128/JVI.00013-15

Combita, A.L., Touzé, A., Bousarghin, L., Sizaret, P.Y., Muñoz, N., Coursaget, P., 2001. Gene transfer using human papillomavirus pseudovirions varies according to virus genotype and requires cell surface heparan sulfate. FEMS Microbiol. Lett. 204, 183–188.

Conrad, M., Bubb, V.J., Schlegel, R., 1993. The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-kilodalton pore-forming protein. J Virol 67, 6170–6178.

Conrad, M., Goldstein, D., Andresson, T., Schlegel, R., 1994. The E5 protein of HPV-6, but not HPV-16, associates efficiently with cellular growth factor receptors. Virology 200, 796–800. https://doi.org/10.1006/viro.1994.1244

Cooper, G.M., 2000. Mitochondria. The Cell: A Molecular Approach. 2nd edition.

Cortese, M.S., Ashrafi, G.H., Campo, M.S., 2010. All 4 di-leucine motifs in the first hydrophobic domain of the E5 oncoprotein of human papillomavirus type 16 are essential for surface MHC class I downregulation activity and E5 endomembrane localization. Int. J. Cancer 126, 1675–1682. https://doi.org/10.1002/ijc.25004

Crosse, K.M., Monson, E.A., Beard, M.R., Helbig, K.J., 2018. Interferon-Stimulated Genes as Enhancers of Antiviral Innate Immune Signaling. JIN 10, 85–93. https://doi.org/10.1159/000484258

Crusius, K., Auvinen, E., Alonso, A., 1997. Enhancement of EGF- and PMA-mediated MAP kinase activation in cells expressing the human papillomavirus type 16 E5 protein. Oncogene 15, 1437–1444. https://doi.org/10.1038/sj.onc.1201312

Crusius, K., Auvinen, E., Steuer, B., Gaissert, H., Alonso, A., 1998. The human papillomavirus type 16 E5-protein modulates ligand-dependent activation of the EGF receptor family in the human epithelial cell line HaCaT. Exp. Cell Res. 241, 76–83. https://doi.org/10.1006/excr.1998.4024

Crusius, K., Rodriguez, I., Alonso, A., 2000. The human papillomavirus type 16 E5 protein modulates ERK1/2 and p38 MAP kinase activation by an EGFR-independent process in stressed human keratinocytes. Virus Genes 20, 65–69.

Cullen, A.P., Reid, R., Campion, M., Lörincz, A.T., 1991. Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. J. Virol. 65, 606–612.

Dall, K.L., Scarpini, C.G., Roberts, I., Winder, D.M., Stanley, M.A., Muralidhar, B., Herdman, M.T., Pett, M.R., Coleman, N., 2008. Characterization of naturally occurring HPV16 integration sites isolated from cervical keratinocytes under noncompetitive conditions. Cancer Res. 68, 8249–8259. https://doi.org/10.1158/0008-5472.CAN-08-1741

Danos, O., Katinka, M., Yaniv, M., 1982. Human papillomavirus 1a complete DNA sequence: a novel type of genome organization among papovaviridae. EMBO J 1, 231–236.

Daud, I.I., Scott, M.E., Ma, Y., Shiboski, S., Farhat, S., Moscicki, A.-B., 2011. Association between toll-like receptor expression and human papillomavirus type 16 persistence. Int. J. Cancer 128, 879–886. https://doi.org/10.1002/ijc.25400

Day, P.M., Baker, C.C., Lowy, D.R., Schiller, J.T., 2004. Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. Proc Natl Acad Sci U S A 101, 14252–14257. https://doi.org/10.1073/pnas.0404229101

Day, P.M., Gambhira, R., Roden, R.B.S., Lowy, D.R., Schiller, J.T., 2008. Mechanisms of Human Papillomavirus Type 16 Neutralization by L2 Cross-Neutralizing and L1 Type-Specific Antibodies. J. Virol. 82, 4638–4646. https://doi.org/10.1128/JVI.00143-08

Day, P.M., Roden, R.B.S., Lowy, D.R., 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.

de Jong, M.D., Galasso, G.J., Gazzard, B., Griffiths, P.D., Jabs, D.A., Kern, E.R., Spector, S.A., 1998. Summary of the II International Symposium on Cytomegalovirus. Antiviral Res. 39, 141–162.

De Vuyst, H., Clifford, G.M., Nascimento, M.C., Madeleine, M.M., Franceschi, S., 2009. Prevalence and type distribution of human papillomavirus in carcinoma and intraepithelial neoplasia of the vulva, vagina and anus: a meta-analysis. Int. J. Cancer 124, 1626–1636. https://doi.org/10.1002/ijc.24116

Denny, L.A., Franceschi, S., de Sanjosé, S., Heard, I., Moscicki, A.B., Palefsky, J., 2012. Human papillomavirus, human immunodeficiency virus and immunosuppression. Vaccine 30 Suppl 5, F168-174. https://doi.org/10.1016/j.vaccine.2012.06.045

DiGiuseppe, S., Bienkowska-Haba, M., Hilbig, L., Sapp, M., 2014. The nuclear retention signal of HPV16 L2 protein is essential for incoming viral genome to transverse the trans-Golgi network. Virology 458–459, 93–105. https://doi.org/10.1016/j.virol.2014.04.024

DiGiuseppe, S., Bienkowska-Haba, M., Sapp, M., 2016. Human Papillomavirus Entry: Hiding in a Bubble. J Virol 90, 8032–8035. https://doi.org/10.1128/JVI.01065-16

DiMaio, D., Petti, L.M., 2013. The E5 proteins. Virology 445, 99–114. https://doi.org/10.1016/j.virol.2013.05.006

Disbrow, G.L., Hanover, J.A., Schlegel, R., 2005. Endoplasmic Reticulum-Localized Human Papillomavirus Type 16 E5 Protein Alters Endosomal pH but Not trans-Golgi pH. J Virol 79, 5839–5846. https://doi.org/10.1128/JVI.79.9.5839-5846.2005

Dixit, E., Boulant, S., Zhang, Y., Lee, A.S.Y., Odendall, C., Shum, B., Hacohen, N., Chen, Z.J., Whelan, S.P., Fransen, M., Nibert, M.L., Superti-Furga, G., Kagan, J.C., 2010. Peroxisomes are

signaling platforms for antiviral innate immunity. Cell 141, 668–681. https://doi.org/10.1016/j.cell.2010.04.018

Doorbar, J., 2005. The papillomavirus life cycle. J. Clin. Virol. 32 Suppl 1, S7-15. https://doi.org/10.1016/j.jcv.2004.12.006

Doorbar, J., Egawa, N., Griffin, H., Kranjec, C., Murakami, I., 2015. Human papillomavirus molecular biology and disease association. Rev. Med. Virol. 25 Suppl 1, 2–23. https://doi.org/10.1002/rmv.1822

Doorbar, J., Ely, S., Sterling, J., McLean, C., 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. https://doi.org/10.1038/352824a0

Dunne, E.F., Unger, E.R., Sternberg, M., McQuillan, G., Swan, D.C., Patel, S.S., Markowitz, L.E., 2007. Prevalence of HPV infection among females in the United States. JAMA 297, 813–819. https://doi.org/10.1001/jama.297.8.813

Egawa, K., 2003. Do Human Papillomaviruses Target Epidermal Stem Cells? DRM 207, 251–254. https://doi.org/10.1159/000073085

Favre, M., Breitburd, F., Croissant, O., Orth, G., 1977. Chromatin-like structures obtained after alkaline disruption of bovine and human papillomaviruses. J. Virol. 21, 1205–1209.

Freiberger, D., Lewis, L., Helfand, L., 2015. Human papillomavirus-related high-grade squamous intraepithelial lesions of the esophagus, skin, and cervix in an adolescent lung transplant recipient: a case report and literature review. Transpl Infect Dis 17, 98–102. https://doi.org/10.1111/tid.12322

Gack, M.U., Shin, Y.C., Joo, C.-H., Urano, T., Liang, C., Sun, L., Takeuchi, O., Akira, S., Chen, Z., Inoue, S., Jung, J.U., 2007. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature 446, 916–920. https://doi.org/10.1038/nature05732

Gage, J.R., Meyers, C., Wettstein, F.O., 1990. The E7 proteins of the nononcogenic human papillomavirus type 6b (HPV-6b) and of the oncogenic HPV-16 differ in retinoblastoma protein binding and other properties. J. Virol. 64, 723–730.

Gao, P., Zheng, J., 2010. High-risk HPV E5-induced cell fusion: a critical initiating event in the early stage of HPV-associated cervical cancer. Virol. J. 7, 238. https://doi.org/10.1186/1743-422X-7-238

García-Vallvé, S., Alonso, A., Bravo, I.G., 2005. Papillomaviruses: different genes have different histories. Trends Microbiol. 13, 514–521. https://doi.org/10.1016/j.tim.2005.09.003

Gautam, D., Moody, C.A., 2016. Impact of the DNA Damage Response on Human Papillomavirus Chromatin. PLoS Pathog 12. https://doi.org/10.1371/journal.ppat.1005613

Genther Williams, S.M., Disbrow, G.L., Schlegel, R., Lee, D., Threadgill, D.W., Lambert, P.F., 2005. Requirement of epidermal growth factor receptor for hyperplasia induced by E5, a high-risk human papillomavirus oncogene. Cancer Res. 65, 6534–6542. https://doi.org/10.1158/0008-5472.CAN-05-0083

Giroglou, T., Florin, L., Schäfer, F., Streeck, R.E., Sapp, M., 2001. Human Papillomavirus Infection Requires Cell Surface Heparan Sulfate. J Virol 75, 1565–1570. https://doi.org/10.1128/JVI.75.3.1565-1570.2001

Globocan 2012 - Home [WWW Document], n.d. URL http://globocan.iarc.fr/ (accessed 1.10.18).

Goldmacher, V.S., Bartle, L.M., Skaletskaya, A., Dionne, C.A., Kedersha, N.L., Vater, C.A., Han, J.W., Lutz, R.J., Watanabe, S., Cahir McFarland, E.D., Kieff, E.D., Mocarski, E.S., Chittenden, T., 1999. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. Proc. Natl. Acad. Sci. U.S.A. 96, 12536–12541.

Goodrum, F.D., Jordan, C.T., High, K., Shenk, T., 2002. Human cytomegalovirus gene expression during infection of primary hematopoietic progenitor cells: a model for latency. Proc. Natl. Acad. Sci. U.S.A. 99, 16255–16260. https://doi.org/10.1073/pnas.252630899

Gravitt, P.E., 2012. Evidence and impact of human papillomavirus latency. Open Virol J 6, 198–203. https://doi.org/10.2174/1874357901206010198

Groves, I.J., Coleman, N., 2015. Pathogenesis of human papillomavirus-associated mucosal disease. J. Pathol. 235, 527–538. https://doi.org/10.1002/path.4496

Gruener, M., Bravo, I.G., Momburg, F., Alonso, A., Tomakidi, P., 2007. The E5 protein of the human papillomavirus type 16 down-regulates HLA-I surface expression in calnexin-expressing but not in calnexin-deficient cells. Virol J 4, 116. https://doi.org/10.1186/1743-422X-4-116

Gu, Z., Matlashewski, G., 1995. Effect of human papillomavirus type 16 oncogenes on MAP kinase activity. J. Virol. 69, 8051–8056.

Hagensee, M.E., Yaegashi, N., Galloway, D.A., 1993. Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. J Virol 67, 315–322.

Hahn, G., Jores, R., Mocarski, E.S., 1998. Cytomegalovirus remains latent in a common precursor of dendritic and myeloid cells. Proc. Natl. Acad. Sci. U.S.A. 95, 3937–3942.

Hasan, U.A., Bates, E., Takeshita, F., Biliato, A., Accardi, R., Bouvard, V., Mansour, M., Vincent, I., Gissmann, L., Iftner, T., Sideri, M., Stubenrauch, F., Tommasino, M., 2007. TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. J. Immunol. 178, 3186–3197.

Hasan, U.A., Zannetti, C., Parroche, P., Goutagny, N., Malfroy, M., Roblot, G., Carreira, C., Hussain, I., Müller, M., Taylor-Papadimitriou, J., Picard, D., Sylla, B.S., Trinchieri, G., Medzhitov, R., Tommasino, M., 2013. The human papillomavirus type 16 E7 oncoprotein induces a transcriptional repressor complex on the Toll-like receptor 9 promoter. J. Exp. Med. 210, 1369–1387. https://doi.org/10.1084/jem.20122394

Havard, L., Rahmouni, S., Boniver, J., Delvenne, P., 2005. High levels of p105 (NFKB1) and p100 (NFKB2) proteins in HPV16-transformed keratinocytes: role of E6 and E7 oncoproteins. Virology 331, 357–366. https://doi.org/10.1016/j.virol.2004.10.030

Hayajneh, W.A., Colberg-Poley, A.M., Skaletskaya, A., Bartle, L.M., Lesperance, M.M., Contopoulos-Ioannidis, D.G., Kedersha, N.L., Goldmacher, V.S., 2001. The sequence and antiapoptotic functional domains of the human cytomegalovirus UL37 exon 1 immediate early

protein are conserved in multiple primary strains. Virology 279, 233–240. https://doi.org/10.1006/viro.2000.0726

Hebner, C.M., Wilson, R., Rader, J., Bidder, M., Laimins, L.A., 2006. Human papillomaviruses target the double-stranded RNA protein kinase pathway. Journal of General Virology 87, 3183–3193. https://doi.org/10.1099/vir.0.82098-0

Herfs, M., Yamamoto, Y., Laury, A., Wang, X., Nucci, M.R., McLaughlin-Drubin, M.E., Münger, K., Feldman, S., McKeon, F.D., Xian, W., Crum, C.P., 2012. A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. Proc. Natl. Acad. Sci. U.S.A. 109, 10516–10521. https://doi.org/10.1073/pnas.1202684109

Hoepfner, D., Schildknegt, D., Braakman, I., Philippsen, P., Tabak, H.F., 2005. Contribution of the endoplasmic reticulum to peroxisome formation. Cell 122, 85–95. https://doi.org/10.1016/j.cell.2005.04.025

Hoesel, B., Schmid, J.A., 2013. The complexity of NF-κB signaling in inflammation and cancer. Mol Cancer 12, 86. https://doi.org/10.1186/1476-4598-12-86

Hong, S., Laimins, L.A., 2017. Manipulation of the innate immune response by human papillomaviruses. Virus Res. 231, 34–40. https://doi.org/10.1016/j.virusres.2016.11.004

Hong, S., Laimins, L.A., 2013. The JAK-STAT Transcriptional Regulator, STAT-5, Activates the ATM DNA Damage Pathway to Induce HPV 31 Genome Amplification upon Epithelial Differentiation. PLoS Pathog 9. https://doi.org/10.1371/journal.ppat.1003295

Hong, S., Mehta, K.P., Laimins, L.A., 2011. Suppression of STAT-1 expression by human papillomaviruses is necessary for differentiation-dependent genome amplification and plasmid maintenance. J. Virol. 85, 9486–9494. https://doi.org/10.1128/JVI.05007-11

Horner, S.M., Liu, H.M., Park, H.S., Briley, J., Gale, M., 2011. Mitochondrial-associated endoplasmic reticulum membranes (MAM) form innate immune synapses and are targeted by hepatitis C virus. Proc. Natl. Acad. Sci. U.S.A. 108, 14590–14595. https://doi.org/10.1073/pnas.1110133108

Hou, F., Sun, L., Zheng, H., Skaug, B., Jiang, Q.-X., Chen, Z.J., 2011. MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. Cell 146, 448–461. https://doi.org/10.1016/j.cell.2011.06.041

Hu, L., Ceresa, B.P., 2009. Characterization of the plasma membrane localization and orientation of HPV16 E5 for cell-cell fusion. Virology 393, 135–143. https://doi.org/10.1016/j.virol.2009.07.034

Hu, L., Plafker, K., Vorozhko, V., Zuna, R.E., Hanigan, M.H., Gorbsky, G.J., Plafker, S.M., Angeletti, P.C., Ceresa, B.P., 2009. Human papillomavirus 16 E5 induces bi-nucleated cell formation by cell-cell fusion. Virology 384, 125–134. https://doi.org/10.1016/j.virol.2008.10.011

Hu, L., Potapova, T.A., Li, S., Rankin, S., Gorbsky, G.J., Angeletti, P.C., Ceresa, B.P., 2010. Expression of HPV16 E5 Produces Enlarged Nuclei and Polyploidy through Endoreplication. Virology 405, 342–351. https://doi.org/10.1016/j.virol.2010.06.025

Hu, Z., Zhu, D., Wang, W., Li, W., Jia, W., Zeng, X., Ding, W., Yu, L., Wang, X., Wang, L., Shen, H., Zhang, C., Liu, H., Liu, X., Zhao, Y., Fang, X., Li, Shuaicheng, Chen, W., Tang, T., Fu, A.,

Wang, Z., Chen, G., Gao, Q., Li, Shuang, Xi, L., Wang, C., Liao, S., Ma, X., Wu, P., Li, K., Wang, S., Zhou, J., Wang, J., Xu, X., Wang, H., Ma, D., 2015. Genome-wide profiling of HPV integration in cervical cancer identifies clustered genomic hot spots and a potential microhomology-mediated integration mechanism. Nat. Genet. 47, 158–163. https://doi.org/10.1038/ng.3178

Huang, S.-M., McCance, D.J., 2002. Down regulation of the interleukin-8 promoter by human papillomavirus type 16 E6 and E7 through effects on CREB binding protein/p300 and P/CAF. J. Virol. 76, 8710–8721.

Hubert, P., Herman, L., Maillard, C., Caberg, J.-H., Nikkels, A., Pierard, G., Foidart, J.-M., Noel, A., Boniver, J., Delvenne, P., 2007. Defensins induce the recruitment of dendritic cells in cervical human papillomavirus-associated (pre)neoplastic lesions formed in vitro and transplanted in vivo. FASEB J 21, 2765–2775. https://doi.org/10.1096/fj.06-7646com

Hwang, E.S., Nottoli, T., Dimaio, D., 1995. The HPV16 E5 protein: expression, detection, and stable complex formation with transmembrane proteins in COS cells. Virology 211, 227–233. https://doi.org/10.1006/viro.1995.1395

Isaacson, M.K., Juckem, L.K., Compton, T., 2008. Virus entry and innate immune activation. Curr. Top. Microbiol. Immunol. 325, 85–100.

Ishikawa, H., Barber, G.N., 2008. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 455, 674–678. https://doi.org/10.1038/nature07317

Jackson, S.E., Mason, G.M., Wills, M.R., 2011. Human cytomegalovirus immunity and immune evasion. Virus Res. 157, 151–160. https://doi.org/10.1016/j.virusres.2010.10.031

Jeon, S., Lambert, P.F., 1995. Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. Proc Natl Acad Sci U S A 92, 1654–1658.

Jiang, F., Ramanathan, A., Miller, M.T., Tang, G.-Q., Gale, M., Patel, S.S., Marcotrigiano, J., 2011. Structural basis of RNA recognition and activation by innate immune receptor RIG-I. Nature 479, 423–427. https://doi.org/10.1038/nature10537

Joyce, J.G., Tung, J.-S., Przysiecki, C.T., Cook, J.C., Lehman, E.D., Sands, J.A., Jansen, K.U., 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. https://doi.org/10.1074/jbc.274.9.5810

Kabsch, K., Alonso, A., 2002. The human papillomavirus type 16 E5 protein impairs TRAIL- and FasL-mediated apoptosis in HaCaT cells by different mechanisms. J. Virol. 76, 12162–12172.

Kabsch, K., Mossadegh, N., Kohl, A., Komposch, G., Schenkel, J., Alonso, A., Tomakidi, P., 2004. The HPV-16 E5 protein inhibits TRAIL- and FasL-mediated apoptosis in human keratinocyte raft cultures. Intervirology 47, 48–56. https://doi.org/10.1159/000076642

Kalejta, Robert F., 2008. Tegument proteins of human cytomegalovirus. Microbiol. Mol. Biol. Rev. 72, 249–265, table of contents. https://doi.org/10.1128/MMBR.00040-07

Kalejta, R. F., 2008. Functions of human cytomegalovirus tegument proteins prior to immediate early gene expression. Curr. Top. Microbiol. Immunol. 325, 101–115.

Kämper, N., Day, P.M., Nowak, T., Selinka, H.-C., Florin, L., Bolscher, J., Hilbig, L., Schiller, J.T., 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. https://doi.org/10.1128/JVI.80.2.759-768.2006

Karim, R., Meyers, C., Backendorf, C., Ludigs, K., Offringa, R., van Ommen, G.-J.B., Melief, C.J.M., van der Burg, S.H., Boer, J.M., 2011. Human papillomavirus deregulates the response of a cellular network comprising of chemotactic and proinflammatory genes. PLoS ONE 6, e17848. https://doi.org/10.1371/journal.pone.0017848

Karim, R., Tummers, B., Meyers, C., Biryukov, J.L., Alam, S., Backendorf, C., Jha, V., Offringa, R., Ommen, G.-J.B. van, Melief, C.J.M., Guardavaccaro, D., Boer, J.M., Burg, S.H. van der, 2013. Human Papillomavirus (HPV) Upregulates the Cellular Deubiquitinase UCHL1 to Suppress the Keratinocyte's Innate Immune Response. PLOS Pathogens 9, e1003384. https://doi.org/10.1371/journal.ppat.1003384

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K.J., Takeuchi, O., Akira, S., 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat. Immunol. 6, 981–988. https://doi.org/10.1038/ni1243

Kawasaki, T., Kawai, T., 2014. Toll-Like Receptor Signaling Pathways. Front Immunol 5. https://doi.org/10.3389/fimmu.2014.00461

Kell, B., Jewers, R.J., Cason, J., Pakarian, F., Kaye, J.N., Best, J.M., 1994. Detection of E5 oncoprotein in human papillomavirus type 16-positive cervical scrapes using antibodies raised to synthetic peptides. J. Gen. Virol. 75 (Pt 9), 2451–2456. https://doi.org/10.1099/0022-1317-75-9-2451

Kenneson, A., Cannon, M.J., 2007. Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection. Rev. Med. Virol. 17, 253–276. https://doi.org/10.1002/rmv.535

Kim, S.-H., Juhnn, Y.-S., Kang, S., Park, S.-W., Sung, M.-W., Bang, Y.-J., Song, Y.-S., 2006. Human papillomavirus 16 E5 up-regulates the expression of vascular endothelial growth factor through the activation of epidermal growth factor receptor, MEK/ ERK1,2 and PI3K/Akt. Cell. Mol. Life Sci. 63, 930–938. https://doi.org/10.1007/s00018-005-5561-x

Kim, S.-H., Oh, J.-M., No, J.-H., Bang, Y.-J., Juhnn, Y.-S., Song, Y.-S., 2009. Involvement of NF-κB and AP-1 in COX-2 upregulation by human papillomavirus 16 E5 oncoprotein. Carcinogenesis 30, 753–757. https://doi.org/10.1093/carcin/bgp066

Knight, G.L., Grainger, J.R., Gallimore, P.H., Roberts, S., 2004. Cooperation between Different Forms of the Human Papillomavirus Type 1 E4 Protein To Block Cell Cycle Progression and Cellular DNA Synthesis. J Virol 78, 13920–13933. https://doi.org/10.1128/JVI.78.24.13920-13933.2004

Kotnik Halavaty, K., Regan, J., Mehta, K., Laimins, L., 2014. Human papillomavirus E5 oncoproteins bind the A4 endoplasmic reticulum protein to regulate proliferative ability upon differentiation. Virology 452–453, 223–230. https://doi.org/10.1016/j.virol.2014.01.013

Kowli, S., Velidandla, R., Creek, K.E., Pirisi, L., 2013. TGF-β regulation of gene expression at early and late stages of HPV16-mediated transformation of human keratinocytes. Virology 447, 63–73. https://doi.org/10.1016/j.virol.2013.08.034

Krawczyk, E., Suprynowicz, F.A., Sudarshan, S.R., Schlegel, R., 2010. Membrane Orientation of the Human Papillomavirus Type 16 E5 Oncoprotein. J. Virol. 84, 1696–1703. https://doi.org/10.1128/JVI.01968-09

Kreimer, A.R., Clifford, G.M., Boyle, P., Franceschi, S., 2005. Human Papillomavirus Types in Head and Neck Squamous Cell Carcinomas Worldwide: A Systematic Review. Cancer Epidemiol Biomarkers Prev 14, 467–475. https://doi.org/10.1158/1055-9965.EPI-04-0551

Kyo, S., Inoue, M., Hayasaka, N., Inoue, T., Yutsudo, M., Tanizawa, O., Hakura, A., 1994. Regulation of Early Gene Expression of Human Papillomavirus Type 16 by Inflammatory Cytokines. Virology 200, 130–139. https://doi.org/10.1006/viro.1994.1171

Lancini, D., Faddy, H.M., Flower, R., Hogan, C., 2014. Cytomegalovirus disease in immunocompetent adults. Med. J. Aust. 201, 578–580.

Lau, L., Gray, E.E., Brunette, R.L., Stetson, D.B., 2015. DNA tumor virus oncogenes antagonize the cGAS-STING DNA-sensing pathway. Science 350, 568–571. https://doi.org/10.1126/science.aab3291

Lazarczyk, M., Pons, C., Mendoza, J.-A., Cassonnet, P., Jacob, Y., Favre, M., 2008. Regulation of cellular zinc balance as a potential mechanism of EVER-mediated protection against pathogenesis by cutaneous oncogenic human papillomaviruses. J. Exp. Med. 205, 35–42. https://doi.org/10.1084/jem.20071311

Lazarow, P.B., Fujiki, Y., 1985. Biogenesis of peroxisomes. Annu. Rev. Cell Biol. 1, 489–530. https://doi.org/10.1146/annurev.cb.01.110185.002421

Leechanachai, P., Banks, L., Moreau, F., 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.

Lembo, D., Donalisio, M., De Andrea, M., Cornaglia, M., Scutera, S., Musso, T., Landolfo, S., 2006. A cell-based high-throughput assay for screening inhibitors of human papillomavirus-16 long control region activity. FASEB J. 20, 148–150. https://doi.org/10.1096/fj.05-3904fje

Lewis, C., Baro, M.F., Marques, M., Grüner, M., Alonso, A., Bravo, I.G., 2008. The first hydrophobic region of the HPV16 E5 protein determines protein cellular location and facilitates anchorage-independent growth. Virol J 5, 30. https://doi.org/10.1186/1743-422X-5-30

Li, S., Labrecque, S., Gauzzi, M.C., Cuddihy, A.R., Wong, A.H., Pellegrini, S., Matlashewski, G.J., Koromilas, A.E., 1999. The human papilloma virus (HPV)-18 E6 oncoprotein physically associates with Tyk2 and impairs Jak-STAT activation by interferon-alpha. Oncogene 18, 5727–5737. https://doi.org/10.1038/sj.onc.1202960

Li, X., 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, X.-D., Wu, J., Gao, D., Wang, H., Sun, L., Chen, Z.J., 2013. Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. Science 341, 1390–1394. https://doi.org/10.1126/science.1244040

Liao, S., Deng, D., Hu, X., Wang, W., Li, L., Li, W., Zhou, J., Xu, G., Meng, L., Wang, S., Ma, D., 2013a. HPV16/18 E5, a promising candidate for cervical cancer vaccines, affects SCPs, cell

proliferation and cell cycle, and forms a potential network with E6 and E7. Int. J. Mol. Med. 31, 120–128. https://doi.org/10.3892/ijmm.2012.1168

Liao, S., Deng, D., Zhang, W., Hu, X., Wang, W., Wang, H., Lu, Y., Wang, S., Meng, L., Ma, D., 2013b. Human papillomavirus 16/18 E5 promotes cervical cancer cell proliferation, migration and invasion in vitro and accelerates tumor growth in vivo. Oncol. Rep. 29, 95–102. https://doi.org/10.3892/or.2012.2106

Limaye, A.P., Kirby, K.A., Rubenfeld, G.D., Leisenring, W.M., Bulger, E.M., Neff, M.J., Gibran, N.S., Huang, M.-L., Santo Hayes, T.K., Corey, L., Boeckh, M., 2008. Cytomegalovirus reactivation in critically ill immunocompetent patients. JAMA 300, 413–422. https://doi.org/10.1001/jama.300.4.413

Liu, C., Lin, J., Li, L., Zhang, Y., Chen, W., Cao, Z., Zuo, H., Chen, C., Kee, K., 2015. HPV16 early gene E5 specifically reduces miRNA-196a in cervical cancer cells. Sci Rep 5. https://doi.org/10.1038/srep07653

Liu, W.J., Gissmann, L., Sun, X.Y., Kanjanahaluethai, A., Müller, M., Doorbar, J., 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. https://doi.org/10.1006/viro.1996.8348

Longworth, M.S., Laimins, L.A., 2004. Pathogenesis of Human Papillomaviruses in Differentiating Epithelia. Microbiol Mol Biol Rev 68, 362–372. https://doi.org/10.1128/MMBR.68.2.362-372.2004

Loo, Y.-M., Gale, M., 2011. Immune signaling by RIG-I-like receptors. Immunity 34, 680–692. https://doi.org/10.1016/j.immuni.2011.05.003

Magalhães, A.C., Ferreira, A.R., Gomes, S., Vieira, M., Gouveia, A., Valença, I., Islinger, M., Nascimento, R., Schrader, M., Kagan, J.C., Ribeiro, D., 2016. Peroxisomes are platforms for cytomegalovirus' evasion from the cellular immune response. Sci Rep 6, 26028. https://doi.org/10.1038/srep26028

Maglennon, G.A., McIntosh, P., Doorbar, J., 2011. Persistence of viral DNA in the epithelial basal layer suggests a model for papillomavirus latency following immune regression. Virology 414, 153–163. https://doi.org/10.1016/j.virol.2011.03.019

Maglennon, G.A., McIntosh, P.B., Doorbar, J., 2014. Immunosuppression Facilitates the Reactivation of Latent Papillomavirus Infections. J Virol 88, 710–716. https://doi.org/10.1128/JVI.02589-13

Marques, M., Ferreira, A.R., Ribeiro, D., 2018. The Interplay between Human Cytomegalovirus and Pathogen Recognition Receptor Signaling. Viruses 10. https://doi.org/10.3390/v10100514

Marur, S., D'Souza, G., Westra, W.H., Forastiere, A.A., 2010. HPV-associated head and neck cancer: a virus-related cancer epidemic. The Lancet Oncology 11, 781–789. https://doi.org/10.1016/S1470-2045(10)70017-6

Masson, M., Hindelang, C., Sibler, A.-P., Schwalbach, G., Travé, G., Weiss, E., 2003. Preferential nuclear localization of the human papillomavirus type 16 E6 oncoprotein in cervical carcinoma cells. Journal of General Virology 84, 2099–2104. https://doi.org/10.1099/vir.0.18961-0

Maufort, J.P., Williams, S.M.G., Pitot, H.C., Lambert, P.F., 2007. Human Papillomavirus 16 E5 Oncogene Contributes to Two Stages of Skin Carcinogenesis. Cancer Res 67, 6106–6112. https://doi.org/10.1158/0008-5472.CAN-07-0921

McBride, A.A., 2008. Replication and Partitioning of Papillomavirus Genomes. Adv Virus Res 72, 155–205. https://doi.org/10.1016/S0065-3527(08)00404-1

McCormick, A.L., Smith, V.L., Chow, D., Mocarski, E.S., 2003. Disruption of Mitochondrial Networks by the Human Cytomegalovirus UL37 Gene Product Viral Mitochondrion-Localized Inhibitor of Apoptosis. Journal of Virology 77, 631–641. https://doi.org/10.1128/JVI.77.1.631-641.2003

McKinney, C.C., Kim, M.J., Chen, D., McBride, A.A., 2016. Brd4 Activates Early Viral Transcription upon Human Papillomavirus 18 Infection of Primary Keratinocytes. mBio 7. https://doi.org/10.1128/mBio.01644-16

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R., Tschopp, J., 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature 437, 1167–1172. https://doi.org/10.1038/nature04193

Mirkovic, J., Howitt, B.E., Roncarati, P., Demoulin, S., Suarez-Carmona, M., Hubert, P., McKeon, F.D., Xian, W., Lee, A., Delvenne, P., Crum, C.P., Herfs, M., 2015. Carcinogenic HPV infection in the cervical squamo-columnar junction. J Pathol 236, 265–271. https://doi.org/10.1002/path.4533

Mogensen, T.H., 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin. Microbiol. Rev. 22, 240–273, Table of Contents. https://doi.org/10.1128/CMR.00046-08

Moody, C.A., Laimins, L.A., 2010. Human papillomavirus oncoproteins: pathways to transformation. Nat. Rev. Cancer 10, 550–560. https://doi.org/10.1038/nrc2886

Morale, M.G., Abjaude, W. da S., Silva, A.M., Villa, L.L., Boccardo, E., 2018. HPV-transformed cells exhibit altered HMGB1-TLR4/MyD88-SARM1 signaling axis. Scientific Reports 8, 3476. https://doi.org/10.1038/s41598-018-21416-8

Morgan, E.L., Wasson, C.W., Hanson, L., Kealy, D., Pentland, I., McGuire, V., Scarpini, C., Coleman, N., Arthur, J.S.C., Parish, J.L., Roberts, S., Macdonald, A., 2018. STAT3 activation by E6 is essential for the differentiation-dependent HPV18 life cycle. PLoS Pathog 14. https://doi.org/10.1371/journal.ppat.1006975

Müller, M., Wasson, C.W., Bhatia, R., Boxall, S., Millan, D., Goh, G.Y.S., Haas, J., Stonehouse, N.J., Macdonald, A., 2015. YIP1 family member 4 (YIPF4) is a novel cellular binding partner of the papillomavirus E5 proteins. Scientific Reports 5, 12523. https://doi.org/10.1038/srep12523

Münger, K., Werness, B.A., Dyson, N., Phelps, W.C., Harlow, E., Howley, P.M., 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. 8, 4099–4105.

Muto, V., Stellacci, E., Lamberti, A.G., Perrotti, E., Carrabba, A., Matera, G., Sgarbanti, M., Battistini, A., Liberto, M.C., Focà, A., 2011. Human Papillomavirus Type 16 E5 Protein Induces Expression of Beta Interferon through Interferon Regulatory Factor 1 in Human Keratinocytes. J. Virol. 85, 5070–5080. https://doi.org/10.1128/JVI.02114-10

Nees, M., Geoghegan, J.M., Hyman, T., Frank, S., Miller, L., Woodworth, C.D., 2001. Papillomavirus type 16 oncogenes downregulate expression of interferon-responsive genes and upregulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes. J. Virol. 75, 4283–4296. https://doi.org/10.1128/JVI.75.9.4283-4296.2001

Nishio, M., Tajima, O., Furukawa, Keiko, Urano, T., Furukawa, Koichi, 2005. Over-expression of GM1 enhances cell proliferation with epidermal growth factor without affecting the receptor localization in the microdomain in PC12 cells. Int. J. Oncol. 26, 191–199.

Odendall, C., Dixit, E., Stavru, F., Bierne, H., Franz, K.M., Fiegen, A., Boulant, S., Gehrke, L., Cossart, P., Kagan, J.C., 2014. Diverse intracellular pathogens activate Type III Interferon expression from peroxisomes. Nat Immunol 15, 717–726. https://doi.org/10.1038/ni.2915

Oelze, I., Kartenbeck, J., Crusius, K., Alonso, A., 1995. Human papillomavirus type 16 E5 protein affects cell-cell communication in an epithelial cell line. J Virol 69, 4489–4494.

Oetke, C., Auvinen, E., Pawlita, M., Alonso, A., 2000. Human papillomavirus type 16 E5 protein localizes to the Golgi apparatus but does not grossly affect cellular glycosylation. Arch. Virol. 145, 2183–2191.

Ogawa-Goto, K., Tanaka, K., Gibson, W., Moriishi, E., Miura, Y., Kurata, T., Irie, S., Sata, T., 2003. Microtubule network facilitates nuclear targeting of human cytomegalovirus capsid. J. Virol. 77, 8541–8547.

Oh, J.-M., Kim, S.-H., Cho, E.-A., Song, Y.-S., Kim, W.-H., Juhnn, Y.-S., 2010. Human papillomavirus type 16 E5 protein inhibits hydrogen-peroxide-induced apoptosis by stimulating ubiquitin-proteasome-mediated degradation of Bax in human cervical cancer cells. Carcinogenesis 31, 402–410. https://doi.org/10.1093/carcin/bgp318

Okamoto, K., Shaw, J.M., 2005. Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. Annu. Rev. Genet. 39, 503–536. https://doi.org/10.1146/annurev.genet.38.072902.093019

Oshiumi, H., Miyashita, M., Matsumoto, M., Seya, T., 2013. A Distinct Role of Riplet-Mediated K63-Linked Polyubiquitination of the RIG-I Repressor Domain in Human Antiviral Innate Immune Responses. PLoS Pathog 9. https://doi.org/10.1371/journal.ppat.1003533

Ozsaran, A.A., Ateş, T., Dikmen, Y., Zeytinoglu, A., Terek, C., Erhan, Y., Ozacar, T., Bilgiç, A., 1999. Evaluation of the risk of cervical intraepithelial neoplasia and human papilloma virus infection in renal transplant patients receiving immunosuppressive therapy. Eur. J. Gynaecol. Oncol. 20, 127–130.

Paludan, S.R., Bowie, A.G., 2013. Immune sensing of DNA. Immunity 38, 870. https://doi.org/10.1016/j.immuni.2013.05.004

Paolini, F., Curzio, G., Cordeiro, M.N., Massa, S., Mariani, L., Pimpinelli, F., de Freitas, A.C., Franconi, R., Venuti, A., 2017. HPV 16 E5 oncoprotein is expressed in early stage carcinogenesis and can be a target of immunotherapy. Hum Vaccin Immunother 13, 291–297. https://doi.org/10.1080/21645515.2017.1264777

Park, J.S., Kim, E.J., Kwon, H.J., Hwang, E.S., Namkoong, S.E., Um, S.J., 2000. Inactivation of interferon regulatory factor-1 tumor suppressor protein by HPV E7 oncoprotein. Implication for the

E7-mediated immune evasion mechanism in cervical carcinogenesis. J. Biol. Chem. 275, 6764–6769.

Patel, D., Huang, S.M., Baglia, L.A., McCance, D.J., 1999. The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300. EMBO J 18, 5061–5072. https://doi.org/10.1093/emboj/18.18.5061

Pedroza-Saavedra, A., Lam, E.W.-F., Esquivel-Guadarrama, F., Gutierrez-Xicotencatl, L., 2010. The human papillomavirus type 16 E5 oncoprotein synergizes with EGF-receptor signaling to enhance cell cycle progression and the down-regulation of p27(Kip1). Virology 400, 44–52. https://doi.org/10.1016/j.virol.2010.01.009

Perea, S.E., Massimi, P., Banks, L., 2000. Human papillomavirus type 16 E7 impairs the activation of the interferon regulatory factor-1. Int. J. Mol. Med. 5, 661–666.

Pett, M., Coleman, N., 2007. Integration of high-risk human papillomavirus: a key event in cervical carcinogenesis? J. Pathol. 212, 356–367. https://doi.org/10.1002/path.2192

Pim, D., Collins, M., Banks, L., 1992. Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. Oncogene 7, 27–32.

Plummer, M., Schiffman, M., Castle, P.E., Maucort-Boulch, D., Wheeler, C.M., ALTS Group, 2007. A 2-year prospective study of human papillomavirus persistence among women with a cytological diagnosis of atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesion. J. Infect. Dis. 195, 1582–1589. https://doi.org/10.1086/516784

Pyeon, D., Pearce, S.M., Lank, S.M., Ahlquist, P., Lambert, P.F., 2009. Establishment of human papillomavirus infection requires cell cycle progression. PLoS Pathog. 5, e1000318. https://doi.org/10.1371/journal.ppat.1000318

Raj, K., Berguerand, S., Southern, S., Doorbar, J., Beard, P., 2004. E1^E4 Protein of Human Papillomavirus Type 16 Associates with Mitochondria. J. Virol. 78, 7199–7207. https://doi.org/10.1128/JVI.78.13.7199-7207.2004

Ranieri, D., Belleudi, F., Magenta, A., Torrisi, M.R., 2015. HPV16 E5 expression induces switching from FGFR2b to FGFR2c and epithelial-mesenchymal transition. Int. J. Cancer 137, 61–72. https://doi.org/10.1002/ijc.29373

Regan, J.A., Laimins, L.A., 2008. Bap31 Is a Novel Target of the Human Papillomavirus E5 Protein. J. Virol. 82, 10042–10051. https://doi.org/10.1128/JVI.01240-08

Reinholz, M., Kawakami, Y., Salzer, S., Kreuter, A., Dombrowski, Y., Koglin, S., Kresse, S., Ruzicka, T., Schauber, J., 2013. HPV16 activates the AIM2 inflammasome in keratinocytes. Arch Dermatol Res 305, 723–732. https://doi.org/10.1007/s00403-013-1375-0

Reinson, T., Henno, L., Toots, M., Ustav, M., Ustav, M., 2015. The Cell Cycle Timing of Human Papillomavirus DNA Replication. PLoS ONE 10, e0131675. https://doi.org/10.1371/journal.pone.0131675

Reiser, J., Hurst, J., Voges, M., Krauss, P., Münch, P., Iftner, T., Stubenrauch, F., 2011. High-risk human papillomaviruses repress constitutive kappa interferon transcription via E6 to prevent pathogen recognition receptor and antiviral-gene expression. J. Virol. 85, 11372–11380. https://doi.org/10.1128/JVI.05279-11

Richards, K.H., Wasson, C.W., Watherston, O., Doble, R., Blair, G.E., Wittmann, M., Macdonald, A., 2015. The human papillomavirus (HPV) E7 protein antagonises an Imiquimod-induced inflammatory pathway in primary human keratinocytes. Sci Rep 5, 12922. https://doi.org/10.1038/srep12922

Richards, R.M., Lowy, D.R., Schiller, J.T., Day, P.M., 2006. Cleavage of the papillomavirus minor capsid protein, L2, at a furin consensus site is necessary for infection. Proc Natl Acad Sci U S A 103, 1522–1527. https://doi.org/10.1073/pnas.0508815103

Rodríguez, A.C., Schiffman, M., Herrero, R., Wacholder, S., Hildesheim, A., Castle, P.E., Solomon, D., Burk, R., 2008. Rapid Clearance of Human Papillomavirus and Implications for Clinical Focus on Persistent Infections. J Natl Cancer Inst 100, 513–517. https://doi.org/10.1093/jnci/djn044

Rodríguez, M.I., Finbow, M.E., Alonso, A., 2000. Binding of human papillomavirus 16 E5 to the 16 kDa subunit c (proteolipid) of the vacuolar H+-ATPase can be dissociated from the E5-mediated epidermal growth factor receptor overactivation. Oncogene 19, 3727–3732. https://doi.org/10.1038/sj.onc.1203718

Ronco, L.V., Karpova, A.Y., Vidal, M., Howley, P.M., 1998. Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. Genes Dev. 12, 2061–2072.

Rozenblatt-Rosen, O., Deo, R.C., Padi, M., Adelmant, G., Calderwood, M.A., Rolland, T., Grace, M., Dricot, A., Askenazi, M., Tavares, M., Pevzner, S.J., Abderazzaq, F., Byrdsong, D., Carvunis, A.-R., Chen, A.A., Cheng, J., Correll, M., Duarte, M., Fan, C., Feltkamp, M.C., Ficarro, S.B., Franchi, R., Garg, B.K., Gulbahce, N., Hao, T., Holthaus, A.M., James, R., Korkhin, A., Litovchick, L., Mar, J.C., Pak, T.R., Rabello, S., Rubio, R., Shen, Y., Singh, S., Spangle, J.M., Tasan, M., Wanamaker, S., Webber, J.T., Roecklein-Canfield, J., Johannsen, E., Barabási, A.-L., Beroukhim, R., Kieff, E., Cusick, M.E., Hill, D.E., Münger, K., Marto, J.A., Quackenbush, J., Roth, F.P., DeCaprio, J.A., Vidal, M., 2012. Interpreting cancer genomes using systematic host network perturbations by tumour virus proteins. Nature 487, 491–495. https://doi.org/10.1038/nature11288

Sahab, Z., Sudarshan, S.R., Liu, X., Zhang, Y., Kirilyuk, A., Kamonjoh, C.M., Simic, V., Dai, Y., Byers, S.W., Doorbar, J., Suprynowicz, F.A., Schlegel, R., 2012. Quantitative measurement of human papillomavirus type 16 e5 oncoprotein levels in epithelial cell lines by mass spectrometry. J. Virol. 86, 9465–9473. https://doi.org/10.1128/JVI.01032-12

Sanchez, V., Greis, K.D., Sztul, E., Britt, W.J., 2000. Accumulation of Virion Tegument and Envelope Proteins in a Stable Cytoplasmic Compartment during Human Cytomegalovirus Replication: Characterization of a Potential Site of Virus Assembly. J Virol 74, 975–986.

Sanders, C.M., 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. https://doi.org/10.1093/emboj/17.23.7044

Sato, H., Watanabe, S., Furuno, A., Yoshiike, K., 1989. Human papillomavirus type 16 E7 protein expressed in Escherichia coli and monkey COS-1 Cells: Immunofluorescence detection of the nuclear E7 protein. Virology 170, 311–315. https://doi.org/10.1016/0042-6822(89)90386-3

Schapiro, F., Sparkowski, J., Adduci, A., Suprynowicz, F., Schlegel, R., Grinstein, S., 2000. Golgi alkalinization by the papillomavirus E5 oncoprotein. J. Cell Biol. 148, 305–315.

Scheffner, M., Huibregtse, J.M., Vierstra, R.D., Howley, P.M., 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75, 495–505.

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

Schelhaas, M., Shah, B., Holzer, M., Blattmann, P., Kühling, L., Day, P.M., Schiller, J.T., Helenius, A., 2012. Entry of human papillomavirus type 16 by actin-dependent, clathrin- and lipid raft-independent endocytosis. PLoS Pathog. 8, e1002657. https://doi.org/10.1371/journal.ppat.1002657

Schiffman, M., Castle, P.E., Jeronimo, J., Rodriguez, A.C., Wacholder, S., 2007. Human papillomavirus and cervical cancer. The Lancet 370, 890–907. https://doi.org/10.1016/S0140-6736(07)61416-0

Schiffman, M., Doorbar, J., Wentzensen, N., de Sanjosé, S., Fakhry, C., Monk, B.J., Stanley, M.A., Franceschi, S., 2016. Carcinogenic human papillomavirus infection. Nat Rev Dis Primers 2, 16086. https://doi.org/10.1038/nrdp.2016.86

Schiffman, M., Herrero, R., Desalle, R., Hildesheim, A., Wacholder, S., Rodriguez, A.C., Bratti, M.C., Sherman, M.E., Morales, J., Guillen, D., Alfaro, M., Hutchinson, M., Wright, T.C., Solomon, D., Chen, Z., Schussler, J., Castle, P.E., Burk, R.D., 2005. The carcinogenicity of human papillomavirus types reflects viral evolution. Virology 337, 76–84. https://doi.org/10.1016/j.virol.2005.04.002

Schrader, M., 2006. Shared components of mitochondrial and peroxisomal division. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, Mitochondrial Dynamics in Cell Life and Death 1763, 531–541. https://doi.org/10.1016/j.bbamcr.2006.01.004

Schuck, S., Stenlund, A., 2005. Assembly of a double hexameric helicase. Mol. Cell 20, 377–389. https://doi.org/10.1016/j.molcel.2005.09.020

Schwartz, S., 2013. Papillomavirus transcripts and posttranscriptional regulation. Virology 445, 187–196. https://doi.org/10.1016/j.virol.2013.04.034

Schwarz, E., Freese, U.K., Gissmann, L., Mayer, W., Roggenbuck, B., Stremlau, A., Hausen, H. zur, 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature 314, 111. https://doi.org/10.1038/314111a0

Scott, M., Nakagawa, M., Moscicki, A.-B., 2001. Cell-Mediated Immune Response to Human Papillomavirus Infection. Clin. Diagn. Lab. Immunol. 8, 209–220. https://doi.org/10.1128/CDLI.8.2.209-220.2001

Seth, R.B., Sun, L., Chen, Z.J., 2006. Antiviral innate immunity pathways. Cell Research 16, 141. https://doi.org/10.1038/sj.cr.7310019

Seth, R.B., Sun, L., Ea, C.-K., Chen, Z.J., 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell 122, 669–682. https://doi.org/10.1016/j.cell.2005.08.012

Sharon-Friling, R., Goodhouse, J., Colberg-Poley, A.M., Shenk, T., 2006. Human cytomegalovirus pUL37x1 induces the release of endoplasmic reticulum calcium stores. Proc. Natl. Acad. Sci. U.S.A. 103, 19117–19122. https://doi.org/10.1073/pnas.0609353103

Simeone, P., Teson, M., Latini, A., Carducci, M., Venuti, A., 2004. Endothelin-1 could be one of the targets of psoriasis therapy. Br. J. Dermatol. 151, 1273–1275. https://doi.org/10.1111/j.1365-2133.2004.06277.x

Smotkin, D., Wettstein, F.O., 1987. The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. J. Virol. 61, 1686–1689.

Spitkovsky, D., Hehner, S.P., Hofmann, T.G., Möller, A., Schmitz, M.L., 2002. The human papillomavirus oncoprotein E7 attenuates NF-kappa B activation by targeting the Ikappa B kinase complex. J. Biol. Chem. 277, 25576–25582. https://doi.org/10.1074/jbc.M201884200

Stern-Ginossar, N., Weisburd, B., Michalski, A., Le, V.T.K., Hein, M.Y., Huang, S.-X., Ma, M., Shen, B., Qian, S.-B., Hengel, H., Mann, M., Ingolia, N.T., Weissman, J.S., 2012. Decoding human cytomegalovirus. Science 338, 1088–1093. https://doi.org/10.1126/science.1227919

Stöppler, M.C., Straight, S.W., Tsao, G., Schlegel, R., McCance, D.J., 1996. The E5 gene of HPV-16 enhances keratinocyte immortalization by full-length DNA. Virology 223, 251–254. https://doi.org/10.1006/viro.1996.0475

Straight, S.W., Herman, B., McCance, D.J., 1995. The E5 oncoprotein of human papillomavirus type 16 inhibits the acidification of endosomes in human keratinocytes. J. Virol. 69, 3185–3192.

Straight, S.W., Hinkle, P.M., Jewers, R.J., McCance, D.J., 1993. The E5 oncoprotein of human papillomavirus type 16 transforms fibroblasts and effects the downregulation of the epidermal growth factor receptor in keratinocytes. J. Virol. 67, 4521–4532.

Sunthamala, N., Thierry, F., Teissier, S., Pientong, C., Kongyingyoes, B., Tangsiriwatthana, T., Sangkomkamhang, U., Ekalaksananan, T., 2014. E2 proteins of high risk human papillomaviruses down-modulate STING and IFN-κ transcription in keratinocytes. PLoS ONE 9, e91473. https://doi.org/10.1371/journal.pone.0091473

Suprynowicz, F.A., Disbrow, G.L., Krawczyk, E., Simic, V., Lantzky, K., Schlegel, R., 2008. HPV-16 E5 oncoprotein upregulates lipid raft components caveolin-1 and ganglioside GM1 at the plasma membrane of cervical cells. Oncogene 27, 1071–1078. https://doi.org/10.1038/sj.onc.1210725

Suprynowicz, F.A., Krawczyk, E., Hebert, J.D., Sudarshan, S.R., Simic, V., Kamonjoh, C.M., Schlegel, R., 2010. The Human Papillomavirus Type 16 E5 Oncoprotein Inhibits Epidermal Growth Factor Trafficking Independently of Endosome Acidification. J Virol 84, 10619–10629. https://doi.org/10.1128/JVI.00831-10

Takami, Y., Nakagami, H., Morishita, R., Katsuya, T., Cui, T.-X., Ichikawa, T., Saito, Y., Hayashi, H., Kikuchi, Y., Nishikawa, T., Baba, Y., Yasuda, O., Rakugi, H., Ogihara, T., Kaneda, Y., 2007. Ubiquitin carboxyl-terminal hydrolase L1, a novel deubiquitinating enzyme in the vasculature, attenuates NF-kappaB activation. Arterioscler. Thromb. Vasc. Biol. 27, 2184–2190. https://doi.org/10.1161/ATVBAHA.107.142505

Takeuchi, O., Akira, S., 2010. Pattern recognition receptors and inflammation. Cell 140, 805–820. https://doi.org/10.1016/j.cell.2010.01.022

Thomsen, P., van Deurs, B., Norrild, B., Kayser, L., 2000. The HPV16 E5 oncogene inhibits endocytic trafficking. Oncogene 19, 6023–6032. https://doi.org/10.1038/sj.onc.1204010

Tomakidi, P., Cheng, H., Kohl, A., Komposch, G., Alonso, A., 2000. Connexin 43 expression is downregulated in raft cultures of human keratinocytes expressing the human papillomavirus type 16 E5 protein. Cell Tissue Res. 301, 323–327.

Tsao, Y.P., Li, L.Y., Tsai, T.C., Chen, S.L., 1996. Human papillomavirus type 11 and 16 E5 represses p21(WafI/SdiI/CipI) gene expression in fibroblasts and keratinocytes. J. Virol. 70, 7535–7539.

Tummers, B., Burg, S.H.V.D., 2015. High-risk human papillomavirus targets crossroads in immune signaling. Viruses 7, 2485–2506. https://doi.org/10.3390/v7052485

Van Tine, B.A., Kappes, J.C., Banerjee, N.S., Knops, J., Lai, L., Steenbergen, R.D.M., Meijer, C.L.J.M., Snijders, P.J.F., Chatis, P., Broker, T.R., Moen, P.T., Chow, L.T., 2004. Clonal selection for transcriptionally active viral oncogenes during progression to cancer. J. Virol. 78, 11172–11186. https://doi.org/10.1128/JVI.78.20.11172-11186.2004

Varnum, S.M., Streblow, D.N., Monroe, M.E., Smith, P., Auberry, K.J., Pasa-Tolic, L., Wang, D., Camp, D.G., Rodland, K., Wiley, S., Britt, W., Shenk, T., Smith, R.D., Nelson, J.A., 2004. Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome. J. Virol. 78, 10960–10966. https://doi.org/10.1128/JVI.78.20.10960-10966.2004

Vartanian, J.-P., Guétard, D., Henry, M., Wain-Hobson, S., 2008. Evidence for Editing of Human Papillomavirus DNA by APOBEC3 in Benign and Precancerous Lesions. Science 320, 230–233. https://doi.org/10.1126/science.1153201

Venuti, A., Salani, D., Poggiali, F., Manni, V., Bagnato, A., 1998. The E5 oncoprotein of human papillomavirus type 16 enhances endothelin-1-induced keratinocyte growth. Virology 248, 1–5. https://doi.org/10.1006/viro.1998.9227

Villarino, A.V., Kanno, Y., O'Shea, J.J., 2015. Mechanisms of Jak/STAT signaling in immunity and disease. J Immunol 194, 21–27. https://doi.org/10.4049/jimmunol.1401867

Wack, A., Terczyńska-Dyla, E., Hartmann, R., 2015. Guarding the frontiers: The biology of type III interferons. Nature immunology 16, 802–9. https://doi.org/10.1038/ni.3212

Wanders, R.J.A., 2004. Peroxisomes, lipid metabolism, and peroxisomal disorders. Mol. Genet. Metab. 83, 16–27. https://doi.org/10.1016/j.ymgme.2004.08.016

Warren, C.J., Xu, T., Guo, K., Griffin, L.M., Westrich, J.A., Lee, D., Lambert, P.F., Santiago, M.L., Pyeon, D., 2015. APOBEC3A Functions as a Restriction Factor of Human Papillomavirus. J. Virol. 89, 688–702. https://doi.org/10.1128/JVI.02383-14

Wiens, M.E., Smith, J.G., 2015. Alpha-Defensin HD5 Inhibits Furin Cleavage of Human Papillomavirus 16 L2 To Block Infection. J. Virol. 89, 2866–2874. https://doi.org/10.1128/JVI.02901-14

Wille, P.T., Wisner, T.W., Ryckman, B., Johnson, D.C., 2013. Human Cytomegalovirus (HCMV) Glycoprotein gB Promotes Virus Entry In Trans Acting as the Viral Fusion Protein Rather than as a Receptor-Binding Protein. mBio 4, e00332-13. https://doi.org/10.1128/mBio.00332-13

Woodby, B.L., Songock, W.K., Scott, M.L., Raikhy, G., Bodily, J.M., 2018. Induction of Interferon Kappa in Human Papillomavirus 16 Infection by Transforming Growth Factor Beta-Induced Promoter Demethylation. J. Virol. 92. https://doi.org/10.1128/JVI.01714-17

Wüstenhagen, E., Boukhallouk, F., Negwer, I., Rajalingam, K., Stubenrauch, F., Florin, L., 2018. The Myb-related protein MYPOP is a novel intrinsic host restriction factor of oncogenic human papillomaviruses. Oncogene 1. https://doi.org/10.1038/s41388-018-0398-6

Xu, H., He, X., Zheng, H., Huang, L.J., Hou, F., Yu, Z., de la Cruz, M.J., Borkowski, B., Zhang, X., Chen, Z.J., Jiang, Q.-X., 2014. Structural basis for the prion-like MAVS filaments in antiviral innate immunity. Elife 3, e01489. https://doi.org/10.7554/eLife.01489

Xu, L.-G., Wang, Y.-Y., Han, K.-J., Li, L.-Y., Zhai, Z., Shu, H.-B., 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. Mol. Cell 19, 727–740. https://doi.org/10.1016/j.molcel.2005.08.014

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y.-M., Gale, M., Akira, S., Yonehara, S., Kato, A., Fujita, T., 2005. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J. Immunol. 175, 2851–2858.

Yu, T., Ferber, M.J., Cheung, T.H., Chung, T.K.H., Wong, Y.F., Smith, D.I., 2005. The role of viral integration in the development of cervical cancer. Cancer Genet. Cytogenet. 158, 27–34. https://doi.org/10.1016/j.cancergencyto.2004.08.021

Zamorano Cuervo, N., Osseman, Q., Grandvaux, N., 2018. Virus Infection Triggers MAVS Polymers of Distinct Molecular Weight. Viruses 10. https://doi.org/10.3390/v10020056

Zeng, W., Sun, L., Jiang, X., Chen, X., Hou, F., Adhikari, A., Xu, M., Chen, Z.J., 2010. Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. Cell 141, 315–330. https://doi.org/10.1016/j.cell.2010.03.029

Zhang, B., Li, P., Wang, E., Brahmi, Z., Dunn, K.W., Blum, J.S., Roman, A., 2003. The E5 protein of human papillomavirus type 16 perturbs MHC class II antigen maturation in human foreskin keratinocytes treated with interferon-gamma. Virology 310, 100–108.

Zhang, B., Spandau, D.F., Roman, A., 2002. E5 protein of human papillomavirus type 16 protects human foreskin keratinocytes from UV B-irradiation-induced apoptosis. J. Virol. 76, 220–231.

Zhang, B., Srirangam, A., Potter, D.A., Roman, A., 2005. HPV16 E5 protein disrupts the c-Cbl-EGFR interaction and EGFR ubiquitination in human foreskin keratinocytes. Oncogene 24, 2585–2588. https://doi.org/10.1038/sj.onc.1208453

Zhong, B., Yang, Y., Li, S., Wang, Y.-Y., Li, Y., Diao, F., Lei, C., He, X., Zhang, L., Tien, P., Shu, H.-B., 2008. The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. Immunity 29, 538–550. https://doi.org/10.1016/j.immuni.2008.09.003

zur Hausen, H., 2002. Papillomaviruses and cancer: from basic studies to clinical application. Nat. Rev. Cancer 2, 342–350. https://doi.org/10.1038/nrc798

zur Hausen, H., 1999. Immortalization of human cells and their malignant conversion by high risk human papillomavirus genotypes. Semin. Cancer Biol. 9, 405–411. https://doi.org/10.1006/scbi.1999.0144

Appendix A

Chemicals and reagents

Acetic Acid, Merck Millipore

Acrylamide, Fisher Scientific

Agar, Formedium

Agarose, Roth

Ammonium Persulfate (APS), Sigma-Aldrich

Ampicillin, Sigma-Aldrich

Bovine serum albumin (BSA), NZYTech

Bromophenol Blue, Sigma-Aldrich

Chloroform, Merck Millipore

Dimetilsulfóxido (DMSO), Sigma-Aldrich

Dithiothreitol (DTT), New England Biolabs

Ethanol, Merck Millipore

Ethylenediaminetetraacetic acid (EDTA), Sigma-Aldrich

Foy (Camostat 305), Schwarz-Pharma

Glycerin, Roth

Glycine, Fisher Scientific

Hydrochloric acid (HCl) 37%, Merck Millipore

Isopropanol, Merck Millipore

Kanamycin (Kan), Sigma-Aldrich

Lysogeny broth (LB), Fisher Scientific

Methanol, Merck Millipore

Midori Green Advance, Nippon Genetics

Milk powder, Nestlé

Mowiol, Applichem

N-propyl-gallate, Fluka

Paraformaldehyde (PFA), Sigma-Aldrich

Potassium chloride (KCl), Sigma-Aldrich

Phenylmethylsulfonyl fluoride (PMSF), Sigma-Aldrich

RNAse free water, Fisher Scientific

Sodium chloride (NaCl), Sigma-Aldrich

Sodium dodecyl sulfate (SDS), Sigma-Aldrich

Sodium phosphate (NaHPO4), Sigma-Aldrich

iTaq™ Universal SYBR® Green Supermix, BioRad

Tetramethylethylenediamine (TEMED), Fluka

Trifast, PeqLab

Tris, Fisher Scientific

Trasylol (Aprotinin), Bayer

Triton, Sigma-Aldrich

Tween-20, Sigma-Aldrich

β-Mercaptoethanol, Sigma-Aldrich

RNase

Appendix B

Solutions, buffers and gels

1x PBS

137 mM NaCl, 8 mM $NaHPO_4$, 2,68 mM KCl, 1,47 mM KH_2PO_4, pH 7,34 (prepared from a 10x PBS solution diluted in dH_2O)

1x TBS-T

10 mM Tris Base, 150 mM NaCl and 0,05% Tween-20, pH 8 (prepared from a 10x TBS-T solution diluted in dH_2O)

1x TAE

40 mM Tris, 20 mM Acetic Acid, 1 mM EDTA, pH 8 (prepared from a TAE 50x solution diluted in dH_2O)

Lysis buffer

1mM Tris-HCl, 50 mM NaCl, 0,5% Deoxylacholat, 1,5 mM Triton X-100, 2,5 mM Trasylol, 0,01 mM Foy stock, 0,1 mM PMSF, diluted in dH_2O

Lysis buffer (for MAVS oligomerization assays)

5 mM NaF, 1 mM Na_3VO_4, 2,5 mM Trasylol, 0,01 mM Foy stock, 0,1 mM PMSF, diluted in PBS

Agarose gel for DNA electrophoresis

1x TAE, 1% agarose, Midori Green DNA stain

Agarose gel for SDD-AGE

1x TAE, 1,5% agarose, 0,1% SDS

Polyacrylamide gels

12.5 % Resolving gel	**4% Stacking gel**
12.5% Acrylamide: Bis-Acrylamide 37.5:1, Fisher BioReagents 2M Tris pH8.8	4% Acrylamide: Bis-Acrylamide 37.5:1, Fisher BioReagents 1M Tris pH6.8

20% SDS 10% APS TEMED	20% SDS 10% APS TEMED

Blocking solution (milk)

5% milk powder in 1x TBS-T

Running buffer (SDS-PAGE)

250 mM Tris, 1,9 M Glycine, 1% SDS

Running buffer (SDD-AGE)

1x TAE, 0,1% SDS

Transfer buffer

0,05 M Tris, 0,4 M Glycine, 0,05% SDS, 20% Methanol

Fixing solution

1x PBS, 4% paraformaldehyde (PFA)

Permeabilization solution

1x PBS, 0,2% Triton X-100

Blocking solution (BSA)

1x PBS, 2% BSA

Mounting Medium

3:1 mixture of Mowiol and n-propyl-gallate

N-propyl-gallate: 1x PBS, 2,5% n-propyl-gallate, 50% glycerol

Mowiol: 12 g Mowiol 4-88, 20 mL Glycerol, 40 mL PBS